VOLUME I *Molecular Biology*

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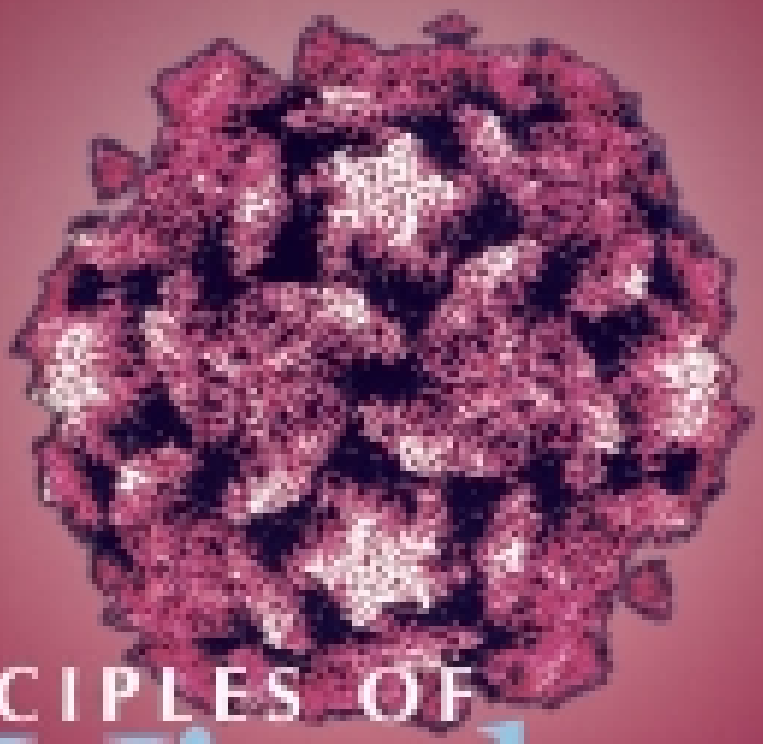

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Front cover illustration: A model of the atomic structure of the poliovirus type 1 Mahoney strain. The model has been highlighted by radial depth cuing so that the portions of the model that are farthest from the center are bright. Prominent surface features include a star-shaped mesa at each of the fivefold axes and a propeller-shaped feature at each of the threefold axes. A deep cleft or canyon surrounds the star-shaped feature. This canyon is the receptor-binding site. Courtesy of Robert Grant, Stéphane Crainic, and James Hogle (Harvard Medical School).

Back cover illustration: Progress in the global eradication of poliomyelitis has been striking, as illustrated by maps showing areas of known or probable circulation of wild-type poliovirus in 1988, 1998, and 2008. Dark red indicates the presence of virus. In 1988, the virus was present on all continents except Australia. By 1998, the Americas were free of wild-type poliovirus, and transmission was interrupted in the western Pacific region (including the People's Republic of China) and in the European region (with the exception of southeastern Turkey). By 2008, the number of countries reporting endemic circulation of poliovirus had been reduced to four: Afghanistan, Pakistan, India, and Nigeria.

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We dedicate this book to the students, current and future scientists and physicians, for whom it was written. We kept them ever in mind.

We also dedicate it to our families:

Jonn, Gethyn, and Amy Leedham

Kathy and Brian

Doris, Aidan, Devin, and Nadia

Rudy, Jeanne, and Chris

Oh, be wiser thou!
Instructed that true knowledge leads to love.

WILLIAM WORDSWORTH
Lines left upon a Seat in a Yew-tree
1888

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Contents

Preface

The enduring goal of scientific endeavor, as of all human enterprise, I imagine, is to achieve an intelligible view of the universe. One of the great discoveries of modern science is that its goal cannot be achieved piecemeal, certainly not by the accumulation of facts. To understand a phenomenon is to understand a category of phenomena or it is nothing. Understanding is reached through creative acts.

A. D. HERSHEY
Carnegie Institution Yearbook 65

The major goal of all three editions of this book has been to define and illustrate the basic principles of animal virus biology. In this information-rich age, the quantity of data describing any given virus can be overwhelming, if not indigestible, for student and expert alike. Furthermore, the urge to write more and more about less and less is the curse of reductionist science and the bane of those who write textbooks meant to be used by students. Consequently, in the third edition, we have continued to distill information with the intent of extracting essential principles, while retaining some descriptions of how the work is done. Our goal is to illuminate process and strategy as opposed to listing facts and figures. We continue to be selective in our choice of topics, viruses, and examples in an effort to make the book readable, rather than comprehensive. Detailed encyclopedic works like *Fields Virology* (2007) have made the best attempt to be all-inclusive, and *Fields* is recommended as a resource for detailed reviews of specific virus families.

What's New

The major change in the third edition is the separation of material into two volumes, each with its unique appendix(es) and general glossary. Volume I covers molecular aspects of the biology of viruses, and Volume II focuses on viral pathogenesis, control of virus infections, and virus evolution. The organization into two volumes follows a natural break in pedagogy and provides considerable flexibility and utility for students and teachers alike. The smaller size and soft covers of the two volumes make them easier for students to carry

and work with than the single hardcover volume of earlier editions. The volumes can be used for two courses, or as parts I and II of a one-semester course. While differing in content, the volumes are integrated in style and presentation. In addition to updating the material for both volumes, we have used the new format to organize the material more efficiently and to keep chapter size manageable.

As in our previous edition, we have tested ideas for inclusion in the text in our own classes. We have also received constructive comments and suggestions from other virology instructors and their students. Feedback from students was particularly useful in finding typographical errors, clarifying confusing or complicated illustrations, and pointing out inconsistencies in content.

For purposes of readability, references again are generally omitted from the text, but each chapter ends with an updated and expanded list of relevant books, review articles, and selected research papers for readers who wish to pursue specific topics. In general, if an experiment is featured in a chapter, one or more references are listed to provide more detailed information.

Principles Taught in Two Distinct, but Integrated Volumes

These two volumes outline and illustrate the strategies by which all viruses are propagated in cells, how these infections spread within a host, and how such infections are maintained in populations. The principles established in Volume I enable understanding of the topics of Volume II: viral disease, its control, and the evolution of viruses.

Volume I: the Science of Virology and the Molecular Biology of Viruses

This volume features the molecular processes that take place in an infected host cell. Chapters 1 and 2 discuss the foundations of virology. A general introduction with historical perspectives as well as definitions of the unique properties of viruses is provided first. The unifying principles that are the foundations of virology, including the concept of a common strategy for viral propagation, are then described. Chapter 2 establishes the principle of the infectious cycle with an introduction to cell biology. The basic techniques for cultivating and assaying viruses are outlined, and the concept of the single-step growth cycle is presented.

Chapter 3 introduces the fundamentals of viral genomes and genetics, and it provides an overview of the perhaps surprisingly limited repertoire of viral strategies for genome replication and mRNA synthesis. Chapter 4 describes the architecture of extracellular virus particles in the context of providing both protection and delivery of the viral genome in a single vehicle. In Chapters 5 through 13, we describe the broad spectrum of molecular processes that characterize the common steps of the reproductive cycle of viruses in a single cell, from decoding genetic information to genome replication and production of progeny virions. We describe how these common steps are accomplished in cells infected by diverse but representative viruses, while emphasizing principles applicable to all.

The appendix in Volume I provides concise illustrations of viral life cycles for the main virus families discussed in the text. It is intended to be a reference resource when one is reading individual chapters and a convenient visual means by which specific topics may be related to the overall infectious cycles of the selected viruses.

Volume II: Pathogenesis, Control, and Evolution

This volume addresses the interplay between viruses and their host organisms. Chapters 1 to 7 focus on principles of virus replication and pathogenesis. Chapter 1 provides a brief history of viral pathogenesis and addresses the basic concepts of how an infection is established in a host as opposed to infection of single cells in the laboratory. In Chapter 2, we focus on how viral infections spread in populations. Chapter 3 presents our growing understanding of crucial autonomous reactions of cells to infection and describes how these actions influence the eventual outcome for the host. Chapter 4 provides a virologist's view of immune defenses and their integration with events that occur when single cells are infected. Chapter 5 describes how a particular virus replication strategy and the ensuing host response influence the outcome of infection such that some are short and others are of long duration. Chapter 6 is devoted entirely to the AIDS virus, not only because it is the causative agent of the most serious current worldwide epidemic, but also because of its unique and informative interactions with the human immune defenses. In Chapter 7, we discuss virus infections that transform cells in culture and promote oncogenesis (the formation of tumors) in animals.

Chapters 8 and 9 outline the principles involved in treatment and control of infection. Chapter 8 focuses on vaccines, and chapter 9 discusses the approaches and challenges of antiviral drug discovery. In Chapter 10, the final chapter, we present a foray into the past and future, providing an introduction to viral evolution. We illustrate important principles taught by zoonotic infections, emerging infections, and humankind's experiences with epidemic and pandemic viral infections.

Appendix A summarizes the pathogenesis of common viruses that infect humans in three "slides" (viruses and diseases, epidemiology, and disease mechanisms) for each virus or virus group. This information is intended to provide a simple snapshot of pathogenesis and epidemiology. Appendix B provides a concise discussion of unusual infectious agents, such as viroids, satellites, and prions, that are not viruses but that (like viruses) are molecular parasites of the cells in which they replicate.

Reference

Knipe, D. M., and P. M. Howley (ed. in chief). 2007. *Fields Virology,* 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.

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Acknowledgments

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Since the inception of this work, our belief has been that the illustrations must complement and enrich the text. Execution of this plan would not have been possible without the support of Jeff Holtmeier (Director, ASM Press) and the technical expertise and craft of our illustrator. The illustrations are an integral part of the exposition of the information and ideas discussed, and credit for their execution goes to the knowledge, insight, and artistic talent of Patrick Lane of ScEYEnce Studios. As noted in the figure legends, many of the figures could not have been completed without the help and generosity of our many colleagues who provided original images. Special thanks go to those who crafted figures tailored specifically to our needs or provided multiple pieces: Mark Andrake (Fox Chase Cancer Center), Edward Arnold (Rutgers University), Bruce Banfield (The University of Colorado), Christopher Basler and Peter Palese (Mount Sinai School of Medicine), Amy Brideau (Peregrine Pharmaceuticals), Roger Burnett (Wistar Institute), Rajiv Chopra and Stephen Harrison (Harvard University), Marie Chow (University of Arkansas Medical Center), Bob Craigie (NIDDK, National Institutes of Health), Richard Compans (Emory University), Friedrich Frischknecht (European Molecular Biology Laboratory), Wade Gibson (Johns Hopkins University School of Medicine), Ramón González (Universidad Autónoma del Estado de Morelos), David Knipe (Harvard Medical School), Thomas Leitner (Los Alamos National Laboratory), Maxine Linial (Fred Hutchinson Cancer Center), Pedro Lowenstein (University of California, Los Angeles), Paul Masters (New York State Department of Health), Rolf Menzel (National Institutes of Health), Thomas Mettenleiter (Federal Institute for Animal Diseases, Insel Reims, Germany), Heather Ongley and Michael Chapman (Oregon Health and Science University), B. V. Venkataram Prasad (Baylor College of Medicine), Botond Roska (Friedrich Miescher Institute, Basel, Switzerland), Michael Rossmann (Purdue University), Alasdair Steven (National Institutes of Health), Phoebe Stewart (Vanderbilt University), Wesley Sundquist (University of Utah), Jose Varghese (Commonwealth Scientific and Industrial Research Organization), Robert Webster (St. Jude's Children's Research Hospital), Thomas Wilk (European Molecular Biology Laboratory), Alexander Wlodawer (National Cancer Institute), and Li Wu (Medical College of Wisconsin).

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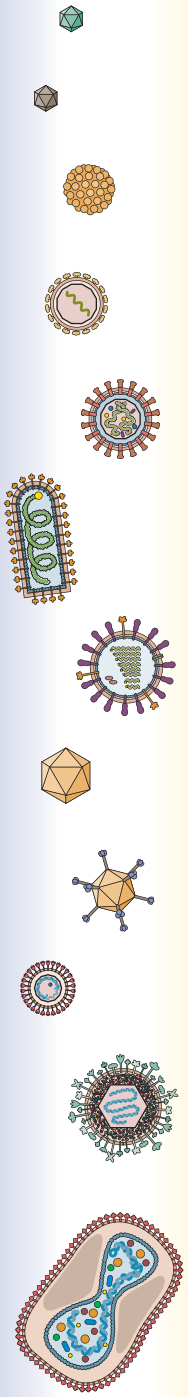
This often-consuming enterprise was made possible by the emotional, intellectual, and logistical support of our families, to whom the two volumes are dedicated.

I The Science of Virology



1

Foundations

Thus, we cannot reject the assumption that the effect of the filtered lymph is not due to toxicity, but rather to the ability of the agent to replicate.

F. Loeffler
1898

Luria's Credo

More than half a century has passed since Salvador Luria wrote the following credo in the introduction to the classic textbook *General Virology:* "There is an intrinsic simplicity of nature and the ultimate contribution of science resides in the discovery of unifying and simplifying generalizations, rather than in the description of isolated situations—in the visualization of simple, overall patterns rather than in the analysis of patchworks."

Despite an explosion of information in biology since Luria wrote these words, his vision of unity in diversity is as relevant now as it was then. That such first principles exist may not be obvious considering the bewildering array of viruses, genes, and proteins recognized in modern virology. Indeed, new viruses are being described regularly (more than 50 since 1988), and viral diseases such as acquired immunodeficiency syndrome (AIDS), hepatitis, and influenza continue to defy our efforts to control them. Yet, as discussed below, Luria's credo still stands: all viruses follow the same simple strategy to ensure their survival. This insight has been hard won over many years of observation, research, and debate; the history of virology is rich and instructive.

Why We Study Viruses

Viruses Are Everywhere

Viruses are all around us, comprising an enormous proportion of our environment, in both number and total mass (Box 1.1). All living things encounter billions of virus particles every day. For example, viruses enter our lungs in the 6 liters of air each of us inhales every minute; they enter our digestive systems with the food we eat; and they are transferred to our eyes, mouths, and other points of entry from the surfaces we touch and the people with whom we interact. Our bodies are reservoirs for viruses that reside in our lungs and our upper respiratory, gastrointestinal, and urogenital tracts, among other places. Our intestinal tracts are loaded with a myriad of plant viruses as well as

BOX 1.1 BACKGROUND
Some astounding numbers

- The biomass on our planet of bacterial viruses *alone* exceeds that of all of Earth's elephants by more than 1,000-fold. There are more than 10^{30} bacteriophage particles in the world's water supply, enough to extend out into space for 200 million light years if arranged head to tail.
- Whales are commonly infected with a member of the virus family *Caliciviridae* that causes rashes, blisters, intestinal problems, and diarrhea and can also infect humans. Whales excrete more than 10^{13} caliciviruses daily.
- With about 10^{16} human immunodeficiency virus (HIV) genomes on the planet today, it is highly probable that somewhere there exist HIV genomes that are resistant to every one of the antiviral drugs that we have now or are likely to have in the future.

hundreds of bacterial species that harbor their own constellations of viruses.

Viruses Cause Human Disease

With such constant exposure, it is nothing short of amazing that the vast majority of viruses that infect us have little or no impact on our health or wellbeing. As described in Volume II, we owe such relative safety to our elaborate immune defense systems, which have evolved precisely to fight microbial infection. When these defenses are compromised, even the most common infection can be lethal. But even with such defenses, some of the most devastating human diseases have been or still are caused by viruses; these diseases include smallpox, yellow fever, poliomyelitis, influenza, measles and, more recently, AIDS. Viral infections can lead to life-threatening diseases of the central nervous system and various vital organs such as the lungs, liver, and intestines. Viruses are responsible for approximately 20% of the human cancer burden, and viral infections of the respiratory and gastrointestinal tracts kill millions of children in the developing world each year. As summarized in Volume II, Appendix A, there is no question about the biomedical importance of these agents.

Viruses Infect All Living Things

Viruses also infect pets, food animals, plants, insects, and wildlife throughout the world. They infect microbes such as algae, fungi, and bacteria, and some even "prey" on other viruses. Viral infection of agricultural plants and animals can have enormous economic and societal impact. Recent outbreaks of infection by foot-and-mouth disease virus and avian influenza virus led to the destruction (**culling**) of millions of cattle, sheep, and poultry to prevent further spread. Losses in the United Kingdom during the 2001 outbreak of foot-and-mouth disease ran into billions of dollars and caused havoc for both farmers and the government. More recent outbreaks of the H5N1 avian influenza virus in Asia have caused similar disruption and economic loss. Viruses that infect crops such as potatoes and fruit trees are common and can lead to serious food shortage as well as financial devastation.

Viruses Can Cross Species Boundaries

Although viruses generally have a limited host range, they can and do spread across species barriers. As the world's human population continues to expand and impinge on the wilderness, cross-species (**zoonotic**) infections of humans are occurring with increasing frequency. In addition to the AIDS pandemic, the highly fatal Ebola hemorrhagic fever and the severe acute respiratory syndrome (SARS) are recent examples of new viral diseases to emerge from zoonotic infections. The current pandemic of H5N1 influenza in avian species has the world riveted by the frightening possibility that a new, highly pathogenic strain of this virus might emerge following transmission from birds to human hosts. Indeed, given the eons over which viruses have had the opportunity to interact with various species, today's "natural" host may simply be a way station in a virus's evolution.

Viruses "R" Us

There are many thousands of copies of retroviral DNA in the human genome, so every cell in our body contains viral DNA. Human endogenous retroviruses, and elements thereof, make up about 5 to 8% of our DNA. Most are inactive, fossil remnants from infections of germ cells that have occurred over millions of years during our evolution, but some are suspected to be associated with specific diseases. Each of us will pass our constellation of these genetic elements to our children.

Viruses Are Uniquely Valuable Tools with Which To Study Biology

Because viruses are dependent on their hosts for propagation, studies of the way in which viral infection induces reprogramming of cellular mechanisms have provided unique

Table 1.1 Bacteriophages: landmarks in molecular biology[a]

Year	Discovery (discoverer[s])
1939	One-step growth of viruses (Ellis and Delbrück)
1946	Mixed phage infection leads to genetic recombination (Delbrück)
1947	Mutation and DNA repair (multiplicity reactivation) (Luria)
1952	Transduction of genetic information (Zinder and Lederberg)
1952	DNA, not protein, found to be the genetic material (Hershey and Chase)
1952	Restriction and modification of DNA (Luria)
1955	Definition of a gene (*cis-trans* test) (Benzer)
1958	Mechanisms of control of gene expression by repressors and activators are established (Pardee, Jacob, and Monod)
1958	Definition of the episome (Jacob and Wollman)
1961	Discovery of mRNA (Brenner, Jacob, and Meselson)
1961	Elucidation of the triplet code by genetic analysis (Crick, Barnett, Brenner, and Watts-Tobin)
1961	Genetic definition of nonsense codons as stop signals for translation (Campbell, Epstein, and Bernstein)
1964	Colinearity of the gene with the polypeptide chain (Sarabhai, Stretton, and Brenner)
1966	Pathways of macromolecular assembly (Edgar and Wood)
1974	Vectors for recombinant DNA technology (Murray and Murray, Thomas, Cameron, and Davis)

[a]Sources: T. D. Brock, *The Emergence of Bacterial Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1990); K. Denniston and L. Enquist, *Recombinant DNA* (*Benchmark Papers in Microbiology*, vol. 15) (Dowden, Hutchinson and Ross, Inc., Stroudsburg, PA, 1981); and C. K. Mathews, E. Kutter, G. Mosig, and P. Berget, *Bacteriophage T4* (American Society for Microbiology, Washington, DC, 1983).

insights into cellular biology and functioning of host defenses. Intensive studies of viruses that infect bacteria, the bacteriophages, laid the foundation of modern molecular biology (Table 1.1), and crystallization of the plant virus tobacco mosaic virus established a landmark in structural biology. Studies of animal viruses established many fundamental principles of cellular function, including the processes of gene replication and transcription and of messenger RNA (mRNA) processing and translation. The study of cancer (transforming) viruses revealed the genetic basis of this disease. It seems clear that studies of viruses will continue to open up such paths of discovery in the future.

Viruses Can Also Be Used To Manipulate Biology

With the development of recombinant DNA technology and our increased understanding of some viral systems, it has become possible to use viral genomes as vehicles for the delivery of selected genes to cells and organisms for both scientific and medical purposes. The use of viral vectors to introduce tagged genes into various cells and organisms to study their function has become a standard method in biology. Viral vectors are also being tested to treat human disease via "gene therapy." Such technological advances also have a dark side, as they make it possible to reprogram viral genomes as agents of bioterrorism. A comprehensive understanding of virus biology and pathogenesis is our only rational defense against such eventualities.

Virus Prehistory

Viruses have been known as distinct biological entities for little more than a century. Consequently, efforts to understand and control these important agents of disease are phenomena of the 20th century. Nevertheless, evidence of viral infection can be found among the earliest recordings of human activity, and methods for combating viral disease were practiced long before the first virus was recognized.

Viral Infections in Antiquity

Reconstruction of the prehistoric past to provide a plausible account of when or how viruses established themselves in human populations is a challenging task. However, extrapolating from current knowledge, we can deduce that some modern viruses undoubtedly were associated with the earliest precursors of mammals and coevolved with humans. Other viruses entered human populations only recently. The last 10,000 years of human history was a time of radical change for humans and our viruses: animals were domesticated, the human population increased dramatically, large population centers appeared, and commerce drove interactions among unprecedented numbers of humans. We can infer from scattered glimpses of ancient history that viruses have long been a part of human experience.

Some viruses that eventually established themselves in human populations were undoubtedly transmitted to early humans from animals, much as still happens today. Early human groups that domesticated and lived with their animals were almost certainly exposed to different viruses than were nomadic hunter societies. Similarly, as many different viruses are **endemic** in the tropics, human societies in that environment must have been exposed to a greater variety of viruses than societies established in temperate climates. When nomadic groups met others with domesticated animals, or individuals from tropical cultures mingled with those from cooler climates, human-to-human contact could have provided new avenues for virus spread. Even so, it seems unlikely that viruses such as those that cause measles or smallpox could have entered a permanent relationship with small groups of

early humans. Such highly virulent viruses, as we now know them to be, either kill their hosts or induce lifelong immunity. Consequently, they can survive only where large, interacting host populations, which offer a sufficient number of naive and sensitive hosts, are available for their continued propagation. Such viruses could not have been established in human populations until large, settled communities appeared. Less virulent viruses that enter into a more benign, long-term relationship with their hosts were therefore more likely to be the first to become adapted to replication in the earliest human populations. These viruses include the modern retroviruses, herpesviruses, and papillomaviruses.

Evidence of several viral diseases can be found in ancient records (Fig. 1.1). The Greek poet Homer characterizes Hector as rabid in *The Iliad*. Mesopotamian laws that outline the responsibilities of the owners of rabid dogs date from before 1,000 B.C. Their existence indicates that the communicable nature of this viral disease was already well known by that time. Egyptian hieroglyphs that illustrate what appear to be the consequences of poliovirus infection (a withered leg typical of poliomyelitis [Fig. 1.1B]) or pustular lesions characteristic of smallpox also date from that period. The smallpox virus, which was probably endemic in the Ganges river basin by the fifth century B.C. and subsequently spread to other parts of Asia and Europe, has played an important part in human history. Its introduction into the previously unexposed native populations of Central and South America by colonists in the 15th century led to lethal epidemics, which are considered an important factor in the conquests achieved by a small number of European soldiers. Other viral diseases known in ancient

Figure 1.1 References to viral diseases abound in the ancient literature. (A) An image of Hector from an ancient Greek vase. Courtesy of the University of Pennsylvania Museum (object 30-44-4). **(B)** An Egyptian stele, or stone tablet, from the 18th dynasty (1580–1350 B.C.) depicting a man with a withered leg and the "drop foot" syndrome characteristic of polio. Panel B is reprinted from W. Biddle, *A Field Guide to Germs* (Henry Holt and Co., LLC, New York, NY, 1995; © 1995 by Wayne Biddle), with permission from the publisher.

A

Here this firebrand, rabid Hector, leads the charge.
HOMER, *The Iliad,*
translated by Robert Fagels
(Viking Penguin)

B

times include mumps and, perhaps, influenza. Yellow fever has been described since the discovery of Africa by Europeans, and it has been suggested that this scourge of the tropical trade was the basis for legends about ghost ships, such as the *Flying Dutchman*, in which an entire ship's crew perished mysteriously.

Humans have not only been subject to viral disease throughout much of their history but have also manipulated these agents, albeit unknowingly, for much longer than might be imagined. One classic example is the cultivation of marvelously patterned tulips, which were of enormous value in 17th-century Holland. Such efforts included deliberate spread of a virus (tulip breaking virus or tulip mosaic virus) that we now know causes the striping of tulip petals so highly prized at that time (Fig. 1.2). Attempts to control viral disease have an even more venerable history.

Figure 1.2 *Three Broken Tulips*. A painting by Nicolas Robert (1624–1685), now in the collection of the Fitzwilliam Museum, Cambridge, United Kingdom. Striping patterns (color breaking) in tulips were described in 1576 in western Europe and were caused by a viral infection. This beautiful image depicts the remarkable consequences of infection with the tulip mosaic virus. Courtesy of the Fitzwilliam Museum, University of Cambridge.

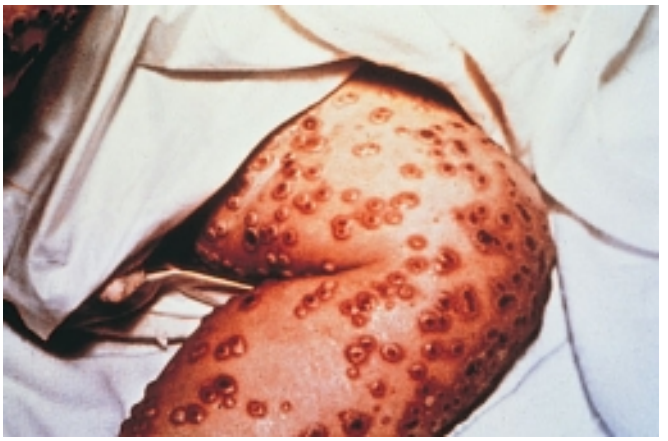


Figure 1.3 Characteristic smallpox lesions in a young victim. Illustrations like these were used to track down individuals infected with the smallpox virus (variola virus) during the World Health Organization campaign to eradicate the disease. Photo courtesy of the Immunization Action Coalition (original source: Centers for Disease Control and Prevention).

The First Vaccines

Measures to control one viral disease have been used with some success for the last millennium. The disease is smallpox (Fig. 1.3), and the practice is called **variolation**, inoculation of healthy individuals with material from a smallpox pustule into a scratch made on the arm. Variolation, which was widespread in China and India by the 11th century, was based on the recognition that smallpox survivors were protected against subsequent bouts of the disease. Variolation later spread to Asia Minor, where its value was recognized by Lady Mary Wortley Montagu, wife of the British ambassador to the Ottoman Empire. She introduced this practice into England in 1721, where it became quite widespread following successful inoculation of children of the royal family. George Washington is said to have introduced variolation among Continental Army soldiers in 1776. However, the consequences of variolation were unpredictable and never pleasant. Serious skin lesions invariably developed at the site of inoculation and might be accompanied by more generalized rash and disease, with a fatality rate of 1 to 2%. From the comfortable viewpoint of a developed country in the 21st century, such a death rate seems unacceptably high. However, in the 18th century, variolation was perceived as a much better alternative than contracting natural smallpox, a disease associated with a fatality rate of 25% in the whole population and 40% in babies and young children.

In the 1790s, Edward Jenner, an English country physician, recognized the principle on which modern

methods of viral immunization are based, even though viruses themselves were not to be identified for another 100 years. Jenner himself was variolated as a boy and also practiced this procedure. He was undoubtedly familiar with its effects and risks. Perhaps this experience spurred his great insight upon observing that milkmaids who had been exposed to cowpox (a mild disease in humans) were protected against smallpox. Jenner followed up this astute observation with direct experiments. In 1794 to 1796, he demonstrated that inoculation with extracts from cowpox lesions induced only mild symptoms but protected against the far more dangerous smallpox disease. It is from these experiments with cowpox that we derive the term **vaccination** (*vacca* = cow in Latin). This term was first given its general meaning by Louis Pasteur in 1881 to honor Jenner's accomplishments.

Initially, the only way to propagate and maintain cowpox vaccine was by serial infection of human subjects. This method was eventually banned, as it was associated with transmission of other diseases such as syphilis and hepatitis at significant frequencies. By 1860, the vaccine had been passaged in cows; later, sheep and water buffaloes were also used. While Jenner's original vaccine was based on the virus that causes cowpox, sometime during the human-to-human or cow-to-cow transfers, the poxvirus now called vaccinia virus replaced the cowpox virus. Vaccinia virus is the basis for the modern smallpox vaccine, but its origins are not known. It exhibits little genetic similarity to the viruses that cause cowpox or smallpox or to many of the known members of the poxvirus family. Scientists have recovered the smallpox vaccine used in New York in 1876 and have verified that it contains vaccinia virus and not cowpox virus. Speculation about when and how the switch occurred has produced some fascinating scenarios (Box 1.2).

The first deliberately attenuated viral vaccine was made by Louis Pasteur. In 1885 he inoculated rabbits with material from the brain of a cow infected with rabies virus and then used aqueous suspensions of dried spinal cords from these animals to infect other rabbits. After several such passages, the resulting preparations caused mild disease (i.e., were **attenuated**) yet produced effective immunity against rabies. Safer and more efficient methods for the production of larger quantities of these first vaccines awaited the recognition of viruses as distinctive biological entities and knowledge about their parasitism of cells in their hosts. Indeed, it took almost 50 years to discover the next antiviral vaccines: a vaccine for yellow fever virus was developed in 1935, and an influenza vaccine was available in 1936. These advances became possible only with radical changes in our knowledge of living organisms and of the causes of disease.

BOX 1.2 DISCUSSION

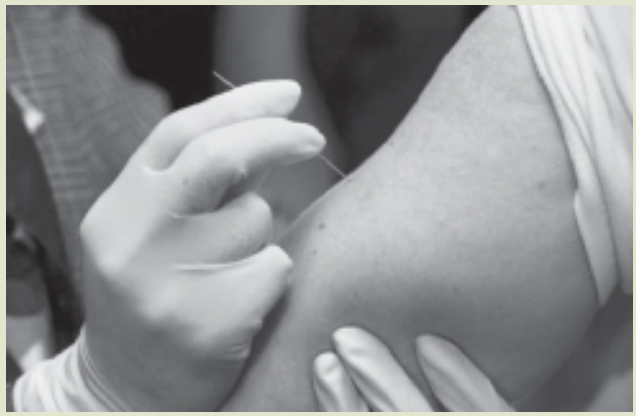
Origin of vaccinia virus

Over the years, at least three hypotheses have been advanced to explain the curious substitution of cowpox virus by vaccinia virus:

1. Recombination of cowpox virus with smallpox virus after variolation of humans
2. Recombination between cowpox virus and animal poxviruses during passage in various animals
3. Genetic drift of cowpox virus after repeated passage in humans and animals

None of these hypotheses has been proven conclusively.

Vaccination against smallpox. The image is from *Public Health Bulletin* vol. 52, no. 3, spring 2003, California County of Orange Health Care Agency (http://www.ochealthinfo.com/newsletters/phbulletin/2003/2003-spring.htm), with permission.



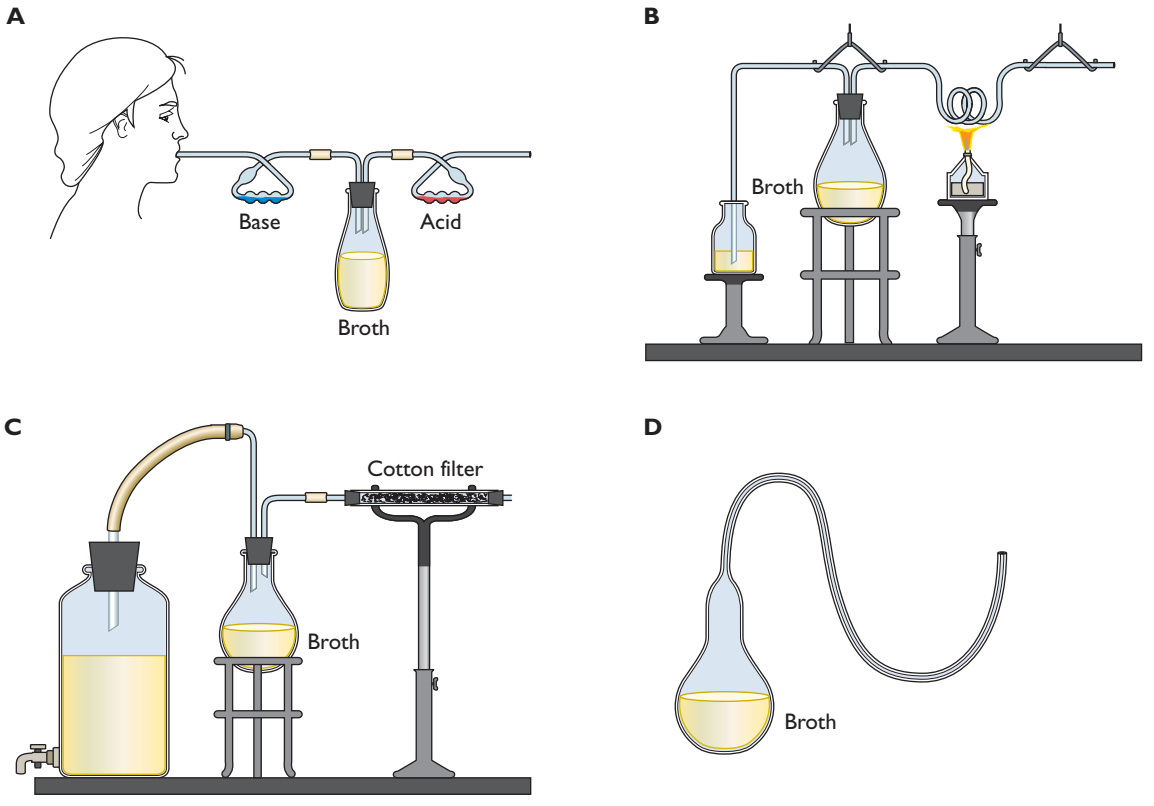
Microorganisms as Pathogenic Agents

The 19th century was a period of revolution in scientific thought, particularly in ideas about the origins of living things. The publication of Charles Darwin's *The Origin of Species* in 1859 crystallized startling (and, to many people, shocking) new ideas about the origin of diversity in plants and animals, until then generally attributed directly to the hand of God. These insights permanently undermined the belief that humans were somehow set apart from all other members of the animal kingdom. From the point of view of the science of virology, the most important changes were in ideas about the causes of disease.

The diversity of macroscopic organisms has been appreciated and cataloged since the dawn of recorded human history. A vast new world of organisms too small to be visible to the naked eye was revealed through the microscopes of Antony van Leeuwenhoek (1632–1723). Among

van Leeuwenhoek's vivid and enthusiastic descriptions of living microorganisms as "wee animalcules," seen in such ordinary materials as rain or seawater, are examples of what we now know to be protozoa, algae, and bacteria. By the early 19th century, the scientific community had accepted the existence of microorganisms and turned to the question of their origin—a topic of fierce debate. Some believed that microorganisms arose spontaneously, for example, in decomposing matter, where they were especially abundant. Others held the view that all were generated by the reproduction of like microorganisms, as were macroscopic organisms. The death knell of the spontaneous-generation hypothesis was sounded with the famous experiments of Pasteur. He demonstrated that boiled (i.e., sterilized) medium remained free of microorganisms as long as it was maintained in special flasks with curved, narrow necks designed to prevent entry of airborne microbes (Fig. 1.4). Pasteur also established that particular microorganisms were associated with specific processes, for example, the production of alcohol, lactic acid, or acetic acid (vinegar) by fermentation. This idea was crucial in the development of modern explanations for the causes of disease.

Figure 1.4 Four experiments to challenge the spontaneous-generation hypothesis. The first step in each experiment was to boil the broth medium very thoroughly to destroy all living organisms. Air was then admitted to the flasks to satisfy the believers in spontaneous generation, who insisted that oxygen must be present for life to originate. However, the air admitted to the broth medium was first freed of living organisms in several ingenious ways. Under these conditions the broths remained perfectly sterile; no microorganisms appeared in them, showing that living things could not be generated spontaneously from the lifeless liquid. **(A)** Sterilizing air by chemical treatment. The set of bulbs next to the person's face contained alkali, and the other set contained concentrated acid. Air was drawn in through the acid to inactivate microbes before it reached the broth. **(B)** Sterilizing air by using heat. Air could enter the flask of broth only by passing through the coiled glass tube kept hot by the flame. **(C)** Sterilizing air by physically trapping particles. The aspiration bottle drew air into the flask through the tube containing cotton at the right. The cotton filtered out the microbes in the air, just as the cotton or foam plugs now used in bacteriological culture tubes protect the culture from air contamination. **(D)** Pasteur's famous swan-neck flasks provided passive exclusion of microbes from the sterilized broth. Although the flask was freely open to the air, the broth remained sterile so long as the microbe-bearing dust that collected in the neck did not reach the liquid.



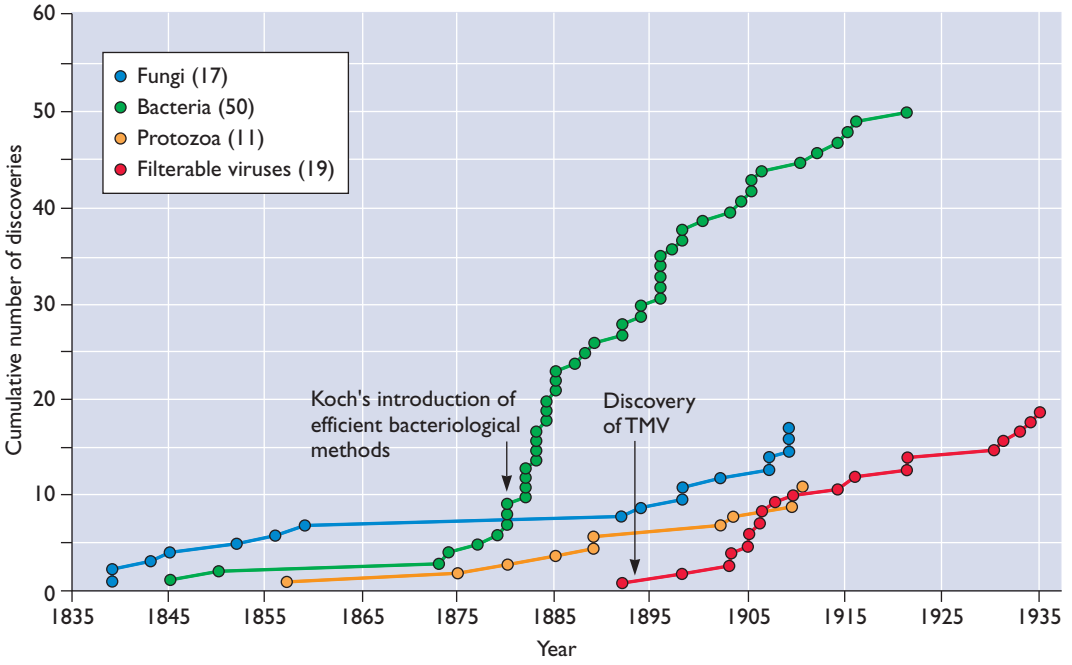


Figure 1.5 The pace of early discovery of new infectious agents. Koch's introduction of efficient bacteriological techniques spawned an explosion of new discoveries of bacterial agents in the early 1880s. Similarly, the discovery of filterable agents launched the field of virology in the early 1900s. Despite an early surge of virus discovery, only 19 distinct human viruses had been reported by 1935. TMV, tobacco mosaic virus. Adapted from K. L. Burdon, *Medical Microbiology* (Macmillan Co., New York, NY, 1939), with permission.

From the earliest times, poisonous air (miasma) was generally invoked to account for **epidemics** of contagious diseases, and there was little recognition of the differences among their causative agents. The association of particular microorganisms, initially bacteria, with specific diseases can be attributed to the ideas of the German physician Robert Koch. He developed and applied a set of criteria for identification of the agent responsible for a specific disease (a **pathogen**). These criteria, **Koch's postulates**, are still applied in the identification of pathogens that can be propagated in the laboratory and tested in an appropriate animal model. The postulates are as follows.

- The organism must be regularly associated with the disease and its characteristic lesions.
- The organism must be isolated from the diseased host and grown in culture.
- The disease must be reproduced when a pure culture of the organism is introduced into a healthy, susceptible host.
- The same organism must be reisolated from the experimentally infected host.

By applying these criteria, Koch demonstrated that anthrax, a common disease of cattle, was caused by a specific bacterium (designated *Bacillus anthracis*) and that a second, distinct bacterial species caused tuberculosis in humans. Guided by these postulates and the methods for the sterile culture and isolation of pure preparations of bacteria developed by Pasteur, Joseph Lister and Koch identified and classified many pathogenic bacteria (as well as yeasts and fungi) during the last part of the 19th century (Fig. 1.5). From these beginnings, investigation into the causes of infectious disease was placed on a secure scientific foundation, the first step toward rational treatment and ultimately control. During the last decade of the 19th century, failures of the paradigm that bacterial or fungal agents are responsible for all diseases led to the identification of a new class of infectious agents—submicroscopic pathogens that came to be called **viruses**.

Discovery of Viruses

The first report of a pathogenic agent smaller than any known bacterium appeared in 1892. The Russian scientist Dimitrii Ivanovsky observed that the causative agent of tobacco mosaic disease was not retained by the unglazed porcelain filters used at that time to remove bacteria from extracts and culture media (Fig. 1.6). Six years later in

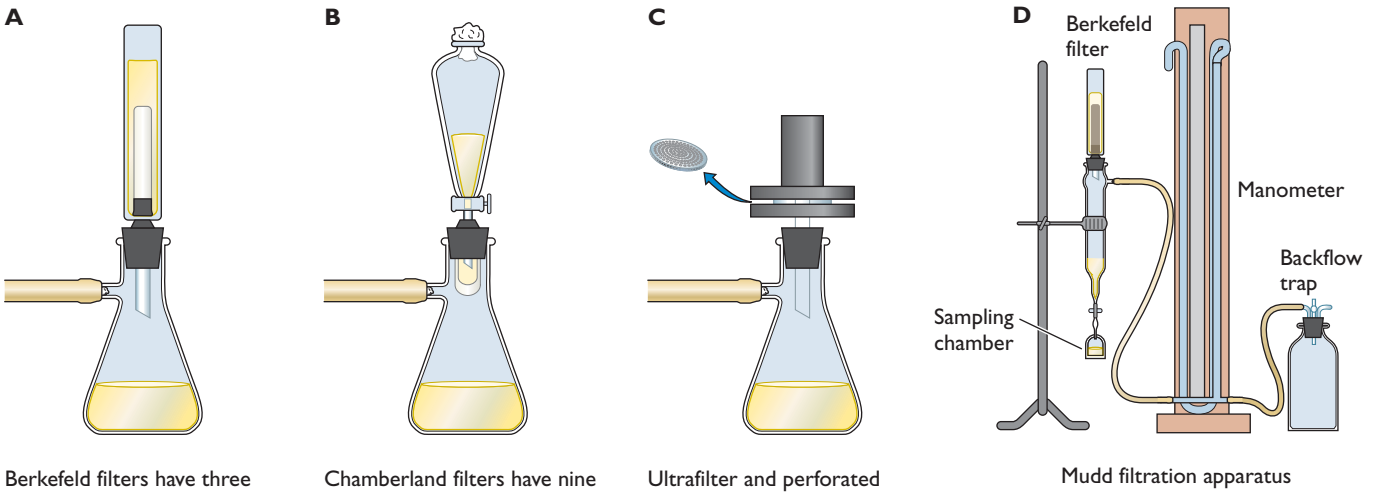


Figure 1.6 Filter systems used to characterize viruses. Filters for making liquids free of cultivatable organisms were instrumental in the first identification of viruses. Several types of filters were used in the early days of virus research, and four are illustrated here. Berkefeld filters **(A)** and Chamberland filters **(B)** are typical of the "candle" style of filter comprising diatomaceous earth pressed into the shape of a hollow candle open at one end. Only the smallest pore sizes retained bacteria and allowed viruses to pass through. For the Chamberland filter **(B)**, the fluid to be filtered was introduced into the open end by means of a funnel. The filter is cut away to illustrate the hollow center. Filters similar to these were probably used by Ivanovsky, Loeffler, and Frosch to isolate the first plant and animal viruses. **(C)** Example of a collodion membrane "ultrafilter." The thin membrane is the filtering surface and is stretched over a filter plate. **(D)** A Mudd filtration apparatus illustrating the typical setup for filtering fluids. All the filters were designed to operate under negative pressure by connection with a suction pump or aspirator. Positive pressure was also used. The trap (on the right) was essential to catch backflow of liquid when the suction was released. The manometer (in the middle) provided an accurate means of measuring and regulating pressure. The filtrate was collected in a graduated buret, from which measured samples were collected and tested.

Holland, Martinus Beijerinck independently made the same observation. More importantly, Beijerinck made the conceptual leap that because the pathogen was so small that it could pass through filters that trapped all known bacteria, it must be a distinctive agent.

The same year (1898), the German scientists Friedrich Loeffler and Paul Frosch, both former students and assistants of Koch, observed that the causative agent of foot-and-mouth disease was also filterable (Box 1.3). Not only were the tobacco mosaic and foot-and-mouth disease pathogens much smaller than any previously recognized microorganism, but also they replicated **only** in their host organisms. For example, extracts of an infected tobacco plant diluted into sterile solution produced no additional infectious agents until introduced into leaves of healthy plants, which subsequently developed tobacco mosaic disease. The serial transmission of infection by diluted extracts established that these diseases were not caused by a bacterial toxin present in the original preparations derived from infected tobacco plants or cattle. The failure of both pathogens to multiply in solutions that readily supported the growth of bacteria, as well as their dependence on host organisms for reproduction, further distinguished these new agents from pathogenic bacteria. Beijerinck termed the submicroscopic agent responsible for tobacco mosaic disease *contagium vivum fluidum* to emphasize its infectious nature and distinctive reproductive and physical properties. Agents passing through filters that retain bacteria came to be called ultrafilterable viruses, appropriating the term *virus* from the Latin for "poison." This term eventually was simplified to viruses.

The discovery of the first virus, tobacco mosaic virus, is often attributed to the work of Ivanovsky in 1892. However, he did not identify the tobacco mosaic disease pathogen as a distinctive agent, nor was he convinced that its passage through bacterial filters was not the result of some technical failure. It may be more appropriate to attribute the founding of the field of virology to the astute

BOX 1.3 DISCUSSION

The first animal virus discovered remains a scourge today

The first animal virus to be discovered, foot-and-mouth disease virus, infects domestic cattle, pigs, and sheep, as well as many species of wild animals. Although mortality is low, morbidity is high and infected domestic animals lose their commercial value. The virus is highly contagious, and the most common and effective method of control is by the slaughter of entire herds in affected areas.

Outbreaks of foot-and-mouth disease were widely reported in Europe, Asia, Africa, and South and North America in the 1800s. The largest epidemic ever recorded in the United States occurred in 1914. After gaining entry into the Chicago stockyards, the virus spread to more than 3,500 herds in 22 states. This calamity accelerated epidemiologic and disease control programs, eventually leading to the field- and laboratory-based systems maintained by the U.S. Department of Agriculture to protect domestic livestock from foreign animal and plant diseases. Similar control systems have been established in other Western countries, but this virus still presents a formidable challenge throughout the world. A 1997 outbreak of foot-and-mouth disease among pigs in Taiwan resulted in economic losses of greater than $10 billion.

In 2001, an epidemic outbreak in the United Kingdom spread to other countries in Europe and led to the slaughter of more than 3 million infected and uninfected farm animals. The associated economic, societal, and political costs threatened to bring down the British government. Images of mass graves and horrific pyres consuming the corpses of dead animals sensitized the public as never before. It is clear that viruses do not have to infect humans to exact a human cost. It is sobering to realize that we have known about this animal virus longer than any other but have still not learned enough to keep it from exacting its toll.

Murphy, F. A., E. P. J. Gibbs, M. C. Horzinek, and M. J. Studdert. 1999. *Veterinary Virology,* 3rd ed. Academic Press, Inc., San Diego, CA.

insights of Beijerinck, Loeffler, and Frosch, who recognized the distinctive nature of the plant and animal pathogens they were studying more than 100 years ago.

The pioneering work on tobacco mosaic virus and foot-and-mouth disease virus was followed by the identification of viruses associated with specific diseases in many other organisms. Important landmarks from this early period include the identification of viruses that cause leukemias or solid tumors in chickens by Vilhelm Ellerman and Olaf Bang in 1908 and by Peyton Rous in 1911, respectively. The study of viruses associated with cancers in chickens, particularly the Rous sarcoma virus, eventually led to an understanding of the molecular basis of cancer (Volume II, Chapter 7).

The fact that bacteria could also be hosts to viruses was first recognized by Frederick Twort in 1915 and Félix d'Hérelle in 1917. d'Hérelle named them **bacteriophages** because of their ability to lyse bacteria on the surface of agar plates (phage is derived from the Greek for "eating"). In an interesting twist of serendipity, Twort made his discovery of bacterial viruses while testing the smallpox vaccine virus to see if it would grow on simple media. He found bacterial contaminants, some of them exhibiting an unusual "glassy transformation," which proved to be the result of lysis by a bacteriophage. Investigation of bacteriophages provided the foundations for the field of molecular biology, as well as fundamental insights into how viruses interact with their host cells.

The Definitive Properties of Viruses

Throughout the early period of virology, in which many viruses of plants, animals, and bacteria were cataloged, the origin and nature of these distinctive infectious agents were quite controversial. Arguments centered on whether viruses represented parts of a cell that had somehow acquired a new kind of existence or whether they were built from virus-specific components. Little progress was made toward resolving these issues and establishing the definitive properties of viruses until new techniques were developed, which allowed their visualization or propagation in cultured cells.

The Structural Simplicity of Viruses

Dramatic confirmation of the structural simplicity of viruses came in 1935, when crystals of tobacco mosaic virus were obtained by Wendell Stanley. At that time, nothing was known of the structural organization of any biologically important macromolecules, such as proteins and DNA. Indeed, the crucial role of DNA as genetic material had not even been recognized. The ability to obtain an infectious agent in crystalline form, a state that is more generally associated with inorganic material, therefore created much wonder and speculation about whether a virus is truly a life form. In retrospect, it is obvious that the relative ease with which tobacco mosaic virus could be crystallized was a direct result of its structural simplicity and the ability of many particles to associate in regular arrays.

The 1930s saw the introduction of the instrument that rapidly revolutionized virology, the electron microscope. The great magnifying power of this instrument (eventually over 100,000-fold) allowed direct visualization of virus particles for the first time. It has always been an exciting experience for investigators to obtain images of viruses,

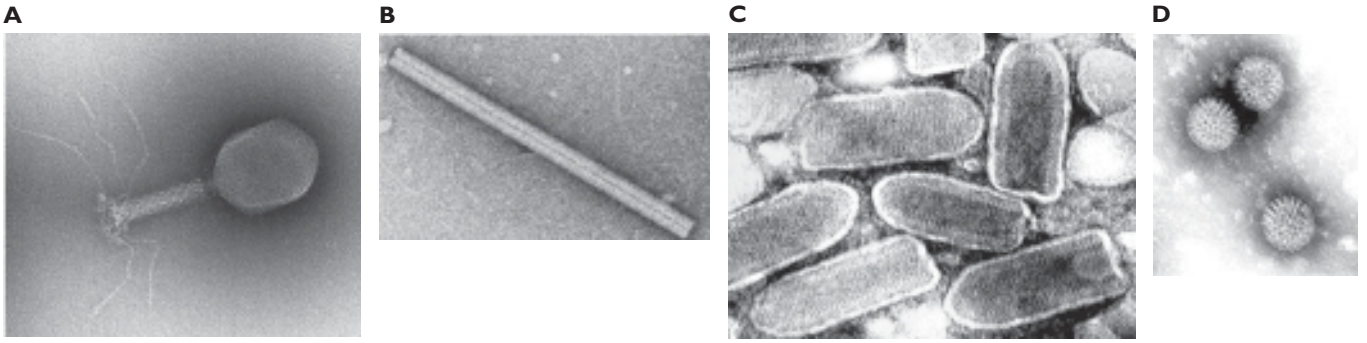


Figure 1.7 Electron micrographs of viruses following negative staining. (A) The complex, nonenveloped virus bacteriophage T4. Note the intricate tail and tail fibers. Courtesy of R. L. Duda, University of Pittsburgh. **(B)** The helical, nonenveloped particle of tobacco mosaic virus. Reprinted from the Universal Virus Database of the International Committee on Taxonomy of Viruses (http://www.ncbi.nlm.nih.gov/ICTVdb/WIntkey/Images/em_tmv.gif), with permission. **(C)** Enveloped particles of the rhabdovirus vesicular stomatitis virus. Courtesy of F. P. Williams, University of California, Davis. **(D)** Nonenveloped, icosahedral human rotavirus particles. Courtesy of F. P. Williams, U.S. Environmental Protection Agency (http://www.epa.gov/nerlcwww/rota.htm).

especially as they often prove to be remarkably elegant in appearance (Fig. 1.7). Images of many different viruses confirmed that these agents are very small (Fig. 1.8) and far simpler in structure than any cellular organism. Many appeared as regular helical or spherical particles. The description of the morphology of virus particles made possible by electron microscopy also opened the way for the first rational classification of viruses.

The Intracellular Parasitism of Viruses

Organisms as Hosts

The fundamental characteristic of viruses is their absolute dependence on a living host for reproduction: they are **obligate parasites**. Transmission of plant viruses such as tobacco mosaic virus can be achieved readily, for example, by applying extracts of an infected plant to a scratch made on the leaf of a healthy plant. Furthermore, as a single infectious particle of many plant viruses is sufficient to induce the characteristic lesion (Fig. 1.9), the concentration of the infectious agent could be measured. Plant viruses were therefore the first to be studied in detail. Viruses of animals could also be propagated under experimental conditions, and methods were developed to quantify these agents by determining the lethal dose. These methods could also be applied to human viruses that were able to infect laboratory animals. The transmission of yellow fever virus to mice by Max Theiler in 1930 was an achievement that led to the isolation of an attenuated strain, still considered one of the safest and most effective ever produced for the vaccination of humans.

After specific viruses and host organisms were identified, it became possible to produce sufficient quantities of virus particles for investigation of their physical and chemical properties. Scientists were also able to determine the consequences of infection for the host. Features such as the incubation period, gross symptoms of infection, and effects on specific tissues and organs were investigated. Laboratory animals remain an essential tool in investigations of the pathogenesis of viruses that cause disease. However, real progress toward understanding the mechanisms of virus replication was made only with the development of tissue and cell culture systems. Among the simplest, but crucial to both virology and molecular biology, were cultures of bacterial cells.

Lessons from Bacteriophages

In the late 1930s and early 1940s, bacteriophages, or "phages," received increased attention as a result of controversy centering on how they were formed. d'Hérelle, one of their discoverers, favored the hypothesis that there was only one phage that attacked all bacteria. This hypothesis was disproved by F. Macfarlane Burnet, who showed that there were a great variety of phages with different physical and biological properties. Nevertheless, the precise nature of these agents remained elusive. John Northrup, a biochemist at the Rockefeller Institute in Princeton, NJ, championed the theory that a phage was a metabolic product of a bacterium. Phage formation was said to be analogous to the autocatalytic formation of enzymes from inactive precursors, a direct rejection of the proposal by d'Hérelle that a phage was a living organism. On the other hand,

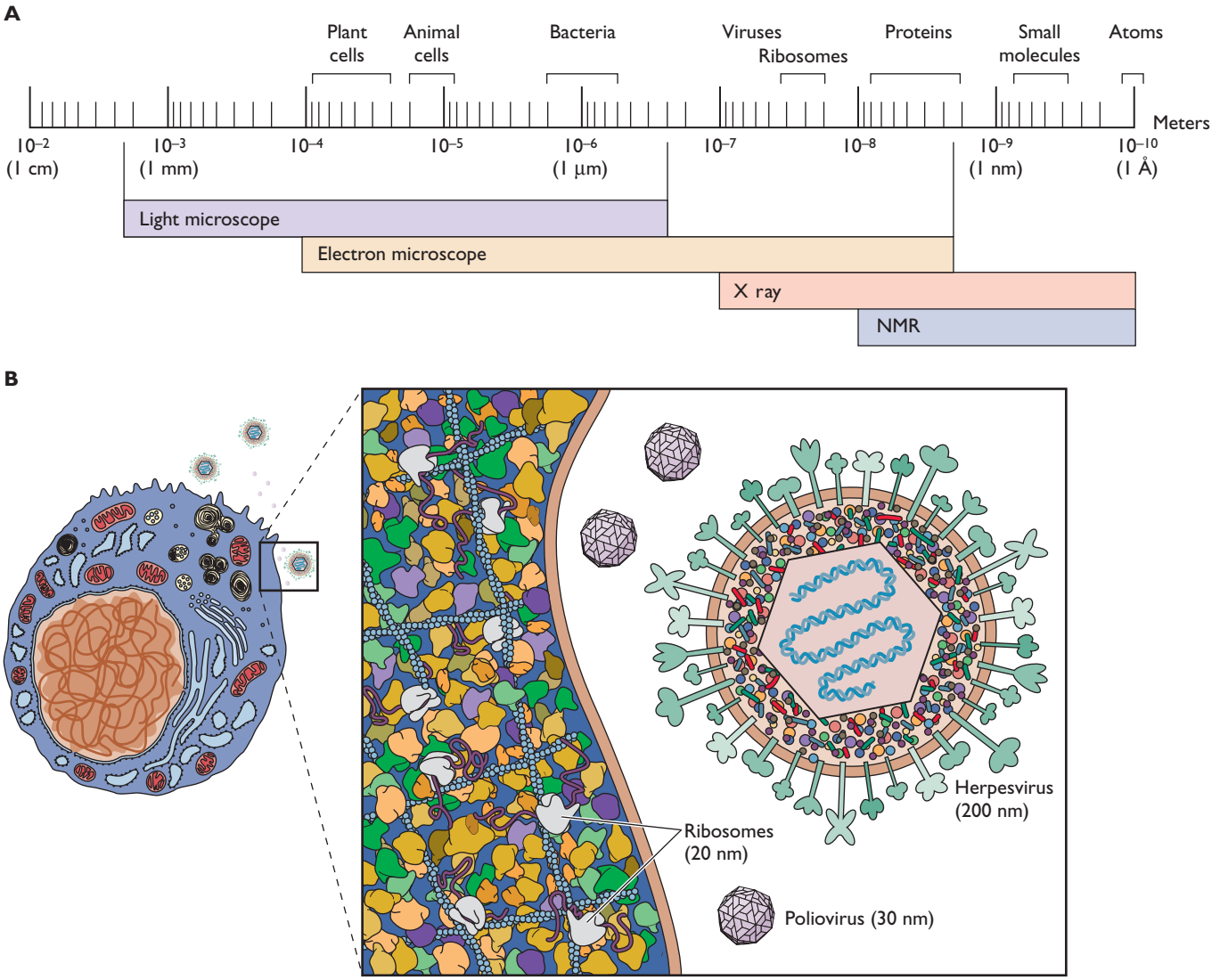


Figure 1.8 Size matters. (A) Sizes of animal and plant cells, bacteria, viruses, proteins, molecules, and atoms are indicated. The resolving powers of various techniques used in virology, including light microscopy, electron microscopy, X-ray crystallography, and nuclear magnetic resonance (NMR) spectroscopy, are indicated. Viruses, which are within the resolving power of the electron microscope, are about 2 orders of magnitude smaller than the smallest bacterium. The units commonly used in descriptions of virus particles or their components are the nanometer (nm [10^{-9} m]) and the angstrom (Å [10^{-10} m]). Adapted from A. J. Levine, *Viruses* (Scientific American Library, New York, NY, 1991); used with permission of Henry Holt and Company, LLC. **(B)** Illustration of the size differences among two viruses and a typical host cell.

Max Delbrück, in his work with Emory Ellis and later with Luria, regarded phages as autonomous, stable, self-replicating entities characterized by heritable traits. According to this paradigm, phages were seen as ideal tools with which to investigate the nature of genes and heredity. Probably the most critical early contribution of Delbrück and Ellis was the perfection of the one-step growth technique for synchronization of the replication of phage, an achievement that allowed analysis of a single cycle of phage growth in a population of bacteria. This approach introduced highly quantitative methods to virology, as well as an unprecedented rigor of analysis. The first experiments showed that phages indeed multiplied in the bacterial host and were liberated in a "burst" by lysis of the cell.

Figure 1.9 Lesions induced by tobacco mosaic virus on an infected tobacco leaf. In 1886, Adolph Mayer first described the characteristic patterns of light and dark green areas on the leaves of tobacco plants infected with tobacco mosaic virus. He demonstrated that the mosaic lesions could be transmitted from an infected plant to a healthy plant by aqueous extracts derived from infected plants. The number of local necrotic lesions that result is directly proportional to the number of infectious particles in the preparation. Photo by J. P. Krausz, from *Lessons in Plant Pathology*, The American Phytopathological Society Education Center (http://www.apsnet.org/education/lessonsPlantPath/TMV/text/symptom.htm), reprinted with permission.

Delbrück was a zealot for phage research and recruited talented scientists to pursue the fundamental issues of what is now known as the field of molecular biology. This group of scientists, working together in what came to be called the "phage school," focused their attention on specific phages of the bacterium *Escherichia coli*. The list of Nobel laureates who were trained as phage workers is a testament to Delbrück's leadership. Progress was rapid, primarily because of the simplicity of the phage infectious cycle. Phages replicate in bacterial hosts, large numbers of which can easily be obtained by overnight culture. By the mid-1950s, it was evident that viruses from bacteria, animals, and plants share many fundamental properties. However, the phages provided a far more tractable experimental system. Consequently, their study had a profound impact on the development of virology.

One critical lesson came from the definitive experiments which established that viral nucleic acid carries genetic information. It was known from studies of the "transforming principle" of *Pneumococcus* by Oswald Avery, Colin MacLeod, and Maclyn McCarty (1944) that nucleic acid was both necessary and sufficient for the transfer of genetic traits of bacteria. However, in the early 1950s, viral protein was still suspected to be an important component of viral heredity. In a brilliantly simple experiment that included the use of a common kitchen item, a food blender, Alfred Hershey and Martha Chase showed that this hypothesis was incorrect (Box 1.4).

Bacteriophages were originally thought to be lethal agents, killing their host cells after infection. In the early 1920s, a previously unknown interaction was discovered, in which the host cell not only survived the infection but also stably inherited the genetic information of the virus. It was also observed that certain bacterial strains not known to be infected could lyse spontaneously and produce bacteriophages after a period of growth in culture. Such strains were called **lysogenic,** and the phenomenon was called **lysogeny**. Studies of lysogeny uncovered many previously unrecognized features of virus-host cell interactions. Recognition of this phenomenon resulted from the work of many scientists, but it began with the elegant experiments of André Lwoff and colleagues at the Institut Pasteur in Paris. Lwoff showed that a viral genome exists in lysogenic cells in the form of a specific genetic element called the **prophage**. This element determined the ability of lysogenic bacteria to produce infectious bacteriophage. Subsequent studies of the *E. coli* phage lambda established a paradigm for one of the many mechanisms of lysogeny, the integration of a phage genome into a specific site on the bacterial chromosome (Box 1.5).

Bacteriophages became inextricably associated with the new field of molecular biology (Table 1.1). Their study also established many fundamental principles. For example, control of the decision to enter a lysogenic or a lytic pathway is encoded in the genome of the virus. The first mechanisms discovered for the control of gene expression, exemplified by the elegant operon theory of Nobel laureates François Jacob and Jacques Monod, were deduced in part from studies of lysogeny by phage lambda. The biology of phage lambda provided a fertile ground for work on gene regulation, but study of virulent T phages (T1 to T7, where T stands for type) of *E. coli* paved the way for many other important advances (Table 1.1). As we shall see, these systems also provided an extensive preview of mechanisms of animal virus replication (Box 1.6).

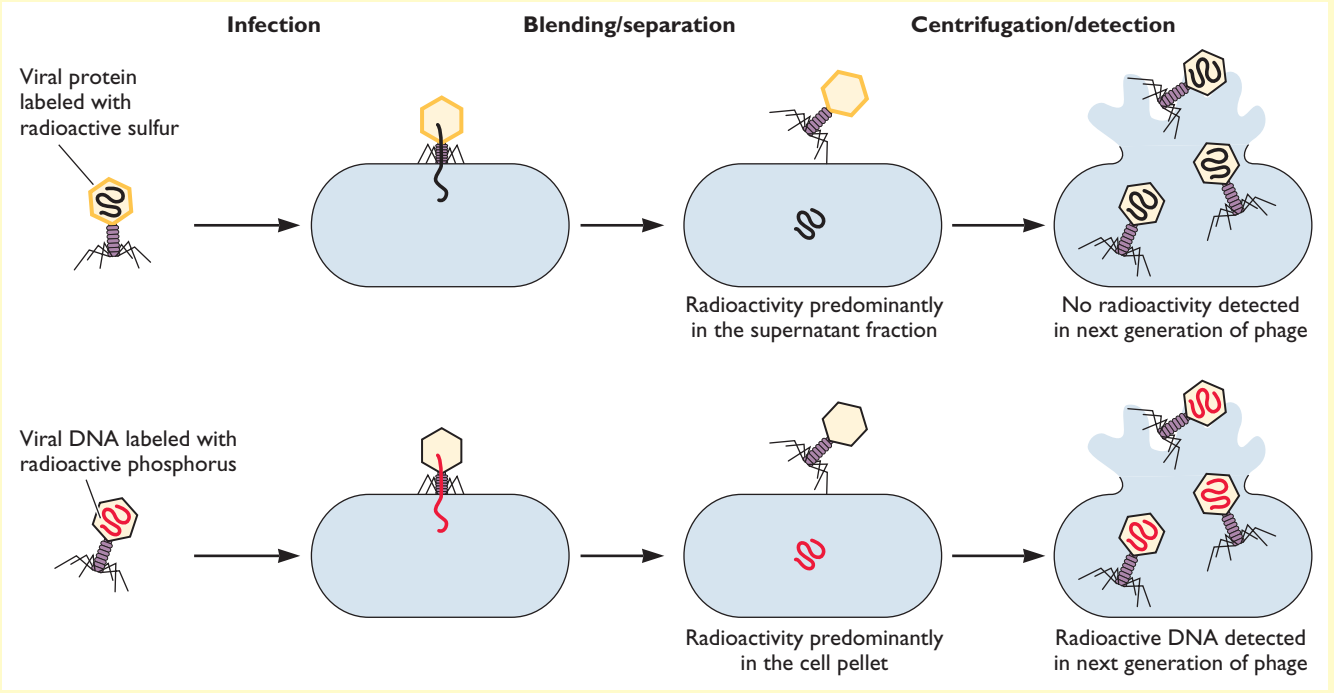
Animal Cells as Hosts

The culture of animal cells in the laboratory was initially more of an art than a science, restricted to cells that grew out of organs or tissues maintained in nutrient solutions under sterile conditions. The finite life span of such **primary cells**, their dependence for growth on natural media such as lymph, plasma, or chicken embryo extracts, and the technical demands of sterile culture prior to the discovery of antibiotics made reproducible experimentation very difficult. By 1955 the work of many investigators had led to a series of important methodological advances. These included the development of defined media optimal

BOX 1.4 EXPERIMENTS

The Hershey-Chase experiment

By differentially labeling the nucleic acid and protein components of virus particles with radioactive phosphorus (^{32}P) and radioactive sulfur (^{35}S), respectively, Alfred Hershey and Margaret Chase showed that the protein coat of the infecting virus could be removed soon after infection by agitating the bacteria for a few minutes in a blender. In contrast, ^{32}P-labeled phage DNA entered and remained associated with the bacterial cells. As such blended cells produced a normal burst of new virus particles, it was clear that this DNA contained all of the information necessary to produce progeny phages.



for growth of mammalian cells, incorporation of antibiotics into tissue culture media, and development of immortal cell lines such as the mouse L and human HeLa cells that are still in widespread use. These advances allowed growth of animal cells in culture to become a routine, reproducible exercise.

The availability of well-characterized cell cultures had several important consequences for virology. It allowed the discovery of several new human viruses, such as adenovirus, measles virus, and rubella virus, for which animal hosts were not available. In 1949, John Enders and colleagues used cell cultures to propagate poliovirus, a feat that led to the development of polio vaccines a few years later. Cell culture technology revolutionized the ability to investigate the replication of viruses. Viral infectious cycles could be studied under precisely controlled conditions by employing the analog of the one-step growth cycle of bacteriophages and simple methods for quantification of infectious particles described in Chapter 2. Our current understanding of the molecular basis of parasitism by viruses, the focus of this Volume (I), is based almost entirely on analyses of one-step growth cycles in cultured cells. Such studies established that viruses are **molecular** parasites: their reproduction depends absolutely on their host cell's biosynthetic machinery for synthesis of the components from which they are built. In contrast to cells, viruses do not reproduce by growth and division. Rather, the infecting genome contains information necessary to redirect cellular systems to the production of many copies of all the components needed for the

BOX 1.5 BACKGROUND

Studies of lysogeny established several general principles in virology

Lytic versus Lysogenic Response to Infection

Some bacterial viruses can enter into either destructive (lytic) or relatively benign (lysogenic) relationships with their host cells. Such bacteriophages were called "temperate." In a lysogenic bacterial cell, viral genetic information persists but viral gene expression is repressed. Such cells are called lysogens, and the quiescent viral genome is called a prophage.

Propagation as a Prophage

For some bacteriophages like lambda and Mu (Mu stands for mutator), prophage DNA is integrated into the host genome of lysogens and passively replicated by the host. Virally encoded enzymes, known as integrase (lambda) and transposase (Mu), mediate the covalent insertion of viral DNA into the chromosome of the host bacterium, establishing it as a prophage.

Transposition by Mu establishes an integrated prophage when viral gene expression is repressed, but it also leads to replication of the viral genome during the lytic cycle.

The prophage DNA of other bacteriophages, such as P1, exists as a plasmid, a self-replicating, autonomous chromosome in a lysogen. Both forms of propagation were subsequently identified in certain animal viruses.

Insertional Mutagenesis

Bacteriophage Mu inserts its genome into many random locations on the host chromosome, causing numerous mutations. This process is called insertional mutagenesis and is a phenomenon subsequently observed with retroviruses.

Gene Repression and Induction

Viral gene expression in lysogens is turned off by the action of viral proteins called repressors. Viral gene expression can be turned on when repressors are inactivated (a process called induction). Elucidation of the mechanisms involved set the stage for later investigation of the control of gene expression in experiments with other viruses and their host cells.

Transduction of Host Genes

Viral genomes can pick up cellular genes and deliver them to new cells (a process known as transduction). The process can be generalized, with the acquisition by the virus of any segment from the host chromosome, or specialized, as is the case for viruses that integrate into specific sites in the host chromosome. For example, occasional mistakes in excision of the lambda prophage after induction result in production of unusual progeny phage that have lost some of their own DNA but have acquired the bacterial DNA adjacent to the prophage. As described in Volume II, Chapter 7, the acute transforming retroviruses also arise via capture of proto-oncogenes in the vicinity of their integration as proviruses. These cancer-inducing cellular genes are then transduced along with viral genes during subsequent infection.

intrinsically programmed, *de novo* assembly of new virus particles.

Viruses Defined

Advances in knowledge of the structure of virus particles and the mechanisms by which viruses are reproduced in their host cells have been accompanied by increasingly accurate definitions of these unique agents. The earliest, pathogenic agents, distinguished by their small size and dependence on a host organism for reproduction, emphasized the importance of viruses as agents of disease. We can now provide a much more precise

BOX 1.6 TERMINOLOGY

The episome

In 1958, François Jacob and Elie Wollman realized that lambda prophage, the *E. coli* F sex factor, and the colicinogenic factor had many common genetic properties. This remarkable insight led to the definition of the episome.

An episome is an exogenous genetic element that is not necessary for cell survival. Its defining characteristic is the ability to reproduce in two alternative states, integrating into the host chromosome or by autonomous replication.

Nowadays this term is often applied to genomes that can be maintained in cells by autonomous replication and never integrate, for example, certain viral DNA genomes.

Jacob and Wollman immediately understood that the episome had value in understanding larger problems, including cancer, as revealed by this quotation: "… in the no man's land between heredity and infection, between physiology and pathology at the cellular level, episomes provide a new link and a new way of thinking about cellular genetics in bacteria, and perhaps in mice, men and elephants" (F. Jacob and E. Wollman, *Viruses and Genes: Readings from* Scientific American [W. H. Freeman & Co., New York, NY, 1961]).

BOX 1.7 WARNING

Viruses don't actually "do" anything!

Many have succumbed to the temptation of ascribing various actions and motives to viruses. While remarkably effective in enlivening a lecture or an article, anthropomorphic characterizations are inaccurate and often misleading.

Here are some examples from the anthropomorphic lexicon that should be avoided.

- Viruses cannot think, employ, ensure, synthesize, exhibit, display, destroy, deploy, depend, reprogram, avoid, retain, evade, exploit, generate, etc.
- Infected cells and hosts do many things in the presence of viruses, but viruses themselves are passive agents, totally at the mercy of their environments.

It is exceedingly difficult to purge such anthropomorphic terms from virology communications. Indeed, hours were spent doing so in preparation of this textbook. A good exercise to appreciate the difficulty of the problem may be to find all the examples that were missed.

definition of viruses, elaborating their relationship with the host cell and the important features of virus particles. The definitive properties of viruses are summarized as follows:

- A virus is an infectious, obligate intracellular parasite.
- The viral genome comprises either DNA or RNA.
- Within an appropriate host cell, the viral genome directs the synthesis by cellular systems of many copies of all the viral components.
- Progeny infectious virus particles, called **virions**, are formed by *de novo* self-assembly from the newly synthesized components within the host cell.
- A progeny virion assembled during the infectious cycle is the vehicle for transmission of the viral genome to the next host cell or organism, where disassembly of the virion leads to the beginning of the next infectious cycle.

With these properties in mind, we can accurately place viruses within the evolutionary continuum of biological agents. They are far simpler than even the smallest microorganisms and lack the complex energy-generating and biosynthetic systems necessary for independent existence (Box 1.7). On the other hand, viruses are **not** the simplest biologically active agents: even the smallest virus, built from a very limited genome and a single type of protein, is significantly more complex than other pathogens. Some of these minimalist molecular pathogens such as **viroids**, which are infectious agents of a variety of economically important plants, comprise a single small molecule of RNA. Others, termed **prions,** are thought to be single protein molecules.

Cataloging Animal Viruses

Around 1960, virus classification was a subject of colorful and quite heated controversy (Box 1.8). New viruses were being discovered and studied by electron microscopy. The virus world consisted of a veritable zoo of particles with different sizes, shapes, and compositions (see, for example, Fig. 1.10). Very strong opinions were advanced concerning classification and nomenclature, and opposing

BOX 1.8 TERMINOLOGY

Complexities of viral nomenclature

No consistent system for naming viruses has been established by their discoverers. For example, among the vertebrate viruses, some are named for the associated diseases (e.g., poliovirus, rabies virus), for the specific type of disease they cause (e.g., murine leukemia virus), or for the sites in the body that are affected or from which they were first isolated (e.g., rhinovirus and adenovirus). Others are named for the geographic locations in which they were first isolated (e.g., Sendai virus [Sendai, Japan] and Coxsackievirus [Coxsackie, New York]) or for the scientists who first discovered them (e.g., Epstein-Barr virus). In these cases the virus names are capitalized. Some viruses are even named for the way in which people imagined they were contracted (e.g., dengue, for "evil spirit," and influenza, for the "influence" of bad air). Finally, combinations of the above designations are also used (e.g., Rous sarcoma virus).

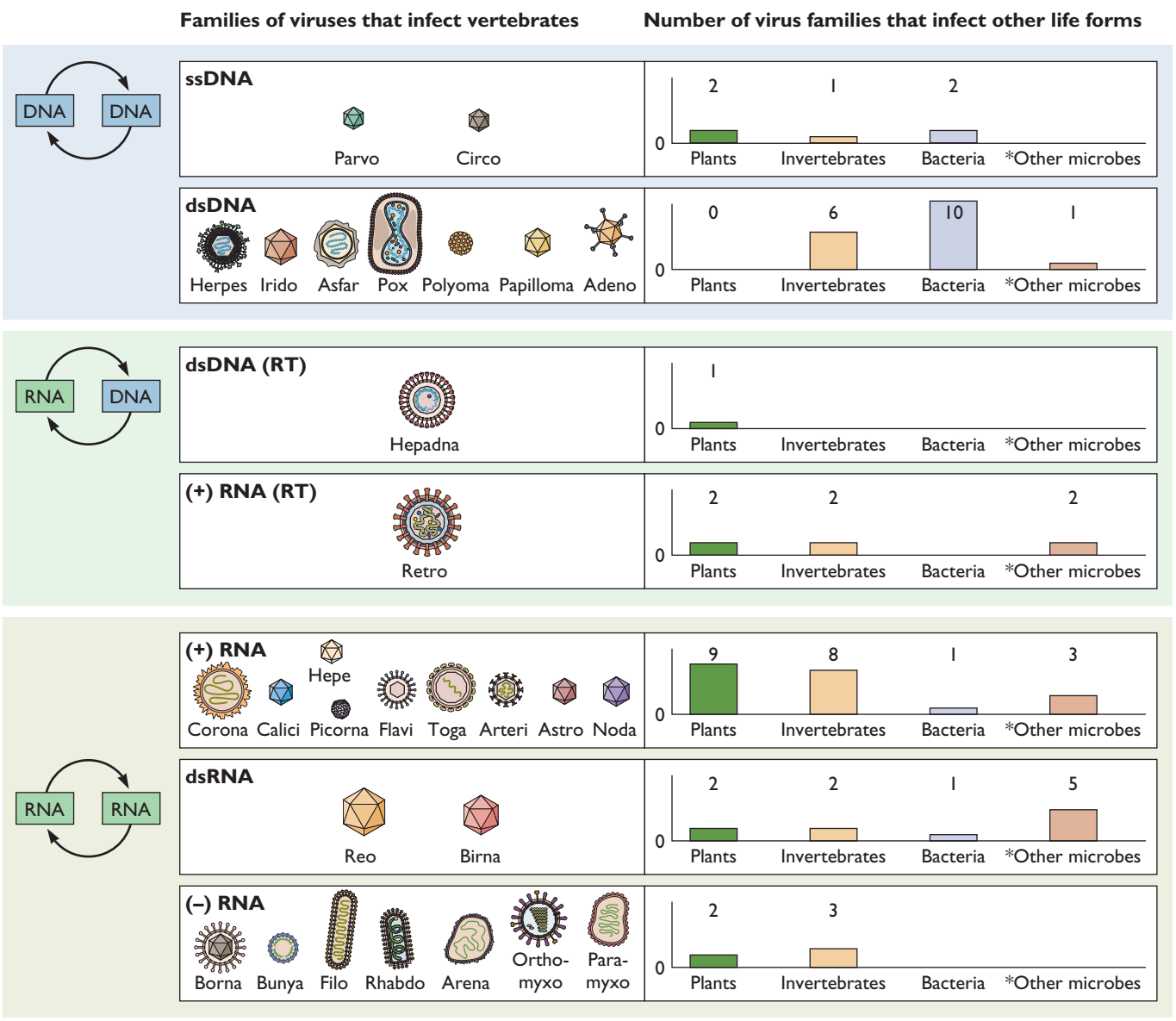


Figure 1.10 Viral families sorted according to the nature of the viral genomes. A wide variety of sizes and shapes are illustrated for the families of viruses that infect vertebrates. Similar diversity exists for the families of viruses that infect other life-forms, but the chart illustrates only the number found to date in each category. As noted, in some categories there are as yet no examples in some life-forms. Adapted from C. M. Fauquet et al. (ed.), *Virus Taxonomy: Classification and Nomenclature of Viruses*, Eighth Report of the International Committee on Taxonomy of Viruses (Academic Press, Inc., San Diego, CA, 2007).

camps developed, as in any controversy involving individuals who tend to focus on differences and those who look for similarities (conventionally known as "splitters" and "lumpers"). Splitters pointed to the inability to infer, from the known properties of viruses, anything about their evolutionary origin or their relationships to one another—the major goal of classical taxonomy. Lumpers maintained that despite such limitations, there were significant practical advantages in grouping isolates with similar properties. Furthermore, it seemed likely that a good classification might actually stimulate fruitful investigation. A major sticking point, however, was

finding agreement on **the** properties that should be considered most important in constructing a scheme for virus classification.

The Classical System

In 1962, Lwoff, Robert Horne, and Paul Tournier advanced a comprehensive scheme for the classification of all viruses (bacterial, plant, and animal) under the classical Linnaean hierarchical system consisting of phylum, class, order, family, genus, and species. Although a subsequently formed international committee on the nomenclature of viruses did not adopt this system *in toto*, its designation of families, genera, and species was used for the classification of animal viruses.

One of the most important principles embodied in the system advanced by Lwoff and his colleagues was that viruses should be grouped according to **their** shared properties rather than the properties of the cells or organisms they infect. A second principle was a focus on the nucleic acid genome as the primary criterion for classification. The importance of the genome had become clear when it was inferred from the Hershey-Chase experiment that viral nucleic acid alone can be infectious (Box 1.4). Four characteristics were to be used in the classification of all viruses:

1. Nature of the nucleic acid in the virion (DNA or RNA)
2. Symmetry of the protein shell (**capsid**)
3. Presence or absence of a lipid membrane (**envelope**)
4. Dimensions of the virion and capsid

Genomics, the elucidation of evolutionary relationships by analyses of nucleic acid and protein sequence similarities, is being used increasingly to assign viruses to a particular family and to order members within a family. For example, human herpesvirus 8 was placed in the subfamily *Gammaherpesvirinae* on the basis of sequence analysis of just a small segment of its DNA. Similarly, hepatitis C virus was classified as a member of the family *Flaviviridae* from the sequence of a cloned DNA copy of its genome. However, as our knowledge of molecular properties of viruses and their replication has increased, it has become apparent that **any** comparison based on one or two criteria can be somewhat misleading. For example, *Hepadnaviridae*, *Retroviridae*, and some plant viruses are classified as different families on the basis of the nature of their genomes, but they are all related by the fact that reverse transcription is an essential step in their reproductive cycles. Moreover, the viral polymerases that perform this task exhibit important similarities in amino acid sequence.

As of the latest report (2005) of the International Committee on Taxonomy of Viruses (ICTV), approximately 40,000 virus isolates from bacteria, plants, and animals had been assigned to one of 3 orders, 73 families, 9 subfamilies, 287 genera, and 1,950 species. Many viruses remain unassigned because they have not yet been characterized adequately, and others are assigned only provisionally. It seems likely that a significant fraction of all existing virus families are now known. However, as we learn more and more about genes, proteins, and reproduction strategies, other relationships will certainly be revealed. Classification refinements can therefore be expected to continue in the future. The ICTV report also includes descriptions of subviral agents (**satellites**, viroids, and prions) and a list of viruses for which information is still insufficient to make assignments. Satellites are composed of nucleic acid molecules that depend for their multiplication on coinfection of a host cell with a helper virus. However, they are not related to this helper. When a satellite encodes the coat protein in which its nucleic acid is encapsidated, it is referred to as a **satellite virus** (e.g., hepatitis delta virus is a satellite virus).

Several years ago, the bacterial virologists who were members of the ICTV agreed to coin similar Latinized family names for the different types of bacteriophages. However, this nomenclature never really took hold, and it has not been widely used by those who do research with bacteriophages. Plant virologists do not classify their viruses into families and genera. Instead, they use group names derived from the prototype virus of each group. For animal viruses, however, the ICTV nomenclature has been applied widely in both the scientific and medical literature, and therefore we adopt it in this text. In this nomenclature, the Latinized virus family names are recognized as starting with capital letters and ending with *-viridae*, as, for example, in the family name *Parvoviridae*. These names are used interchangeably with their common derivatives, as, for example, parvoviruses.

Classification by Genome Type

Because the viral genome carries the entire blueprint for virus propagation, molecular virologists have long considered it the most important characteristic for classification purposes. Therefore, although individual virus families are known by their classical designations, they are more commonly placed in groups according to their genome types, as illustrated in Fig. 1.10. There are seven genome types for all families of viruses, and all seven are represented in viruses that infect vertebrates, although single-stranded RNA genomes are the most numerous. One or more of these types appear to be missing among viruses that infect other life-forms, and the general distributions can vary. For example, double-stranded DNA genomes are most

numerous among the bacterial viruses, but there are as yet no known plant viruses with such genomes.

The Baltimore Classification System

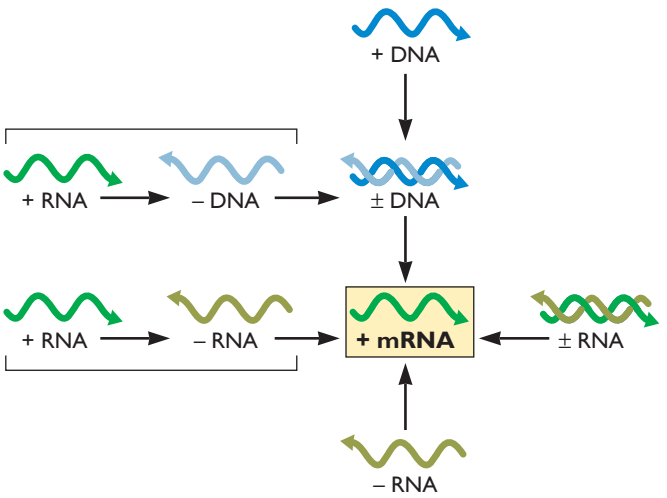
The past three decades have seen an enormous increase in knowledge of the molecular biology of animal viruses and cells. Among the most significant advances has been the elucidation of pathways by which viral genomes are expressed. We know that cellular genes are encoded in double-stranded nuclear DNA and that this genetic information is expressed via single-stranded mRNAs. These mRNAs, which are made in the nucleus, are transported to the cytoplasm, where they are translated by ribosomes and associated machinery. This is the so-called central dogma conceptualized by Francis Crick:

$$\text{DNA} \rightarrow \text{RNA} \rightarrow \text{protein}$$

Because viral protein synthesis is completely dependent on the cell's translational machinery, all viruses must direct the synthesis of mRNA to produce proteins. Appreciation of the central role of the translational machinery and of the importance of mRNA molecules in the programming of viral protein synthesis inspired an alternative classification scheme devised by David Baltimore (Fig. 1.11). This classification highlights the obligatory relationship between the viral genome and its mRNA and describes the pathways for formation of mRNA that must be followed by viruses with either RNA or DNA genomes.

By the molecular biologist's convention, mRNA is defined as a **positive [(+)] strand** because it contains immediately translatable information. In the Baltimore classification, a strand of DNA that is of equivalent sequence is also designated a (+) strand. The RNA and DNA complements of (+) strands are designated **negative [(–)] strands**. The principles embodied in this classification have proved to be extremely valuable, especially for viruses with single-stranded RNA genomes. Knowledge of strand polarity provides virologists with immediate insight into the steps that must take place to initiate replication and expression of the viral genome.

Figure 1.11 The Baltimore classification. All viruses must produce mRNA that can be translated by cellular ribosomes. In this classification system, the unique pathways from various viral genomes to mRNA define specific virus classes on the basis of the nature and polarity of their genomes.



A Common Strategy for Viral Propagation

The basic thesis of this textbook is that **all** viral propagation can be described in the context of three fundamental properties.

- All viral genomes are packaged inside particles that mediate their transmission from host to host.
- The viral genome contains the information for initiating and completing an infectious cycle within a susceptible, permissive cell. An infectious cycle includes attachment and entry of the particle, decoding of genome information, translation of viral mRNA by host ribosomes, genome replication, and assembly and release of particles containing the genome.
- All successful viruses are able to establish themselves in a host population so that virus survival is ensured.

Modern virology is both fascinating and challenging, because of the many and varied ways this strategy is executed. Furthermore, as viruses are obligate molecular parasites, every tactical solution must of necessity tell us something about the host as well as the virus. The intellectual satisfaction of discovering and understanding new principles is as rewarding as the practical consequences of providing solutions to problems of disease.

Perspectives

The viral lifestyle is unique, and understanding virus biology has generated a new appreciation of the molecular biology of host cells and organisms. Some of the important landmarks in animal virology are summarized in Figure 1.12. We have learned that viruses are not primitive entities but, rather, are highly evolved molecular parasitic systems that are subject to checks and

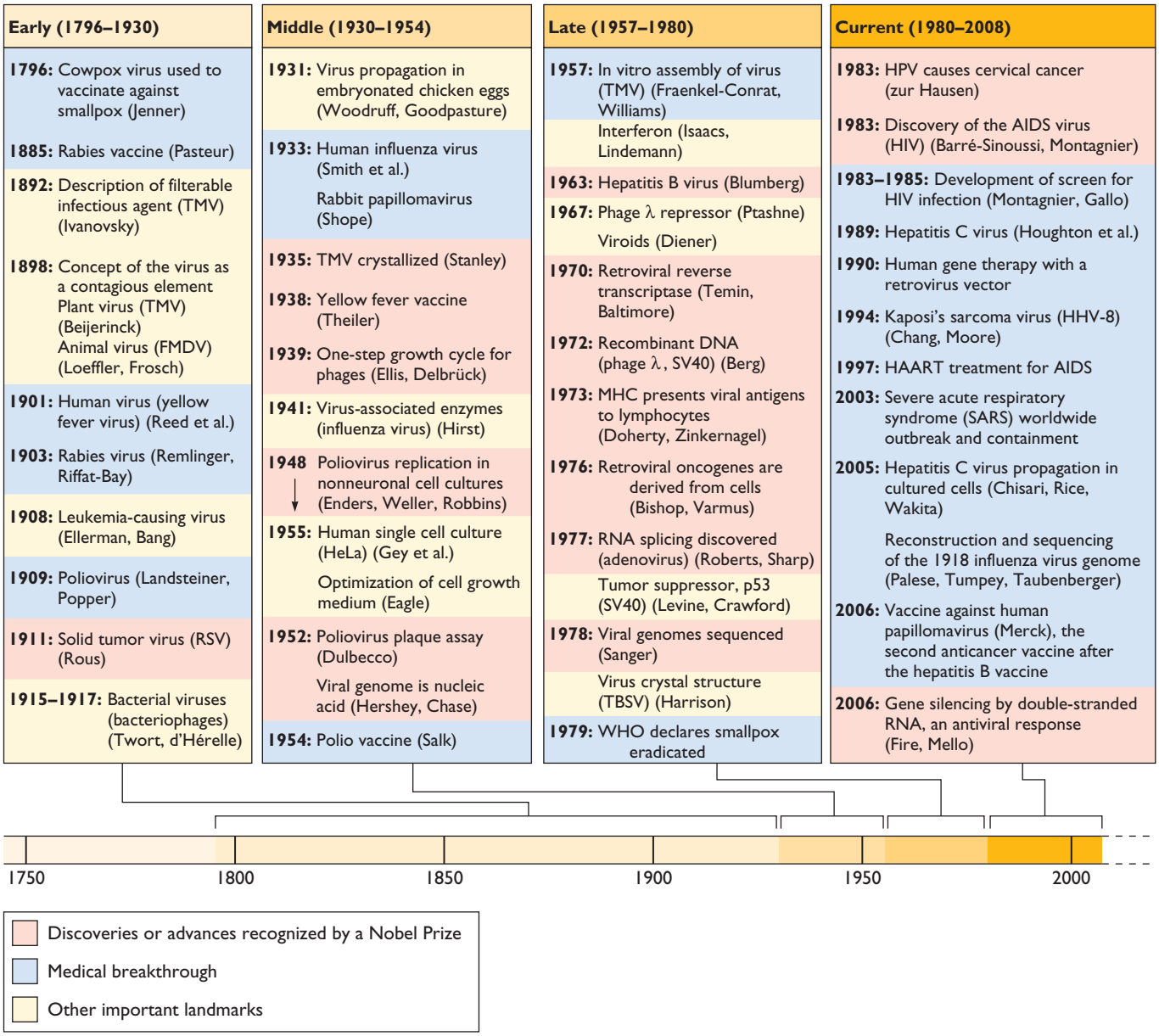


Figure 1.12 Landmarks in the study of animal viruses. Key discoveries and technical advances are listed for each time interval. The pace of discovery has increased exponentially over time. Abbreviations: HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; TBSV, tomato bushy stunt virus; TMV, tobacco mosaic virus; SV40, simian virus 40; FMDV, foot-and-mouth disease virus; WHO, World Health Organization; MHC, major histocompatibility complex; HHV-8, human herpesvirus 8; RSV, Rous sarcoma virus.

balances and selected for survival. For example, if viruses are too successful (virulent) and kill all of their hosts, they face annihilation, but if they are too passive they can also be eliminated. Much has been discovered about the biology of viruses and about host defenses against them. Yet the more we learn, the more we appreciate that much is still unknown. It is our conviction that the fundamental principles elaborated in this volume, and its companion Volume II, will serve as a guide to virologists of the future.

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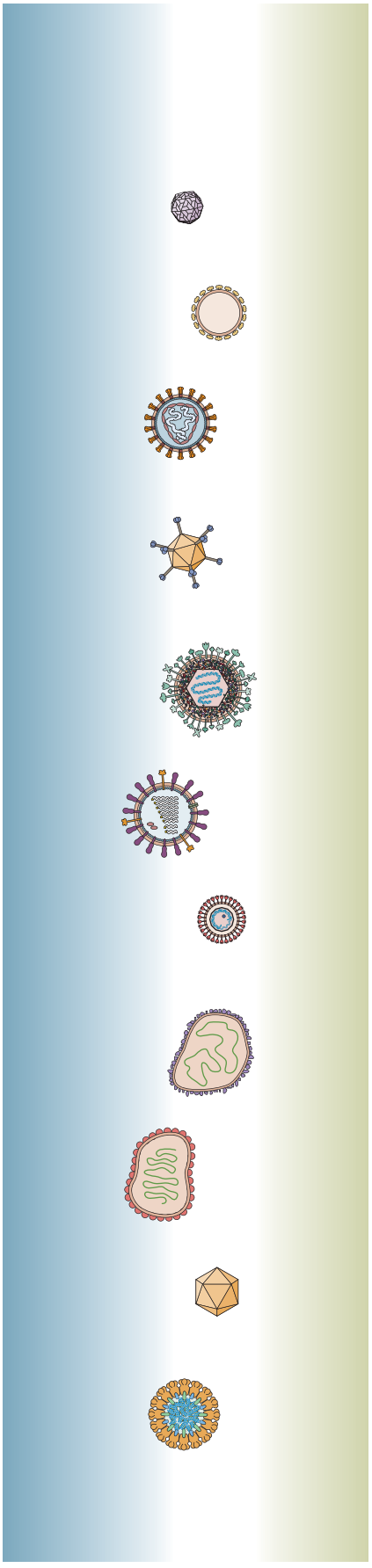
http://www.ncbi.nlm.nih.gov/ICTVdb/ *ICTV-approved virus names and other information as well as links to virus databases.*

http://www.tulane.edu/~dmsander/garryfavweb.html *Has links to almost all the virology sites on the Web.*



2

The Infectious Cycle

You know my methods, Watson.
SIR ARTHUR CONAN DOYLE

Introduction

Viruses are unique: they are exceedingly small, often made up of nothing more than a nucleic acid molecule within a protein shell, yet when they enter cells, they parasitize the cellular machinery to produce thousands of progeny. This simplicity is misleading: viruses can infect all known life forms, and they comprise a variety of structures and genomes. Despite this complexity, viruses are amenable to study because all viral propagation can be described in the context of three fundamental properties, as described in Chapter 1: all viral genomes are packaged inside particles that mediate their transmission from cell to cell; the viral genome contains the information for initiating and completing an infectious cycle; and all viruses can establish themselves in a host population to ensure virus survival.

The objective of research in virology is to understand how viruses enter individual cells, replicate, and assemble new infectious particles. These studies are usually carried out with cell cultures rather than with animals, because cell cultures provide a much simpler and more homogeneous experimental system. Cell cultures can be infected in such a way as to ensure that a single replication cycle occurs synchronously in every infected cell, the **one-step growth curve**. Because all viral infections take place within the cell, a full understanding of viral life cycles also requires knowledge of cell biology and cellular architecture. In this chapter we review the cell surface (the site at which viruses enter and exit cells), methods for quantifying viral growth, and one-step growth analysis.

The Infectious Cycle

Viruses cannot reproduce extracellularly; the production of new infectious viruses takes place within a cell (Fig. 2.1). Virologists divide the viral infectious cycle into discrete steps to facilitate their study, although in virus-infected cells no such artificial boundaries occur. The infectious cycle comprises attachment and entry of the particle, translation of viral mRNA by host ribosomes, genome

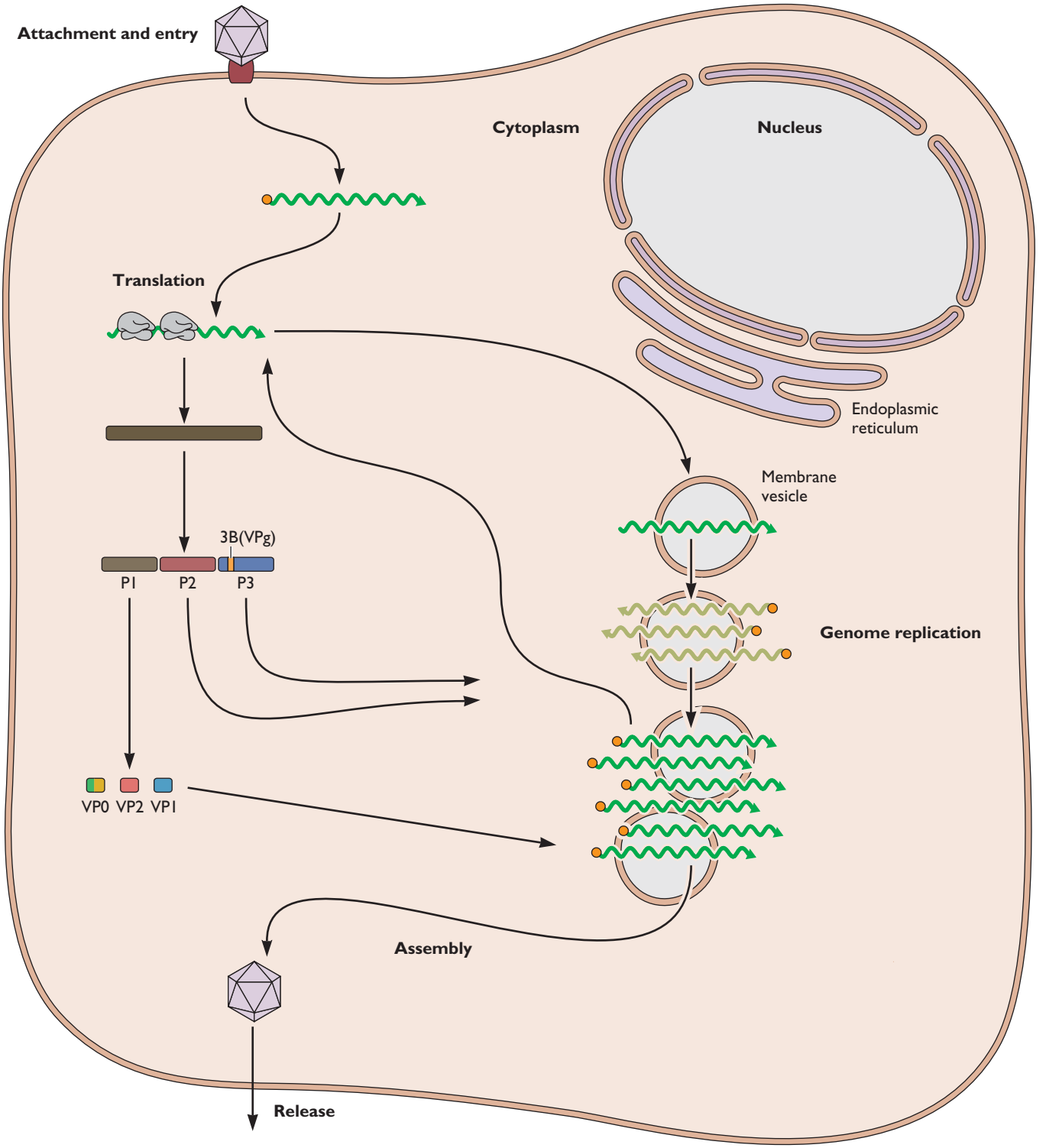


Figure 2.1 The viral infectious cycle. The infectious cycle of poliovirus is shown as an example, illustrating the steps common to all viral life cycles: attachment and entry, translation, genome replication, assembly, and release.

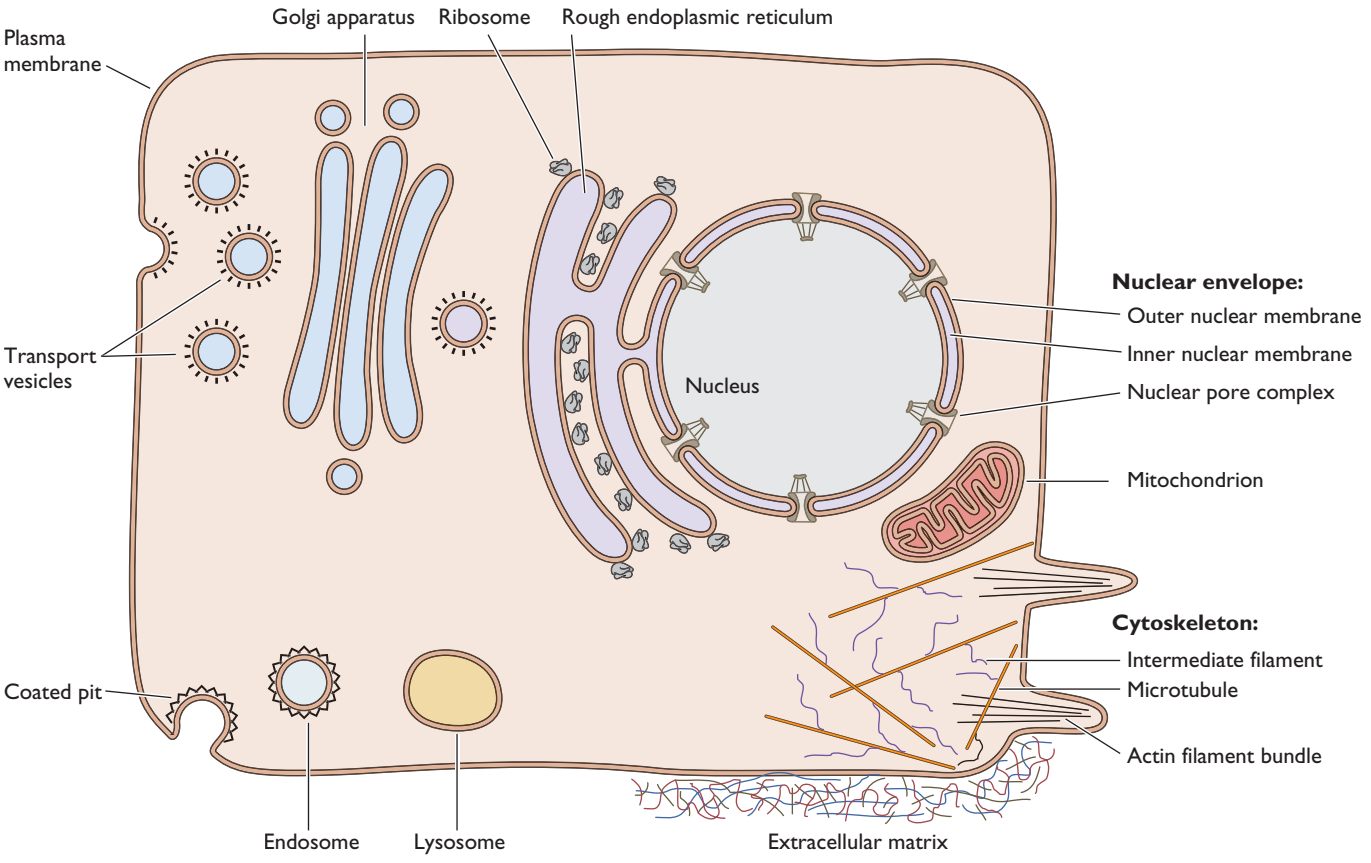
replication, and assembly and release of particles containing the genome. New virus particles produced during the infectious cycle may then infect new cells. The term **virus replication** is another name for the sum total of all events that occur during the infectious cycle.

Because all viral infections begin in a single cell, the virologist can use cultured cells to study stages of the infectious cycle. There are events common to virus replication in animals and in cultured cells, but there are also many important differences. While viruses readily attach to cells in culture, in nature a virus particle must encounter a host, no mean feat for nanoparticles without any means of locomotion. After encountering a host, the virus particle must pass through physical host defenses, such as dead skin, mucous layers, and the extracellular matrix. Host defenses such as antibodies and immune cells, which exist to combat virus infections, are not found in cultured cells. Virus infection of cultured cells has been a valuable tool for understanding viral life cycles, but the differences compared with infection of a living animal must always be considered.

The Cell

Viruses require many different functions of the host cell (Fig. 2.2) for propagation. Cells provide the machinery for translation of viral mRNAs, sources of energy, enzymes for genome replication, and sites of nucleic acid replication and viral assembly. The cellular transport apparatus brings viral genomes to the correct cellular compartment and ensures that viral subunits reach locations where they may be assembled into virus particles. Subsequent chapters include a discussion of cellular functions that are important for individual steps in the viral replication cycle. In the following section we consider in detail the architecture of cell surfaces. The cell membrane merits this special focus because it is not only the portal of entry for all animal viruses, but also the site from which many viruses leave the cell.

Figure 2.2 The mammalian cell. Illustrated schematically are the nucleus and major membrane-bound compartments of the cytoplasm, and components of the cytoskeleton that play important roles in virus replication. The figure is not drawn to scale.



The Architecture of Cell Surfaces

In animals, viral infections usually begin at the epithelial surfaces of the body that are exposed to the environment (Fig. 2.3). Cells cover these surfaces, and the part of these cells exposed to the environment is called the **apical surface**. Conversely, the **basolateral surfaces** of such cells are in contact with adjacent or underlying cells or tissues. These cells exhibit a differential (polar) distribution of proteins and lipids in the plasma membranes that creates the two distinct surface domains. As illustrated in Fig. 2.3, these cell layers differ in thickness and organization. Movement of macromolecules between the cells in the epithelium is prevented by **tight junctions**, which circumscribe the cells at the apical edges of their lateral membranes. Many viral infections are initiated upon entry into epithelial or endothelial cells at their exposed apical surfaces, often by attaching to cell surface molecules specific for this domain. Viruses that both enter and are released at apical membranes can be transmitted laterally from cell to cell without ever transversing the epithelial or endothelial layers; they generally cause localized infections. In other cases, progeny virions are transported to the basolateral surface and released into the underlying cells and tissues, a process that facilitates viral spread to other sites of replication.

There are also more specialized pathways by which viruses reach susceptible cells. For example, some epithelial tissues contain M cells, specialized cells that overlie the collections of lymphoid cells in the gut known as Peyer's patches. M cells function in the transport of intestinal contents to Peyer's patches. Certain viruses, such as poliovirus and human immunodeficiency virus type 1, can also be transported through them by a mechanism called **transcytosis** to gain access to underlying tissues. Such specialized pathways of invasion are considered in Volume II, Chapter 1. Below we describe briefly the structures that surround cells and tissues, as well as the membrane components that mediate the interaction of cells with their environments.

The Extracellular Matrix: Components and Biological Importance

Extracellular matrices hold the cells and tissues of the body together. The extracellular matrix is made up of two main classes of macromolecules (Fig. 2.4). The first class comprises glycosaminoglycans (such as heparan sulfate and chondroitin sulfate), which are unbranched polysaccharides made of repeating disaccharides. Glycosaminoglycans are usually linked to proteins to form **proteoglycans**. The second class of macromolecules in the extracellular matrix consists of fibrous proteins with structural **(collagen** and **elastin)** or adhesive **(fibronectin** and **laminin)**

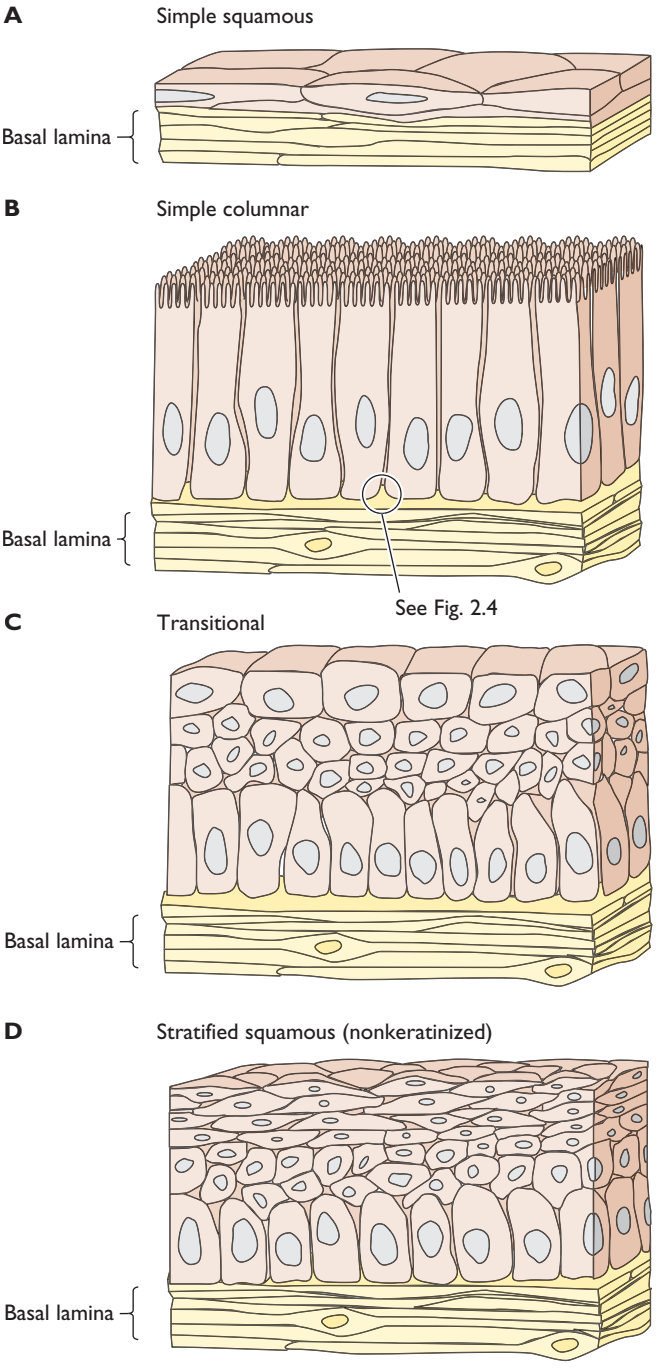


Figure 2.3 Major types of epithelia. (A) Simple squamous epithelium made up of thin cells such as those lining blood vessels and many body cavities. **(B)** Simple columnar epithelium found in the stomach, cervical tract, and small intestine. **(C)** Transitional epithelium, which lines cavities, such as the urinary bladder, that are subject to expansion and contraction. **(D)** Stratified, nonkeratinized epithelium lining surfaces such as the mouth and vagina. Adapted from H. Lodish et al., *Molecular Cell Biology,* 3rd ed. (W. H. Freeman & Co., New York, NY, 1995), with permission.

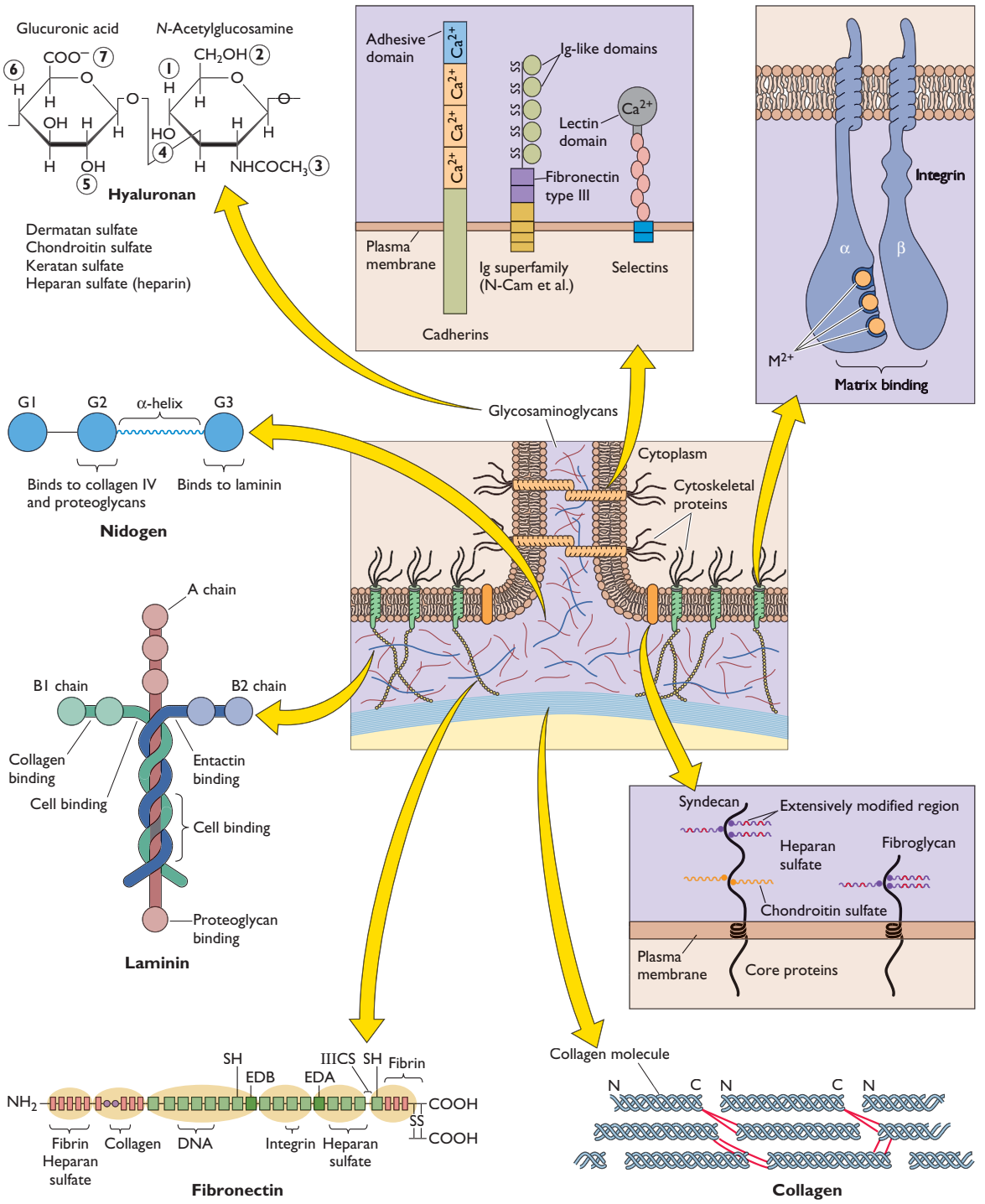


Figure 2.4 Cell adhesion molecules and components of the extracellular matrix. The diagram in the center (expanded from Fig. 2.3B) illustrates the variety of cell surface components that contribute to cell-cell adhesion and attachment to the extracellular matrix. Different glycosaminoglycans such as dermatan sulfate and chondroitin sulfate are produced by covalent linkage of SO_3 at the numbered positions on the hyaluronan molecule. Ig, immunoglobulin; M^{2+}, divalent cation. Adapted from H. Lodish et al., *Molecular Cell Biology,* 3rd ed. (W. H. Freeman & Co., New York, NY, 1995), and G. M. Cooper, *The Cell: a Molecular Approach* (ASM Press, Washington, DC, and Sinauer Associates, Sunderland, MA, 1997), with permission.

functions. The proteoglycan molecules in the matrix form hydrated gels in which the fibrous proteins are embedded, providing strength and resilience to the matrix. The gel provides resistance to compression and allows the diffusion of nutrients between blood and tissue cells. The extracellular matrix of each cell type is specialized for the particular function required, varying in thickness, strength, elasticity, and degree of adhesion.

Most organized groups of cells, like epithelial cells of the skin (Fig. 2.3 and 2.5), are bound tightly on their basal surface to a thin layer of extracellular matrix called the **basal lamina**. This matrix is linked to the basolateral membrane by specific receptor proteins called **integrins** (which are discussed in "Cell Membrane Proteins" below). Integrins are anchored to the intracellular structural network (the **cytoskeleton**) at the inner surface of the cell membrane. The basal lamina is attached to collagen and other material in the underlying loose connective tissue found in many organs of the body (Fig. 2.5). Capillaries, glands, and specialized cells are embedded in this connective tissue. Several viruses gain access to susceptible cells by attaching specifically to components of the extracellular matrix, including some cell adhesion proteins or proteoglycans.

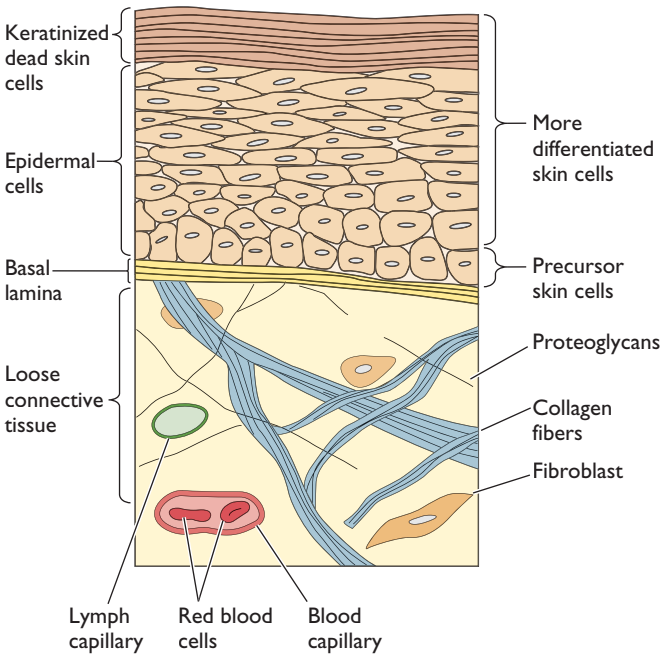


Figure 2.5 Cross section through skin. In this diagram of skin from a pig, the precursor epidermal cells rest on a thin layer of extracellular matrix called the basal lamina. Underneath is loose connective tissue consisting mostly of extracellular matrix. Fibroblasts in the connective tissue synthesize the connective tissue proteins, hyaluronan, and proteoglycans. Blood and lymph capillaries are also located in the loose connective tissue layer. Adapted from H. Lodish et al., *Molecular Cell Biology,* 3rd ed. (W. H. Freeman & Co., New York, NY, 1995), with permission.

Properties of the Plasma Membrane

The plasma membrane of every cell type is composed of a similar phospholipid/glycolipid bilayer, but different sets of membrane proteins and lipids allow the cells of different tissues to carry out their specialized functions. The lipid bilayer consists of molecules that possess both hydrophilic and hydrophobic portions; they are known as **amphipathic** molecules, from the Greek word *amphi* (meaning "on both sides") (Fig. 2.6). They form a sheetlike structure in which polar head groups face the aqueous environment of the cell's cytoplasm (inner surface) or the surrounding environment (outer surface). The polar head groups of the inner and outer leaflets bear side chains with different lipid compositions. The fatty acyl side chains form a continuous hydrophobic interior about 3 nm thick. Hydrophobic interactions are the driving force for formation of the bilayer. However, hydrogen bonding and electrostatic interactions among the polar groups and water molecules or membrane proteins also stabilize the structure.

Thermal energy permits the phospholipid and glycolipid molecules comprising natural cell membranes to rotate freely around their long axes and diffuse laterally. If unencumbered, a lipid molecule can diffuse the length of an animal cell in only 20 s at 37°C. In most cases, phospholipids and glycolipids do not flip-flop from one side of a bilayer to the other, and the outer and inner leaflets of the bilayer remain separate. Similarly, membrane proteins not anchored to the extracellular matrix and/or the underlying structural network of the cell can diffuse rapidly, moving laterally like icebergs in this fluid bilayer. In this way, certain membrane proteins can form functional aggregates. Intracellular organelles such as the nucleus, endoplasmic reticulum, and lysosomes are also enclosed in lipid bilayers, although their composition and physical properties differ.

For many years the plasma membrane was viewed as a uniform and fluid sea, in which lipid and protein components diffused randomly in the plane of the membrane. This simplistic model has been dispelled by experimental findings during the past 15 years. It is now recognized that plasma membranes comprise **microdomains**, regions with distinct lipid and protein composition (Box 2.1). The **lipid raft** is one type of microdomain that is important for virus replication. Lipid rafts are enriched in cholesterol and saturated fatty acids and consequently

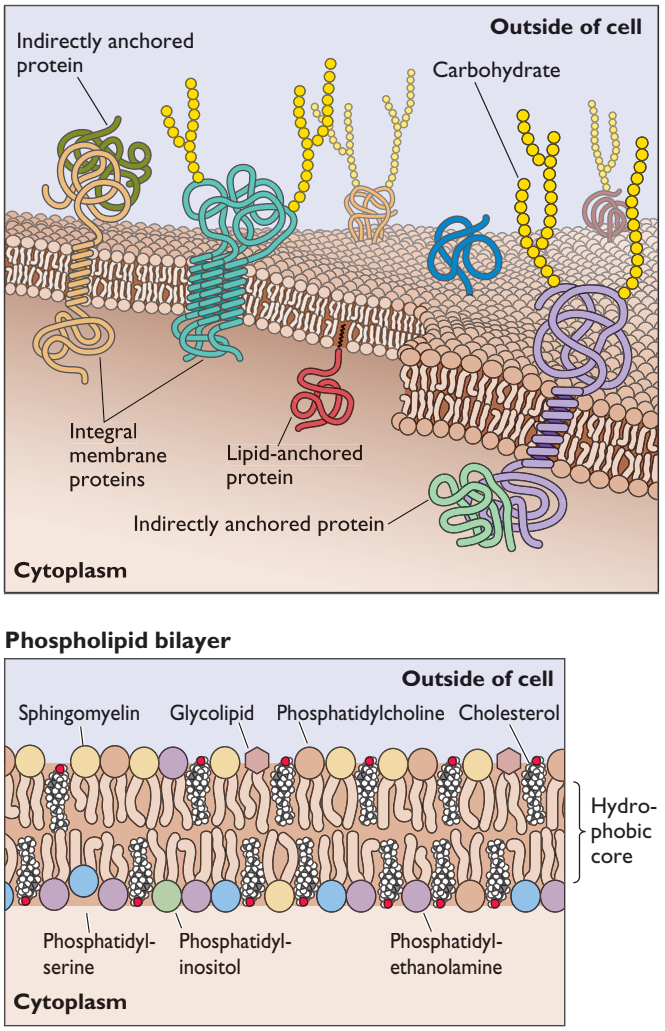


Figure 2.6 The plasma membrane. (Top) Different types of membrane proteins are illustrated. Some integral membrane proteins are transmembrane proteins and are exposed on both sides of the bilayer. **(Bottom)** Lipid components of the plasma membrane. The membrane consists of two layers (leaflets) of phospholipid and glycolipid molecules. Their fatty acid tails converge to form the hydrophobic interior of the bilayer; the polar hydrophilic head groups (shown as balls) line both surfaces. Adapted from G. M. Cooper, *The Cell: a Molecular Approach* (ASM Press, Washington, DC, and Sinauer Associates, Sunderland, MA, 1997), with permission.

are more densely packed and less fluid than other regions of the membrane. The assembly of a variety of viruses takes place at lipid rafts (see Chapter 13). Furthermore, the entry of some viruses requires lipid rafts. For example, virions of human immunodeficiency virus type 1 and Ebola virus enter cells at lipid rafts. Treatment of cells with compounds that disrupt these microdomains blocks entry. One explanation for this requirement might be that cell membrane proteins required for entry are present only in lipid rafts: receptors and coreceptors for human immunodeficiency virus are preferentially located in these domains.

Cell Membrane Proteins

Membrane proteins are classified into two broad categories, **integral membrane proteins** and **indirectly anchored proteins**, names that describe the nature of their interactions with the plasma membrane (Fig. 2.6).

Integral membrane proteins are embedded in the lipid bilayer, because they contain one or more **membrane-spanning domains**, as well as portions that protrude out into the exterior and interior of the cell (Fig. 2.6). Many membrane-spanning domains consist of an α-helix typically 3.7 nm long. It includes 20 to 25 generally hydrophobic or uncharged residues embedded in the membrane, with the hydrophobic side chains protruding outward to interact with the fatty acyl chains of the lipid bilayer. The first and last residues are often positively charged amino acids (lysine or arginine) that can interact with the negatively charged polar head groups of the phospho- or glycolipids to stabilize the membrane-spanning domain. Proteins with membrane spanning domains enable the cell to respond to signals from outside the cell. Such membrane proteins are designed to bind external ligands (e.g., hormones, cytokines, or membrane proteins on the same cell or on other cells) and to signal the occurrence of such interactions to molecules in the interior of the cell. Some proteins with multiple membrane-spanning domains (Fig. 2.6) form critical components of molecular pores or pumps, which mediate the internalization of required nutrients or the expulsion of undesirable material from the cell, or maintain homeostasis with respect to cell volume, pH, and ion concentration.

In many cases, the external portions of membrane proteins are decorated by complex or branched **carbohydrate chains** linked to the peptide backbone. Linkage can be either through nitrogen (**N linked**) in the side chain of asparagine residues or through oxygen (**O linked**) in the side chains of serine or threonine residues. Such membrane **glycoproteins**, as they are called, quite frequently serve as viral receptors. Many viruses attach specifically to one or more of the external components of glycoproteins.

Some membrane proteins do not span the lipid bilayer but are anchored in the inner or outer leaflet by covalently attached hydrocarbon chains (see Chapter 12). Indirectly anchored proteins are bound to the plasma membrane lipid bilayer by interacting either with integral membrane

BOX 2.1 BACKGROUND

Plasma membrane microdomains

According to the Singer-Nicholson fluid mosaic model of membrane structure proposed in 1972, membranes are two-dimensional fluids with proteins inserted into the lipid bilayers (Fig. 2.6). Although the model accurately predicts the general organization of membranes, one of its conclusions has proven incorrect: that proteins and lipids are randomly distributed because they can freely rotate and laterally diffuse within the plane of the membrane. Beginning in the 1990s, the results of a series of experiments indicated that the movement of most proteins in the plasma membrane is partially restricted. In particular, these studies provided evidence for the existence of plasma membrane microdomains that are enriched in glycosphingolipids, cholesterol, glycosylphosphatidylinositol-anchored proteins, and certain intracellular signaling proteins. These microdomains, called lipid rafts, are experimentally defined as being resistant to extraction in cold 1% Triton X-100 and floating in the top half of a 5 to 30% sucrose density gradient. A major component of lipid rafts was found to be caveolin-1, a major coat protein of caveolae. These flask-shaped invaginations of the plasma membrane are involved in the uptake of sphingolipids and integrins, as well as viruses, bacteria, and toxins. Detergent-insoluble microdomains are also present in cells that lack caveolin-1, and it is now known that there are many noncaveolar lipid raft domains in the plasma membrane.

proteins or with the charged sugars of the glycolipids within the membrane lipid. Fibronectin, a protein in the extracellular matrix that binds to integrins (Fig. 2.4), is an example.

Entering Cells

Viral infection is initiated by a collision between the virus particle and the cell, a process that is governed by chance. Therefore, a higher concentration of virus particles increases the probability of infection. However, a virion may not infect every cell it encounters; it must come in contact with the cells and tissues in which it can replicate. Such cells are normally recognized by means of a specific interaction of a virion with a cell surface receptor. This process can be either promiscuous or highly selective, depending on the virus and the distribution of the cell receptor. The presence of such receptors determines whether the cell will be **susceptible** to the virus. However, whether a cell is **permissive** for the replication of a particular virus depends on other, intracellular components found only in certain cell types. Cells must be both susceptible **and** permissive if an infection is to be successful.

In general, viruses have no means of locomotion, but their small size facilitates diffusion driven by Brownian movement. Propagation of viruses is dependent on essentially random encounters with potential hosts and host cells. In this sense, they can be thought of as tiny, opportunistic "Darwinian machines." For such machines, features that increase the probability of favorable encounters are very important. In particular, viral propagation is critically dependent on the production of large numbers of progeny virions with surfaces composed of many copies of structures that enable the virions to attach to susceptible cells.

Successful entry of a virus into a host cell requires that the virus cross the plasma membrane and in some cases the nuclear membrane. The virus particle must also disassemble to make the viral genome accessible in the cytoplasm, and the nucleic acid must be targeted to the correct cellular compartment. These are not simple processes. Cell membranes are not permeable to virus particles. Virions or critical subassemblies are brought across such barriers by specific transport pathways. To survive in the extracellular environment, the viral genome must be encapsidated in a protective coat that shields viral nucleic acid from the variety of potentially harsh conditions that a virion may meet in the environment. For example, mechanical shearing, ultraviolet (UV) irradiation (from sunlight), extremes of pH (in the gastrointestinal tract), dehydration (in the air), and enzymatic attack (in body fluids) are all capable of damaging viral nucleic acids. However, once in the host cell, the protective structures must become sufficiently unstable to release the genome. Virus particles cannot be viewed only as passive vehicles: they must be able to undergo structural transformations that are important for attachment and entry into a new host cell and for the subsequent disassembly required for viral replication.

Making Viral RNA

Although the genomes of viruses come in a number of configurations, they share a common requirement: they must be efficiently copied into progeny genomes for assembly and mRNAs for the synthesis of viral proteins. The synthesis of RNA molecules by RNA viruses is a unique process

that has no counterpart in the cell. With the exception of retroviruses, all RNA viruses encode an RNA-dependent RNA polymerase to catalyze the synthesis of mRNAs and genomes. For the majority of DNA viruses and retroviruses, synthesis of mRNA is accomplished by the cellular enzyme that produces mRNA, RNA polymerase II. Much of our current understanding of the mechanisms of cellular transcription comes from study of the transcription of cellular templates. Analysis of the various means of producing viral mRNA has taught us much about the cell itself.

Making Viral Proteins

Because viruses are parasites of translation, all viral RNAs must be translated by the host's cytoplasmic protein-synthesizing machinery (see Chapter 11). Viral infection often results in modification of the host's translational apparatus so that viral mRNAs are selectively translated. The study of such modifications has revealed a great deal about mechanisms of mRNA translation. Analysis of viral translation has also revealed new strategies, such as internal ribosome binding and leaky scanning, that have been subsequently found to occur in uninfected cells.

Making Viral Genomes

Many viral genomes are copied by the cell's synthetic machinery in cooperation with viral proteins (see Chapters 6 through 9). The cell provides nucleotide substrates, energy, enzymes, and other proteins. In all cases, the internal compartmentalization of the cell must be reckoned with, because essential components are found only in the nucleus, are restricted to the cytoplasm, or are present in cellular membranes. Study of the mechanisms of viral genome replication has established fundamental principles of cell biology and nucleic acid synthesis.

Forming Progeny Virions

The various components of a virion—the nucleic acid genome, capsid protein(s), and in some cases envelope proteins—are often synthesized in different cellular compartments. Their trafficking through and among the cell's compartments and organelles requires that they be equipped with the proper homing signals (see Chapter 12). Virion components must be assembled at some central location, and the information for assembly must be preprogrammed in the component molecules (see Chapter 13). The primary sequences of virion proteins contain sufficient information to specify assembly; this property is exemplified by the remarkable *in vitro* assembly of tobacco mosaic virus from coat protein and RNA (Box 2.2).

Viral Pathogenesis

Viruses command our attention because of their association with animal and plant diseases. The process by which viruses cause disease is called **viral pathogenesis**. To study this process, we must investigate not only the relationships of viruses with the specific cells that they infect but also the consequences of infection to the host organism. The nature of viral disease depends on the effects of viral replication on host cells, the responses of the host's defense systems, and the ability of the virus to spread in and among hosts (Volume II, Chapters 1, 4, and 5).

Overcoming Host Defenses

Organisms have evolved many physical barriers to protect themselves from dangers in their environment such as invading parasites. In addition, vertebrates possess an effective immune system to defend against anything recognized as nonself or dangerous. Studies of the interactions between viruses and the immune system are particularly instructive, because of the many viral countermeasures

BOX 2.2 EXPERIMENTS

In vitro assembly of tobacco mosaic virus

The ability of the primary sequence of virion proteins to specify assembly is exemplified by the coat protein of tobacco mosaic virus. Heinz Fraenkel-Conrat and Robley Williams showed in 1955 that purified tobacco mosaic virus RNA and capsid protein, when mixed and incubated for 24 h, assemble into infectious viruses. When examined by electron microscopy, the particles produced in vitro were identical to the rod-shaped virions produced from infected tobacco plants (Figure 1.7B). Neither the purified viral RNA nor the capsid protein was infectious; no virions were observed in these preparations. These results indicate that the viral coat protein contains all the information needed for assembly of an infectious virion. The spontaneous formation of tobacco mosaic virions in vitro from protein and RNA components is the paradigm for self-assembly in biology.

Fraenkel-Conrat, H., and R. C. Williams. 1955. Reconstitution of active tobacco mosaic virus from its inactive protein and nucleic acid components. *Proc. Natl. Acad. Sci. USA* **40:**690–698.

that can defeat this system. Elucidation of these measures teaches us much about the basis of immunity (Volume II, Chapters 3 and 4).

Cultivation of Viruses

Cell Culture

Types of Cell Culture

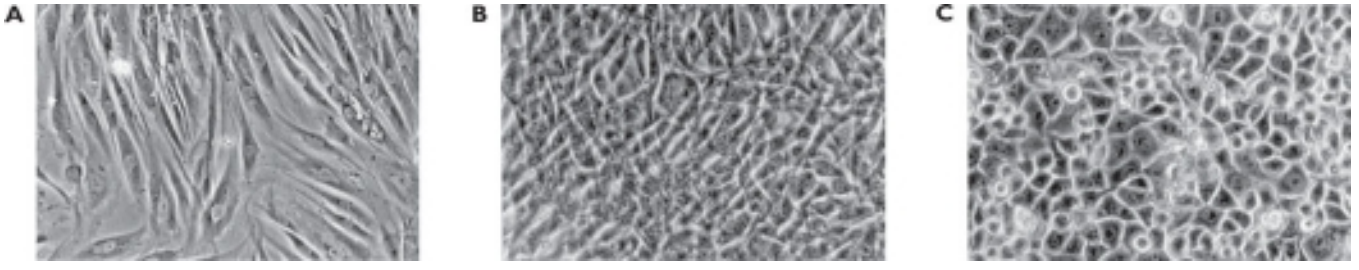
Although human and other animal cells were first cultured in the early 1900s, contamination with bacteria, mycoplasmas, and fungi initially made routine work with such cultures extremely difficult. For this reason, most viruses were grown in laboratory animals. In 1949, John Enders, Thomas Weller, and Frederick Robbins made the discovery that poliovirus could multiply in cultured cells not of neuronal origin. As noted in Chapter 1, this revolutionary finding, for which these three investigators were awarded the Nobel Prize in physiology or medicine in 1954, led the way to the propagation of many other viruses in cultured cells, the discovery of new viruses, and the development of viral vaccines such as those against poliomyelitis, measles, and rubella. The ability to infect cultured cells synchronously permitted studies of the biochemistry and molecular biology of viral replication. Large-scale growth and purification allowed studies of the composition of virus particles, leading to the solution of high-resolution, three-dimensional structures, as discussed in Chapter 4.

Cell culture is still the most common method for the propagation of animal viruses. To prepare a cell culture, tissues are dissociated into a single-cell suspension by mechanical disruption followed by treatment with proteolytic enzymes. The cells are then suspended in culture medium and placed in plastic flasks or covered plates. As the cells divide, they cover the plastic surface. Epithelial and fibroblastic cells attach to the plastic and form a **monolayer**, whereas blood cells such as lymphocytes settle, but do not adhere. The cells are grown in a chemically defined and buffered medium optimal for their growth. Commonly used cell lines double in number in 24 to 48 h in such media. Most cells retain viability after being frozen at low temperatures (−70 to −196°C).

There are three main kinds of cell cultures (Fig. 2.7). **Primary cell cultures** are prepared from animal tissues as described above. They include several cell types and have a limited life span, usually no more than 5 to 20 cell divisions. The most commonly used primary cell cultures are derived from monkey kidneys, human embryonic amnion, human embryonic kidneys, human foreskins, and chicken or mouse embryos. Such cells are used for experimental virology when the state of cell differentiation is important or when appropriate cell lines are not available. They are also used in vaccine production: for example, live attenuated poliovirus vaccine strains may be propagated in primary monkey kidney cells. Primary cell cultures were mandated for the growth of viruses to be used as human vaccines to avoid contamination of the product with potentially oncogenic DNA from continuous cell lines (see below). Some viral vaccines are now prepared in **diploid cell strains**, which consist of a homogeneous population of a single type and can divide up to 100 times before dying. Despite the numerous divisions, these cell strains retain the diploid chromosome number. The most widely used diploid cells are those established from human embryos, such as the WI-38 strain derived from human embryonic lung.

Continuous cell lines consist of a single cell type that can be propagated indefinitely in culture. These immortal lines are usually derived from tumor tissue or by treating a primary cell culture or a diploid strain with a mutagenic chemical or a tumor virus. Such cell lines often do not resemble the cell of origin; they are less differentiated

Figure 2.7 Different types of cell culture used in virology. Confluent cell monolayers photographed by low-power light microscopy. **(A)** Primary human foreskin fibroblasts; **(B)** established line of mouse fibroblasts (3T3); **(C)** continuous line of human epithelial cells (HeLa [Box 2.3]). The ability of transformed HeLa cells to overgrow one another is the result of a loss of contact inhibition. Courtesy of R. Gonzalez, Princeton University.



BOX 2.3 BACKGROUND

The cells of Henrietta Lacks

The most widely used continuous cell line in virology, the HeLa cell line, was derived from Henrietta Lacks. In 1951, the 31-year-old mother of five visited a physician at Johns Hopkins Hospital in Baltimore and found that she had a malignant tumor of the cervix. A sample of the tumor was taken and given to George Gey, head of tissue culture research at Hopkins. Dr. Gey had been attempting for years, without success, to produce a line of human cells that would live indefinitely. When placed in culture, Henrietta Lacks's cells propagated as no other cells had before.

On the day in October that Henrietta Lacks died, George Gey appeared on national television with a vial of her cells, which he called HeLa cells. He said, "It is possible that, from a fundamental study such as this, we will be able to learn a way by which cancer can be completely wiped out." Soon after, HeLa cells were used to propagate poliovirus, which was causing poliomyelitis throughout the world, and they played an important role in the development of poliovirus vaccines. Henrietta Lacks's HeLa cells started a medical revolution: not only was it possible to propagate many different viruses in these cells, but also continuous cell lines could be produced from many human tissues. Sadly, the family of Henrietta Lacks did not learn about HeLa cells, or the revolution they started, until 24 years after her death. Her family members were shocked that cells from Henrietta lived in so many laboratories, and hurt that they had not been told that any cells had been taken from her.

The story of HeLa cells is a sad commentary on the lack of informed consent that pervaded medical research in the 1950s. Since then, biomedical ethics have changed greatly, and now there are strict regulations about clinical research: physicians may not take samples from patients without permission.

For additional information, see http://www.jhu.edu/~jhumag/0400web/01.html.

(having lost the morphology and biochemical features that they possessed in the organ), are often abnormal in chromosome morphology and number **(aneuploid)**, and can be tumorigenic (i.e., they produce tumors when inoculated into nude mice). Examples of commonly used continuous cell lines include HeLa (Henrietta Lacks) cells (Box 2.3), derived from human carcinomas, and L and 3T3 cells, derived from mice. Continuous cell lines provide a uniform population of cells that can be infected synchronously for growth curve analyses (see "The One-Step Growth Cycle" below) or biochemical studies of virus replication.

In contrast to cells that grow in monolayers on plastic dishes, others can be maintained in **suspension cultures**, in which a spinning magnet continuously stirs the cells. The advantage of suspension culture is that a large number of cells can be grown in a relatively small volume. This culture method is well suited for applications that require large quantities of virus particles, such as X-ray crystallography or production of vectors.

Initially, it was thought that viruses, as obligatory intracellular parasites, could not replicate outside a living cell. This dictum was nullified in 1991 by the demonstration that infectious poliovirus could be produced in a cell extract of human cells incubated with viral RNA. Despite this finding, most work on viruses is done *in vivo*, using cultured cells, embryonated eggs, or laboratory animals (Box 2.4).

Evidence of Viral Growth in Cultured Cells

Some viruses kill the cells in which they replicate, and the infected cells may eventually detach from the cell culture plate. As more cells are infected, the changes become visible and are called **cytopathic effects** (Table 2.1). Many types of cytopathic effect can be seen with a simple light or phase-contrast microscope at low power,

BOX 2.4 TERMINOLOGY

In vitro and in vivo

The terms "*in vitro*" and "*in vivo*" are common in the virology literature. *In vitro* means "in glass" and refers to experiments carried out in an artificial environment, such as a glass test tube. Unfortunately, the phrase "experiments performed *in vitro*" is used to designate not only work done in the cell-free environment of a test tube but also work done within cultured cells. The use of the phrase *in vitro* to describe living cultured cells leads to confusion and is inappropriate.

In this textbook, descriptions of experiments being carried out *in vitro* signify the absence of cells, e.g., *in vitro* translation.

Table 2.1 Some examples of cytopathic effects of viral infection of animal cells

Cytopathic effect(s)	Virus(es)
Morphological alterations	
Nuclear shrinking (pyknosis), proliferation of membrane	Picornaviruses
Proliferation of nuclear membrane	Alphaviruses, herpesviruses
Vacuoles in cytoplasm	Polyomaviruses, papillomaviruses
Syncytium formation (cell fusion)	Paramyxoviruses, coronaviruses
Margination and breaking of chromosomes	Herpesviruses
Rounding up and detachment of cultured cells	Herpesviruses, rhabdoviruses, adenoviruses, picornaviruses
Inclusion bodies	
Virions in nucleus	Adenoviruses
Virions in the cytoplasm (Negri bodies)	Rabies virus
"Factories" in the cytoplasm (Guarnieri bodies)	Poxviruses
Clumps of ribosomes in virions	Arenaviruses
Clumps of chromatin in nucleus	Herpesviruses

without fixing or staining the cells. These changes include the rounding up and detachment of cells from the culture dish, cell lysis, swelling of nuclei, and sometimes the formation of a group of fused cells called a syncytium (Fig. 2.8). Observation of other cytopathic effects requires high-power microscopy. These cytopathic effects include the development of intracellular masses of virions or unassembled viral components in the nucleus and/or cytoplasm (inclusion bodies), formation of crystalline arrays of viral proteins, membrane blebbing, duplication of membranes, and fragmentation of organelles. The time required for the development of cytopathology varies greatly among animal viruses. For example, depending on the size of the inoculum, enteroviruses and herpes simplex virus can cause cytopathic effects in 1 to 2 days and destroy the cell monolayer in 3 days. In contrast, cytomegalovirus, rubella virus, and some adenoviruses may not produce such effects for several weeks.

The development of characteristic cytopathic effects in infected cell cultures is frequently monitored in diagnostic virology during isolation of viruses from specimens obtained from infected patients or animals. However, cytopathic effect is also of value in the research laboratory: it can be used to monitor the progress of an infection, and it is often one of the phenotypic traits by which mutant viruses are characterized.

Some viruses multiply in cells without causing obvious cytopathic effects. For example, many members of the families *Arenaviridae*, *Paramyxoviridae*, and *Retroviridae* do not cause obvious damage to cultured cells. The growth of such viruses in cells must therefore be assayed using alternative methods, as described in "Assay of Viruses" below.

Embryonated Eggs

Before the advent of cell culture, many viruses were propagated in embryonated chicken eggs (Fig. 2.9). At 5 to 14 days after fertilization, a hole is drilled in the shell and virus is injected into the site appropriate for its replication. This method of virus propagation is now routine only for influenza virus. The robust yield of this virus from chicken eggs has led to their widespread use in research laboratories and for vaccine production.

Laboratory Animals

In the early 1900s, when viruses were first isolated, freezers and cell cultures were not available and it was necessary to maintain virus stocks by continuous passage from animal to animal. This practice not only was inconvenient but also, as we shall see in Volume II, Chapter 8, led to the selection of viral mutants. For example, monkey-to-monkey intracerebral passage of poliovirus selected a mutant that could no longer infect chimpanzees by the oral route, the natural means of infection. Cell culture has largely supplanted the use of animals for propagating viruses, but some viruses, such as Norwalk virus, cannot yet be grown in this way.

Experimental infection of laboratory animals has always been, and will continue to be, obligatory for studying the processes by which viruses cause disease. The use of monkeys in the study of poliomyelitis, the paralytic disease caused by poliovirus, led to an understanding of the basis of this disease and was instrumental in the development

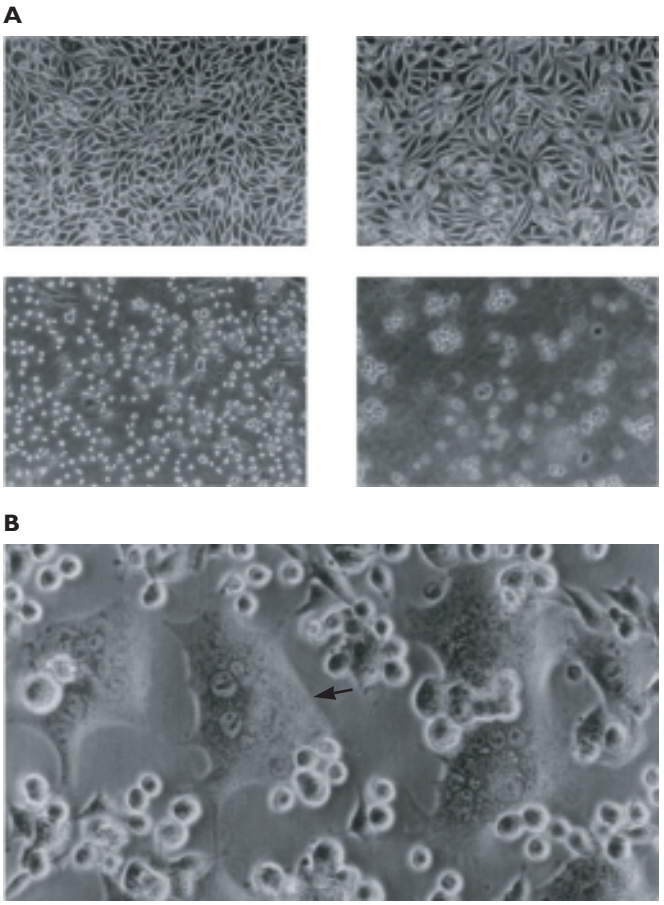


Figure 2.8 Development of cytopathic effect. (A) Cell rounding and lysis during poliovirus infection. (Upper left) Uninfected cells; (upper right) 5 1/2 h after infection; (lower left) 8 h after infection; (lower right) 24 h after infection. **(B)** Syncytium formation induced by murine leukemia virus. The field shows a mixture of individual small cells and syncytia, indicated by the arrow, which are large, multinucleate cells. Courtesy of R. Compans, Emory University School of Medicine.

of a successful vaccine. Similarly, the development of vaccines against hepatitis B virus would not have been possible without experimental studies with chimpanzees. Understanding how the immune system or any complex organ reacts to a virus cannot be achieved without research on living animals. The development of viral vaccines, antiviral drugs, and diagnostic tests for veterinary medicine has also benefited from research on diseases in laboratory animals.

Assay of Viruses

There are two main types of assays for detecting viruses: biological and physical. Because viruses were first recognized by their infectivity, the earliest assays focused on this most sensitive and informative property. However, biological assays such as the plaque assay and end-point titration methods do not measure noninfectious particles. Such particles can be measured by physical assays such as electron microscopy or by immunological methods. Knowledge of the number of noninfectious particles is useful for assessing the quality of a virus preparation.

Measurement of Infectious Units

One of the most important procedures in virology involves measuring the concentration of a virus in a sample, the **virus titer**. This parameter is determined by inoculating serial dilutions of virus into host cell cultures, chicken embryos, or laboratory animals and monitoring for evidence of virus multiplication. The response may be quantitative (as in assays for plaques, fluorescent foci, infectious centers, or transformation) or all-or-none, in which the presence or absence of infection is measured (as in an end-point dilution assay).

Plaque Assay

In 1952, Renato Dulbecco modified the plaque assay developed to determine the titers of bacteriophage stocks for use in animal virology. The plaque assay was adopted

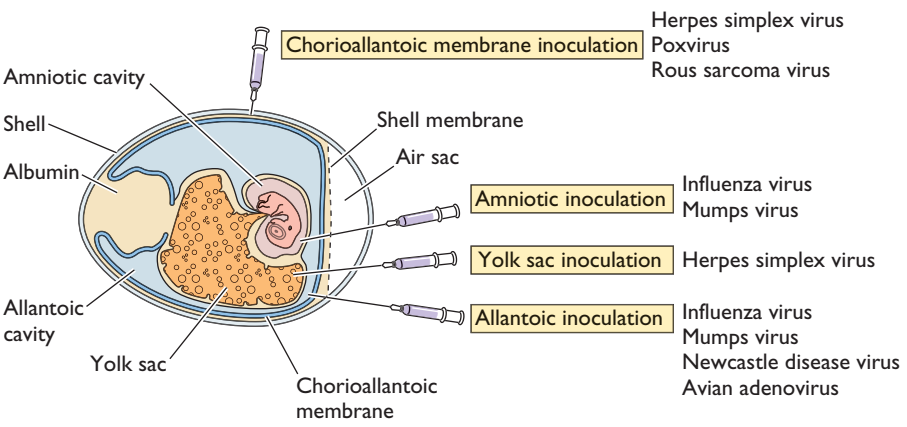
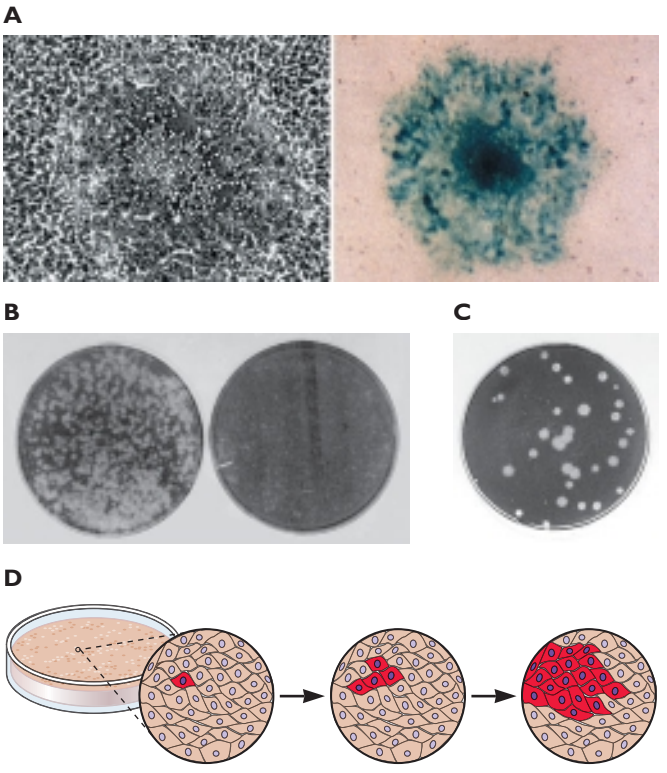


Figure 2.9 Growth of viruses in embryonated eggs. The cutaway view of an embryonated chicken egg shows the different routes by which viruses are inoculated into eggs and the different compartments in which viruses may grow. Adapted from F. Fenner et al., *The Biology of Animal Viruses* (Academic Press, New York, NY, 1974), with permission.

rapidly for reliable determination of the titers of a wide variety of viruses. In this assay, monolayers of cultured cells are incubated with a preparation of virus to allow adsorption to cells. After removal of the inoculum, the cells are covered with nutrient medium containing a supplement, most commonly agar, that results in the formation of a gel. When the original infected cells release new progeny viruses, the spread of viruses to neighboring uninfected cells is restricted by the gel. As a result, each infectious particle produces a circular zone of infected cells, a **plaque**. If the infected cells are damaged, the plaque can be distinguished from the surrounding monolayer. In time, the plaque becomes large enough to be seen with the naked eye (Fig. 2.10). Only viruses that cause visible damage of cultured cells can be assayed in this way.

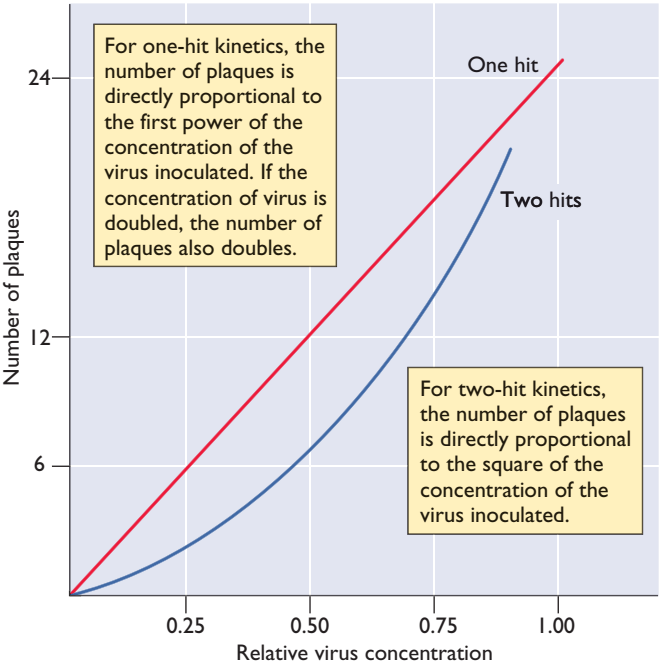
Figure 2.10 Plaques formed by different animal viruses. Plaque sizes reflect the life cycle of a virus in a particular cell type. **(A)** Photomicrograph of a single plaque formed by pseudorabies virus in Georgia bovine kidney cells. (Left) Unstained cells. (Right) Cells stained with the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), which is converted to a blue compound by the product of the *lacZ* gene carried by the virus. Courtesy of B. Banfield, Princeton University. **(B)** Different plaque morphology of influenza C virus strains. Monolayers were stained with the vital dye crystal violet. **(C)** Plaques formed by poliovirus on human HeLa cells stained with crystal violet. **(D)** Illustration of the spread of virus from an initial infected cell to neighboring cells, resulting in a plaque.



For the majority of animal viruses, there is a linear relationship between the number of infectious virus particles and the plaque count (Fig. 2.11). One infectious particle is therefore sufficient to initiate infection, and the virus is said to infect cells with **one-hit kinetics**. Some examples of **two-hit kinetics**, in which two different types of virus particle must infect a cell to ensure replication, have been recognized. For example, the genomes of some (+) strand RNA viruses of plants consist of two RNA molecules that are encapsidated separately. Both RNAs are required for infectivity. The dose-response curve in plaque assays for these viruses is parabolic rather than linear (Fig. 2.11).

The titer of a virus stock can be calculated in **plaque-forming units (PFU) per milliliter** (Box 2.5) When one infectious virus particle initiates a plaque, the viral progeny within the plaque are clones, and virus stocks prepared from a single plaque are known as **plaque purified**. The tip of a small pipette is plunged into the overlay above the

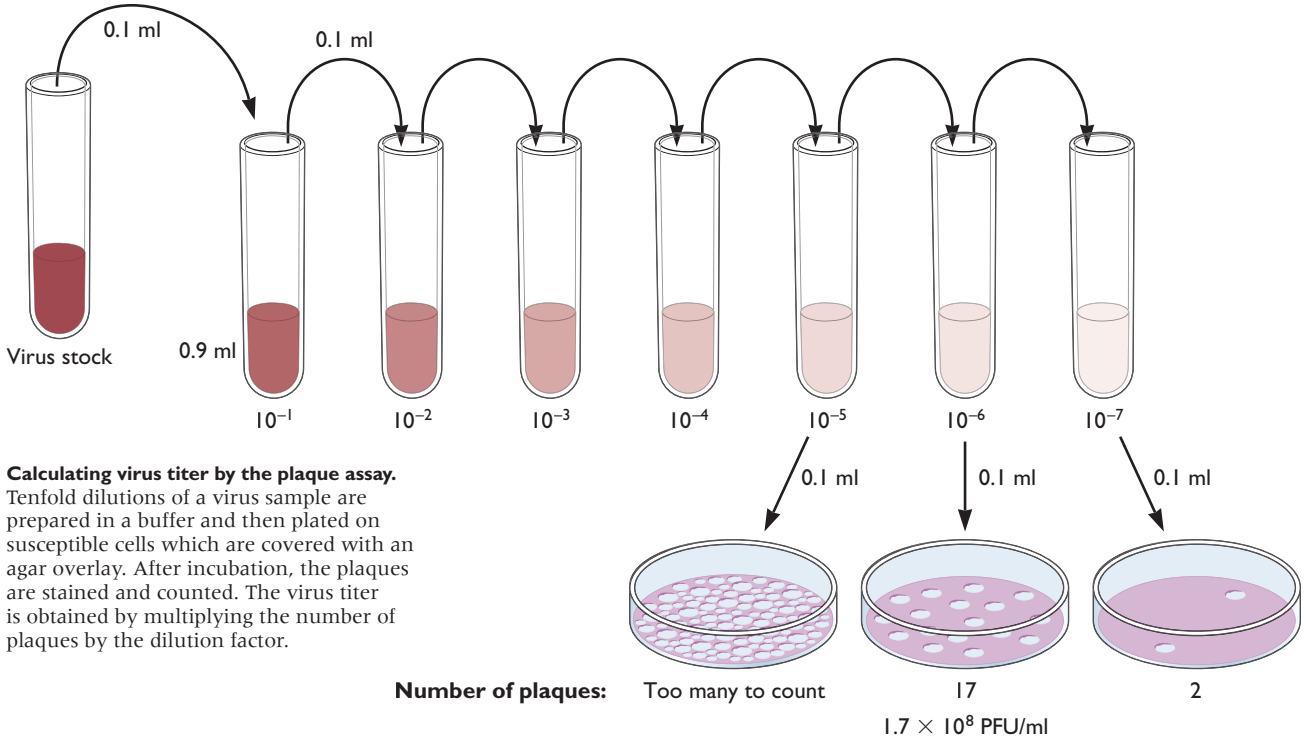
Figure 2.11 The dose-response curve of the plaque assay. The number of plaques produced by a virus with one-hit kinetics (red) or two-hit kinetics (blue) is plotted against the relative concentration of the virus. In two-hit kinetics, there are two classes of uninfected cells, those receiving one particle and those receiving none. The Poisson distribution can be used to determine the proportion of cells in each class: they are e^{-m} and me^{-m} (Box 2.8). Because one particle is not sufficient for infection, $P(0) = e^{-m}(1 + m)$. At a very low multiplicity of infection, this equation becomes $P(i) = (1/2)m^2$ (where i = infection) which gives a parabolic curve. Adapted from B. D. Davis et al., *Microbiology* (J. B. Lippincott Co., Philadelphia, PA, 1980), with permission.



BOX 2.5 METHODS

Calculating virus titer from the plaque assay

To calculate the titer of a virus in plaque-forming units per milliliter, 10-fold serial dilutions of a virus stock are prepared, and 0.1-ml aliquots are inoculated onto susceptible cell monolayers (see figure). After a suitable incubation period, the monolayers are stained and the plaques are counted. To minimize error in calculating the virus titer, only plates containing between 10 and 100 plaques are counted, depending on the area of the cell culture vessel. According to statistical principles, when 100 plaques are counted, the sample titer varies by ±10%. For accuracy, each dilution is plated in duplicate or triplicate (not shown in the figure). Plates with more than 100 plaques are generally not counted because the plaques may overlap, causing inaccuracies. In the example shown in the figure, 17 plaques are observed on the plate produced from the 10^{-6} dilution. Therefore, the 10^{-6} dilution tube contains 17 PFU per 0.1 ml, or 170 PFU per ml, and the titer of the virus stock is 170×10^6 or 1.7×10^8 PFU/ml.



Calculating virus titer by the plaque assay. Tenfold dilutions of a virus sample are prepared in a buffer and then plated on susceptible cells which are covered with an agar overlay. After incubation, the plaques are stained and counted. The virus titer is obtained by multiplying the number of plaques by the dilution factor.

plaque, and the plug of agar containing the virus is recovered. The virus within the agar plug is eluted into buffer and used to prepare virus stocks. To ensure purity, this process is usually repeated at least one more time. Plaque purification is employed widely in virology to establish clonal virus stocks.

Fluorescent-Focus Assay

The fluorescent-focus assay, a modification of the plaque assay, is useful in determining the titers of viruses that do not kill cells. The initial procedure is the same as in the plaque assay. However, after a period sufficient for adsorption and gene expression, cells are permeabilized and incubated with an antibody raised against a viral protein. A second antibody, which recognizes the first, is then added. This second antibody is usually conjugated to a fluorescent indicator, such as fluorescein. The cells are then examined under a microscope at an appropriate wavelength. The titer of the virus stock is expressed in fluorescent-focus-forming units per milliliter.

Infectious-Centers Assay

Another modification of the plaque assay, the infectious-centers assay, is used to determine the fraction of cells in a culture that are infected with a virus. Monolayers of infected cells are suspended before progeny viruses are produced. Dilutions of a known number of infected cells are then plated on monolayers of susceptible cells, which are covered with an agar overlay. The number of plaques that form on the indicator cells is a measure of the number of cells infected in the original population. The fraction of infected cells can therefore be determined. A typical use of the infectious-centers assay is to measure the proportion of infected cells in persistently infected cultures.

Transformation Assay

The transformation assay is useful for determining the titers of some retroviruses that do not form plaques. For example, Rous sarcoma virus transforms chicken embryo cells. As a result, the cells lose their contact inhibition (the property that governs whether cultured cells grow as a single monolayer [see Volume II, Chapter 7]) and become heaped up on one another. The transformed cells form small piles, or **foci**, that can be distinguished easily from the rest of the monolayer (Fig. 2.12). Infectivity is expressed in focus-forming units per milliliter.

End-Point Dilution Assay

The end-point dilution assay provided a measure of virus titer before the development of the plaque assay. It is still used for measuring the titers of certain viruses that do not form plaques or for determining the virulence of a virus in animals. Serial dilutions of a virus stock are inoculated into replicate test units (typically 8 to 10), which can be cell cultures, eggs, or animals. The number of test units that have become infected is then determined for each virus dilution. When cell culture is used, infection is determined by the development of cytopathic effect; in eggs or animals, infection is gauged by death or disease. An example of an end-point dilution assay using cell cultures is shown in Box 2.6. At high dilutions, none of the cell cultures are infected because no infectious particles are delivered to the cells; at low dilutions, every culture is infected. The **end point** is the dilution of virus that affects 50% of the test units. This number can be calculated from the data and expressed as 50% infectious dose (ID_{50}) per milliliter. The first preparation illustrated in Box 2.6 contains 10^5 ID_{50} per ml.

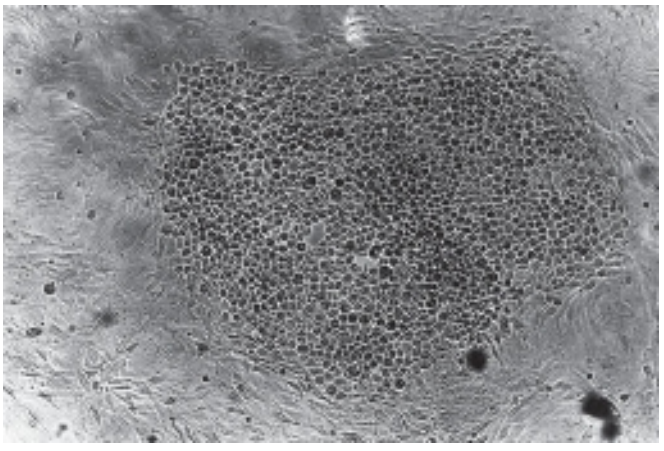
When the end-point dilution assay is used to assess the virulence of a virus or its capacity to cause disease, as defined in Volume II, Chapter 1, the result of the assay can be expressed in terms of 50% lethal dose (LD_{50}) per milliliter or 50% paralytic dose (PD_{50}) per milliliter, end points of death and paralysis, respectively. If the virus titer can be determined separately by plaque assay, the 50% end point determined in an animal host can be related to this parameter. In this way, the effects of the route of inoculation or specific mutations on viral virulence can be quantified.

Efficiency of Plating

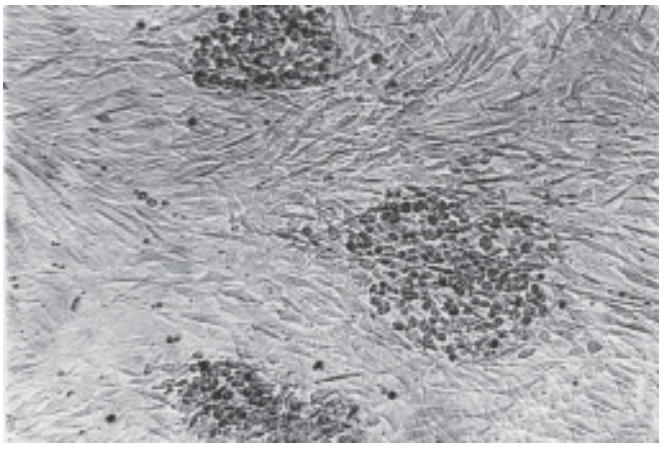
The term **relative efficiency of plating** was coined to assign a value to the plaque count determined with the

Figure 2.12 Transformation assay. Chicken cells transformed by two different strains of Rous sarcoma virus are shown. Loss of contact inhibition causes cells to pile up rather than grow as a monolayer. One focus is seen in panel **A**, and three foci are seen in panel **B** at the same magnification. Courtesy of H. Hanafusa, Osaka Bioscience Institute.

A



B



BOX 2.6 METHODS

End-point dilution assays

In the first example, 10 monolayer cell cultures were infected with each virus dilution. After the incubation period, plates that displayed cytopathic effect were scored +. Fifty percent of the cell cultures displayed cytopathic effect at the 10^{-5} dilution, and therefore the virus stock contains 10^5 ID_{50} units.

In most cases the 50% end point does not fall on a dilution tested as shown in the example; for this reason, various statistical procedures have been developed to calculate the end point of the titration. In one popular method, the dilution containing the ID_{50} is identified by interpolation between the dilutions on either side of this value. The assumption is made that the location of the 50% end point varies linearly with the log of the dilution. Because the number of test units used at each dilution is usually small, the accuracy of this method is relatively low. For example, if six test units are used at each 10-fold dilution, differences in virus titer of only 50-fold or more can be detected reliably. The method is illustrated in the following example, in which the lethality of poliovirus in mice is the end point. Eight mice were inoculated per dilution.

Virus dilution	Cytopathic effect									
10^{-2}	+	+	+	+	+	+	+	+	+	+
10^{-3}	+	+	+	+	+	+	+	+	+	+
10^{-4}	+	+	−	+	+	+	+	+	+	+
10^{-5}	−	+	+	−	+	−	−	+	−	+
10^{-6}	−	−	−	−	−	−	+	−	−	−
10^{-7}	−	−	−	−	−	−	−	−	−	−

In the method of Reed and Muench, the results are pooled, as shown in the table. The interpolated value of the 50% end point, which in this case falls between the fifth and sixth dilutions, is calculated to be $10^{-6.5}$. The virus sample therefore contains $10^{6.5}$ LD_{50}s. The LD_{50} may also be calculated as the concentration of the stock virus in PFU per milliliter (1×10^9) times the 50% end-point titer. In the example shown, the LD_{50} is 3×10^2 PFU.

Dilution	Alive	Dead	Total alive	Total dead	Mortality ratio	Mortality (%)
10^{-2}	0	8	0	40	0/40	100
10^{-3}	0	8	0	32	0/32	100
10^{-4}	1	7	1	24	1/25	96
10^{-5}	0	8	1	17	1/18	94
10^{-6}	2	6	3	9	3/12	75
10^{-7}	5	3	8	3	8/11	27

same bacteriophage but with different strains of bacteria. The value is a ratio of viral titers obtained on two different host cells. This number may be more or less than 1, depending on how well the virus grows in different host cells. A very different value is the **absolute efficiency of plating**, which is defined as the plaque titer divided by the number of virus particles in the sample. The **particle-to-PFU ratio**, a term more commonly used today, is the inverse value (Table 2.2). For many bacteriophages, the particle-to-PFU ratio approaches 1, the lowest value that can be obtained. However, for animal viruses this value can be much higher, ranging from 1 to 10,000. These high values have complicated the study of animal viruses. For example, when the particle-to-PFU ratio is high, it is never certain whether properties measured biochemically are in fact those of the infectious particle or those of the noninfectious component.

Although the linear nature of the dose-response curve indicates that a single particle is capable of initiating an

Table 2.2 Particle-to-PFU ratios of some animal viruses

Virus	Particle/PFU ratio
Adenoviridae	20–100
Alphaviridae	
Semliki Forest virus	1–2
Herpesviridae	
Herpes simplex virus	50–200
Orthomyxoviridae	
Influenza virus	20–50
Papillomaviridae	
Papillomavirus	10,000
Picornaviridae	
Poliovirus	30–1,000
Polyomaviridae	
Polyomavirus	38–50
Simian virus 40	100–200
Poxviridae	1–100
Reoviridae	
Reovirus	10

infection (one-hit kinetics) (Fig. 2.11), the high particle-to-PFU ratio for many viruses demonstrates that not all virions are successful. The high value of this ratio is sometimes caused by the presence of noninfectious particles with genomes that harbor lethal mutations or that have been damaged during growth or purification. An alternative explanation is that although all viruses in a preparation are in fact capable of initiating infection, not all of them succeed because of the complexity of the infectious cycle. Failure at any one step in the cycle prevents completion. A high particle-to-PFU ratio does not indicate that most particles are defective but, rather, indicates that they failed to complete the infection.

Measurement of Virus Particles and Their Components

Although the numbers of virus particles and infectious units are often not equal, assays for particle number are frequently used to approximate the number of infectious particles present in a sample. For example, the concentration of viral DNA or protein can be used to estimate the particle number, assuming that the ratio is constant. Biochemical or physical assays are usually more rapid and easier to carry out than assays for infectivity, which may be slow, cumbersome, or not possible. Assays for subviral components also provide information on particle number if the stoichiometry of these components in the virus particle is known.

Imaging Particles

Electron microscopy. With few exceptions, virus particles are too small to be observed directly by light microscopy. However, they can be seen readily in the electron microscope. If a sample contains only one type of virus, the particle count can be determined. First, a virus preparation is mixed with a known concentration of latex beads. The numbers of virus particles and beads are then counted, allowing the concentration of the virus particles in the sample to be determined by comparison.

Live-cell imaging of single fluorescent virions. The discovery of green fluorescent protein revolutionized the study of the cell biology of virus infection. This protein, isolated from the jellyfish *Aequorea victoria*, is a convenient reporter for monitoring transcription or translation, because it is directly visible in living cells without the need for fixation, substrates, or coenzymes. Similar proteins isolated from different organisms, which emit light of different wavelengths, are also widely used in virology. The use of fluorescent proteins has allowed visualization of single virus particles in living cells. The coding sequence for the fluorescent protein is inserted into the viral genome, often fused to the coding region of a virion protein. The fusion protein is incorporated into the viral particle, which is visible in cells by fluorescence microscopy (Fig. 2.13). Using this approach, entry, uncoating, replication, assembly, and egress of single particles can all theoretically be observed in living cells.

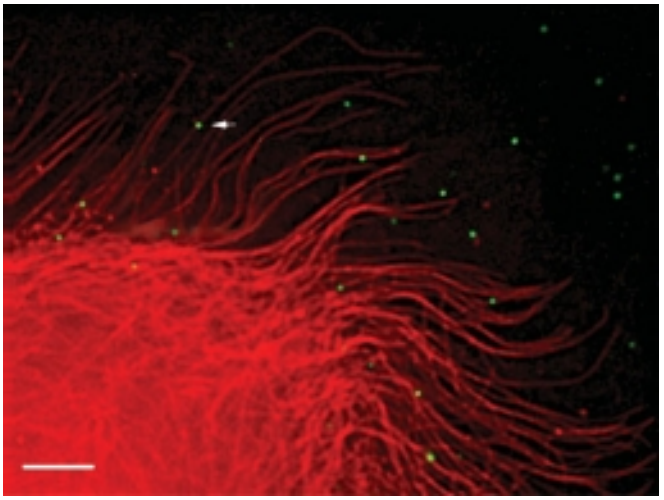


Figure 2.13 Live-cell imaging of single virus particles by fluorescence. Single-virus-particle imaging with green fluorescent protein illustrates microtubule-dependent movement of human immunodeficiency virus type one particles in cells. Rhodamine-tubulin was injected into cells to label microtubules (red). The cells were infected with virus particles that contain a fusion of green fluorescent protein with Vpr. Virus particles can be seen as green dots. Bar, 5 μm. Courtesy of David McDonald, University of Illinois.

Hemagglutination

Members of the *Adenoviridae*, *Orthomyxoviridae*, and *Paramyxoviridae*, among others, contain proteins that can bind to erythrocytes (red blood cells); these viruses can link multiple cells, resulting in formation of a lattice. This property is called **hemagglutination**. For example, influenza viruses contain an envelope glycoprotein called hemagglutinin, which binds to *N*-acetylneuraminic acid-containing glycoproteins on erythrocytes. In practice, two-fold serial dilutions of the virus stock are prepared, mixed with a defined quantity of red blood cells, and added to small wells in a plastic tray (Fig. 2.14). Unadsorbed red blood cells tumble to the bottom of the well and form a sharp dot or button. In contrast, agglutinated red blood cells form a diffuse lattice that coats the well. Because the assay is rapid (30 min), it is often used as a quick indicator of the relative quantities of virus particles. However, it is not sufficiently sensitive to detect small numbers of particles.

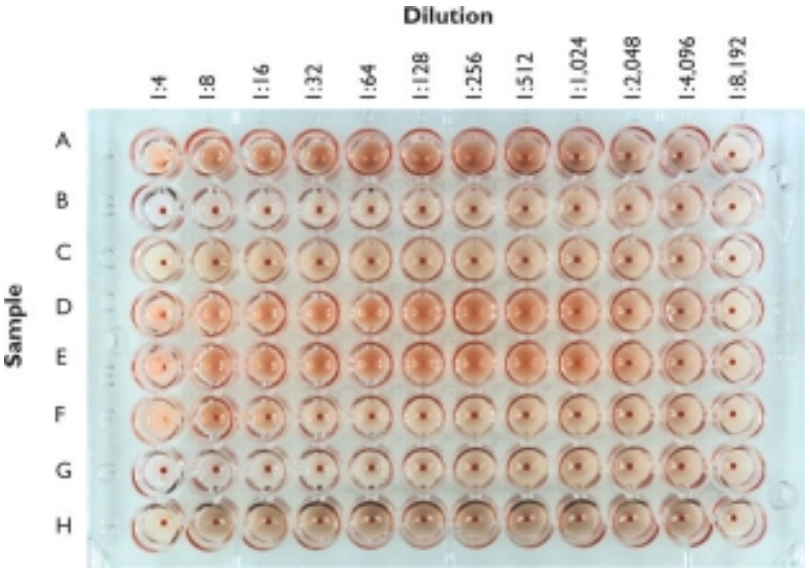


Figure 2.14 Hemagglutination assay. Samples of different influenza viruses were diluted, and a portion of each dilution was mixed with a suspension of chicken red blood cells and added to the wells. After 30 min at 4°C, the wells were photographed. Sample A causes hemagglutination until a dilution of 1:256 and therefore has a hemagglutination titer of 256. Elution of the virus from red blood cells as seen in column 1, rows D and E, is caused by neuraminidase in the virus particle. This enzyme cleaves *N*-acetylneuraminic acid from glycoprotein receptors and elutes bound viruses from red blood cells. Courtesy of C. Basler and P. Palese, Mount Sinai School of Medicine of the City University of New York.

Measurement of Viral Enzyme Activity

Some animal virus particles contain nucleic acid polymerases, which can be assayed by mixing permeabilized particles with radioactively labeled precursors and measuring the incorporation of radioactivity into nucleic acid. This type of assay is used most frequently for retroviruses, many of which do not transform cells or form plaques. The reverse transcriptase incorporated into the virus particle is assayed by mixing cell culture supernatants with a mild detergent (to permeabilize the viral envelope), an appropriate template and primer, and a radioactive nucleoside triphosphate. If reverse transcriptase is present, a radioactive product will be produced by priming on the poly(rC) template. This product can be detected by precipitation or bound to a filter and quantified. Because enzymatic activity is proportional to particle number, this assay allows rapid tracking of virus production in the course of an infection.

Serological Methods

Many virological techniques are based on the specificity of the antibody-antigen reaction. Some of the techniques, such as immunostaining, immunoprecipitation, immunoblotting, and the enzyme-linked immunosorbent assay, are by no means limited to the detection of viruses and viral proteins. All these approaches have been used extensively to study the structures and functions of cellular proteins.

Virus neutralization. When a virus preparation is inoculated into an animal, an array of antibodies is produced. These antibodies can bind to virus particles, but not all of them can block infectivity **(neutralize)**, as discussed in Volume II, Chapter 4. Virus neutralization assays are usually conducted by mixing dilutions of antibodies with virus, incubating them, and assaying for remaining infectivity in cultured cells, eggs, or animals. The end point is defined as the highest dilution of antibody that inhibits the development of cytopathic effect in cells or virus replication in eggs or animals.

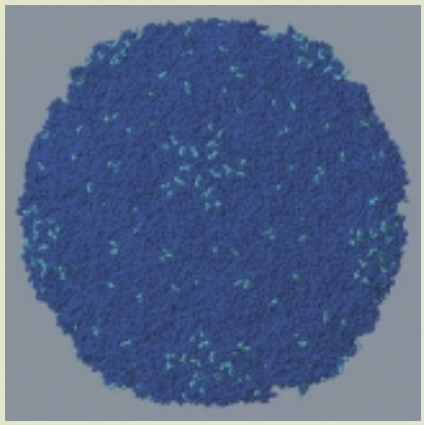
Some neutralizing antibodies define **type-specific antigens** on the virus particle. For example, the three **serotypes** of poliovirus are distinguished on the basis of neutralization tests; type 1 poliovirus is neutralized by antibodies to type 1 virus but not by antibodies to type 2 or type 3 poliovirus, and so forth. Neutralization tests have therefore been valuable for virus classification. These antibodies may also be used to map the three-dimensional structure of neutralization antigenic sites on the virion (Box 2.7).

Hemagglutination inhibition. Antibodies against viral proteins with hemagglutination activity can block the ability of virus to bind red blood cells. In this assay, dilutions of antibodies are incubated with virus, and erythrocytes are added as outlined above. After incubation, the hemagglutination inhibition titer is read as the highest dilution of antibody that inhibits hemagglutination. This test is sensitive, simple, inexpensive, and rapid; it is the method of choice for assaying antibodies to any virus that causes hemagglutination. It can be used to detect antibodies to viral hemagglutinin in animal and human sera or to identify the origin of the hemagglutinin of influenza viruses produced in cells coinfected with two parent viruses.

BOX 2.7 DISCUSSION

Neutralization antigenic sites

Knowledge of the antigenic structure of a virus is useful in understanding the immune response to these agents and in designing new vaccination strategies. The use of **monoclonal antibodies** (antibodies of a single specificity made by a clone of antibody-producing cells) in neutralization assays permits mapping of antigenic sites on a virus particle, or of the amino acid sequences that are recognized by neutralizing antibodies. Each monoclonal antibody binds specifically to a short amino acid sequence (8 to 12 residues) that fits into the antibody-combining site. This amino acid sequence, which may be linear or nonlinear, is known as an **epitope**. In contrast, **polyclonal antibodies** comprise the repertoire produced in an animal against the many epitopes of an antigen. Antigenic sites may be identified by cross-linking the monoclonal antibody to the virus and determining which protein is the target of the antibody. Epitope mapping may also be performed by assessing the abilities of monoclonal antibodies to bind synthetic peptides representing viral protein sequences. When the monoclonal antibody recognizes a linear epitope, it may react with the protein in Western blot analysis, facilitating direct identification of the viral protein harboring the antigenic site. The most elegant understanding of antigenic structures has come from the isolation and study of variant viruses that are resistant to neutralization with specific monoclonal antibodies (called **monoclonal antibody-resistant variants**). By identifying the amino acid change responsible for this phenotype, the antibody-binding site can be located and, together with three-dimensional structural information, can provide detailed information on the nature of antigenic sites that are recognized by neutralizing antibodies (see figure).



Locations of neutralization antigenic sites on the capsid of human rhinovirus type 14. Amino acids that change in viral mutants selected for resistance to neutralization by monoclonal antibodies are shown in cyan on a model of the viral capsid. These amino acids are in VP1, VP2, and VP3 on the surface of the virion.

Complement fixation. The complement fixation assay can be used to determine if antibodies against a virus are present in serum. The interaction of viral antigen and antibody can cause complement fixation, which leads to membrane lysis, as discussed in Volume II, Chapter 4. Red blood cells are used as targets, because lysis of their membranes is readily observed (Fig. 2.15).

Because crude preparations of viral antigens are often used in the complement fixation test, this test is not highly specific. The antigen preparations may include proteins or specific epitopes shared by related groups of viruses. As a result, the test detects most serotypes within a given family of viruses and is therefore said to recognize group-specific antigens. For example, the three serotypes of poliovirus share a complement-fixing, group-specific antigen. Complement fixation is not as sensitive as neutralization or hemagglutination inhibition. Nevertheless, it is often the first assay performed on sera from infected patients to identify the family to which the infecting virus belongs.

Viral Growth: the Burst Concept

A fundamental and important concept is that viruses replicate by the assembly of preformed components into particles. The parts are first made in cells and then assembled into the final product. The growth of viruses is very different from the growth of cells, which multiply by binary fission. This simple build-and-assemble strategy is unique to viruses, but the details for members of different virus families are astoundingly different. There are many ways to build a virus particle, and each one tells us something new about virus structure and assembly.

Modern studies of viruses have their origins in the work of Delbrück and colleagues, who studied the T-even bacteriophages starting in 1937. Delbrück believed that these bacteriophages were perfect models for understanding virus replication. He also thought that phages were excellent models for studying the gene: they were self-replicating (a hallmark of a gene), their mutations were inherited, and they were small, easily manipulated entities that grew rapidly.

In the late 1930s, Delbrück focused his attention on the fact that one bacterial cell usually makes hundreds of progeny viruses. The yield from one cell is one viral generation; it was called the **burst** because viruses literally burst from the infected cell. According to the burst concept, an infected cell either produces virus or it does not. Under carefully controlled laboratory conditions, most cells make on average about the same number of bacteriophages per cell. For example, in one of Delbrück's experiments, the number of bacteriophage T4 particles produced from individual

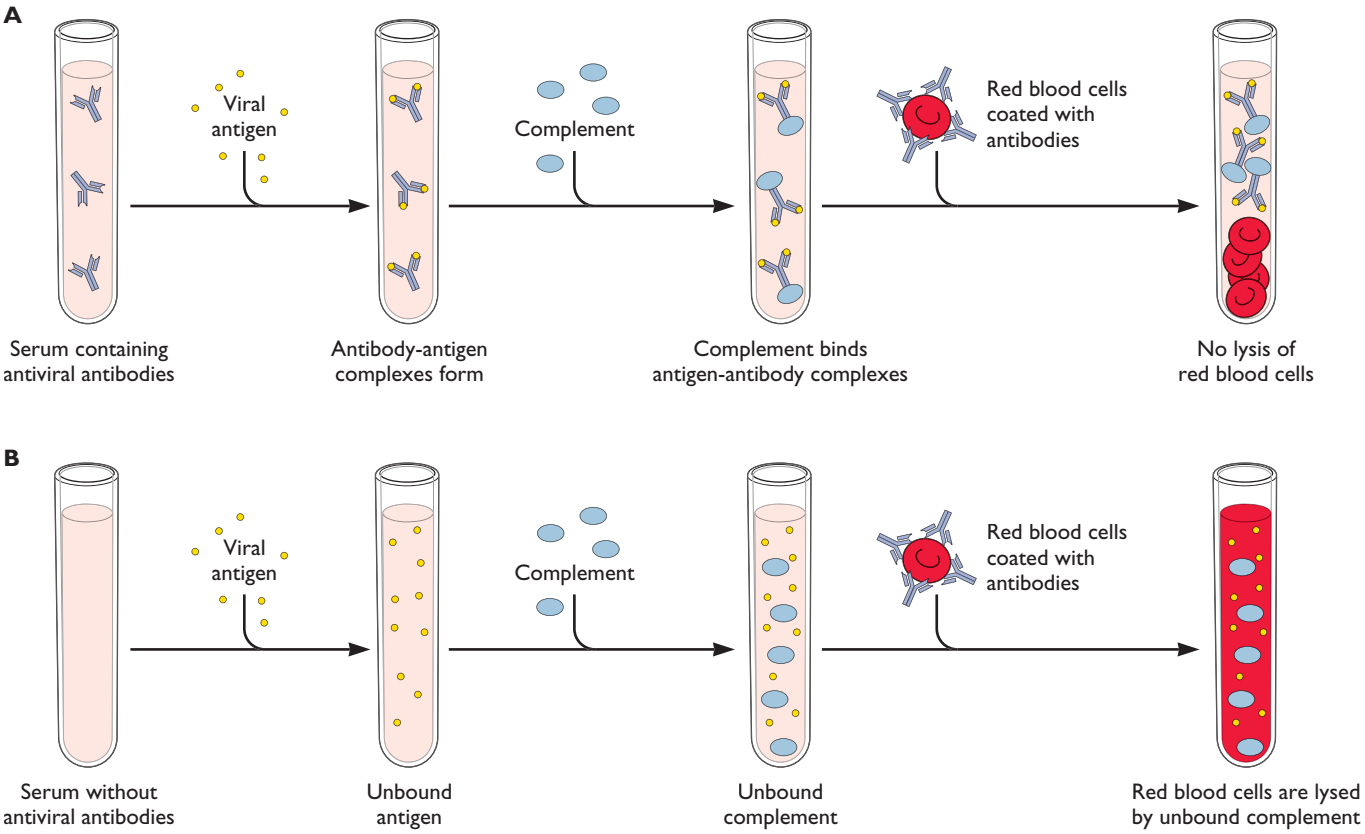


Figure 2.15 Detection of viral antigens by complement fixation. Samples of patients' sera are heated to inactivate endogenous complement and then incubated with preparations of viral antigen and a standardized quantity of guinea pig complement. If an antibody-antigen reaction takes place, complement fixation will occur. The latter is detected by adding sheep red blood cells that have been coated with rabbit anti-red blood cell antibodies. If complement has been fixed by binding of viral antigen to antibody, the red blood cells remain intact **(A)**; if complement is not fixed, the red blood cells are lysed by the action of free complement **(B)**.

single-cell bursts from four *Escherichia coli* cells was 101, 127, 57, and 316. The average burst is 150 particles per cell; if this experiment were done today, using similar experimental conditions, the average burst would be similar. The variation of the burst size from cell to cell is of interest, as it shows that the replication machinery is not precise.

Another important component of the burst concept is that a cell has a finite capacity to produce virus. A number of factors limit the number of particles produced per cell, such as metabolic resources, the number of sites for replication in the cell, the regulation of virion release, and host defenses. The burst principle holds for most viruses. In general, larger cells (e.g., eukaryotic cells) produce more virus particles per cell; yields of 1,000 to 10,000 virions per eukaryotic cell are not uncommon.

The burst concept applies only to viruses that kill the cell after infection, namely, the cytopathic viruses. However, there are viruses that do not kill their host cells; virus particles are produced as long as the cell is alive. There is no burst; instead, there is a continuous release of virus particles. Examples include filamentous bacteriophages, some retroviruses, and hepatitis viruses.

The One-Step Growth Cycle

Initial Concept

The idea that one-step growth analysis can be used to study the single-cell life cycle of viruses originated from the work on bacteriophages by Ellis and Delbrück. In their classic experiment, they added virus particles to a culture of rapidly growing *E. coli* cells; these particles adsorbed quickly to the cells. The infected culture was then diluted, preventing further adsorption of unbound particles. This simple dilution step is the key to the experiment: it reduces further

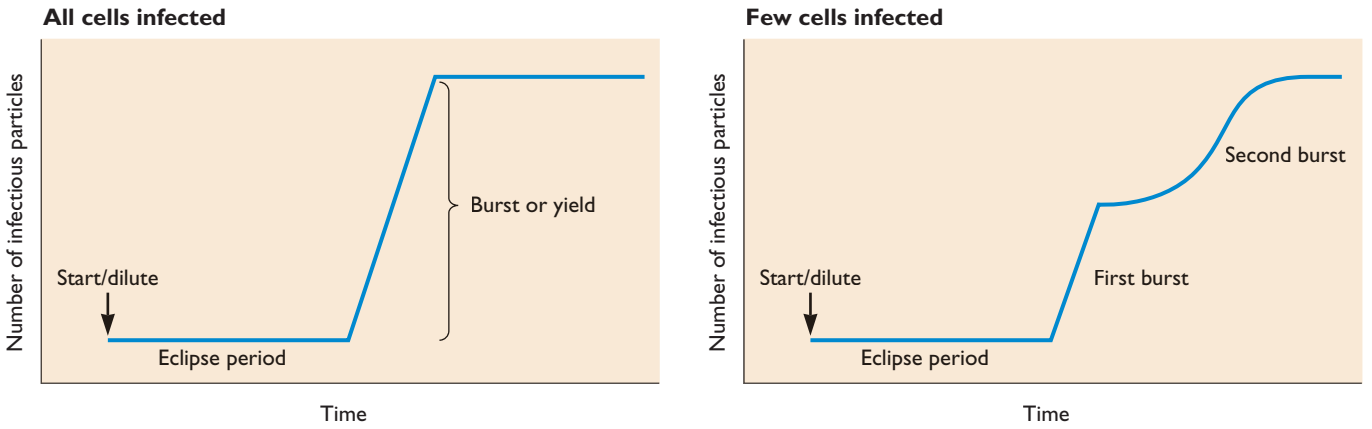


Figure 2.16 One-step growth curves of bacteriophages. The figure shows the growth of a bacteriophage in *E. coli* under conditions when all cells are infected **(A)** and when only a few cells are infected **(B)**.

binding of virus to cells and effectively synchronizes the infection. Samples of the diluted culture were then taken every few minutes and analyzed for the number of infectious bacteriophages. When the results were plotted, several key observations emerged (Fig. 2.16). Numbers of new viruses did not increase in a linear fashion from the start of the infection. There was an initial lag, followed by a rapid increase in virus production, which then plateaued. This single cycle of virus replication produces the "burst" of virus progeny. If the experiment is repeated, so that only a few cells are initially infected, the graph looks different (Fig. 2.16). Instead of a single cycle, there is a stepwise increase in numbers of new viruses with time. Each step represents one cycle of virus infection.

Once the nature of the viral growth cycle was explored using the one-step growth curve, questions emerged about what was happening in the cell before the burst. What was the fate of the incoming virus? Did it disappear? How was more virus produced? These questions were answered by A. Doermann, who in 1951 found an elegant way of looking inside the infected cell. Instead of sampling the diluted culture for virus after various periods of infection, Doermann prematurely lysed the infected cells as the infection proceeded and then assayed the lysate for infectious virus. The results were extremely informative. Immediately after dilution, there was a complete loss, or eclipse, of infectious virus for 10 to 15 min (Fig. 2.16). In other words, input infectious virions disappeared and no new phage particles were detected during this period. The loss of infectivity is a consequence of the release of the genome from the virion, to allow for subsequent transcription of viral genes. Particle infectivity is lost during this phase because the released genome is not infectious under the conditions of the plaque assay. Next, new infectious viruses were detected inside the cell, before they were released into the medium (Fig. 2.16). These were newly assembled virions that had not yet been released by cell lysis. The results of these experiments defined two new terms in virology: the **eclipse period**, the phase in which infectivity is lost when virions are disassembled after penetrating cells, and the **latent period**, the time it takes to replicate, assemble, and release new virus, approximately 20 to 25 min for *E. coli* bacteriophages.

Synchronous infection, the key to the one-step growth cycle, is usually accomplished by infecting cells with a sufficient number of virus particles to ensure that most of the cells are infected rapidly.

One-Step Growth Analysis: a Valuable Tool for Studying Animal Viruses

One-step growth analysis soon became adapted for studying the replication of animal viruses. The experiment begins with removal of the medium from the cell monolayer and addition of virus in a small volume to promote rapid adsorption. One cell monolayer is infected for each time point. After approximately 1 h, the unadsorbed inoculum is removed, the cells are washed, and fresh medium is added. At different times after infection, samples of the cell culture supernatant are collected and the virus titer is determined. The kinetics of intracellular virus production can be monitored by removing the medium containing extracellular particles, scraping the cells into fresh medium, and lysing them by repeated cycles of freeze-thawing. A cell extract is prepared after removal of cellular debris by centrifugation, and the virus titer in the extract is measured.

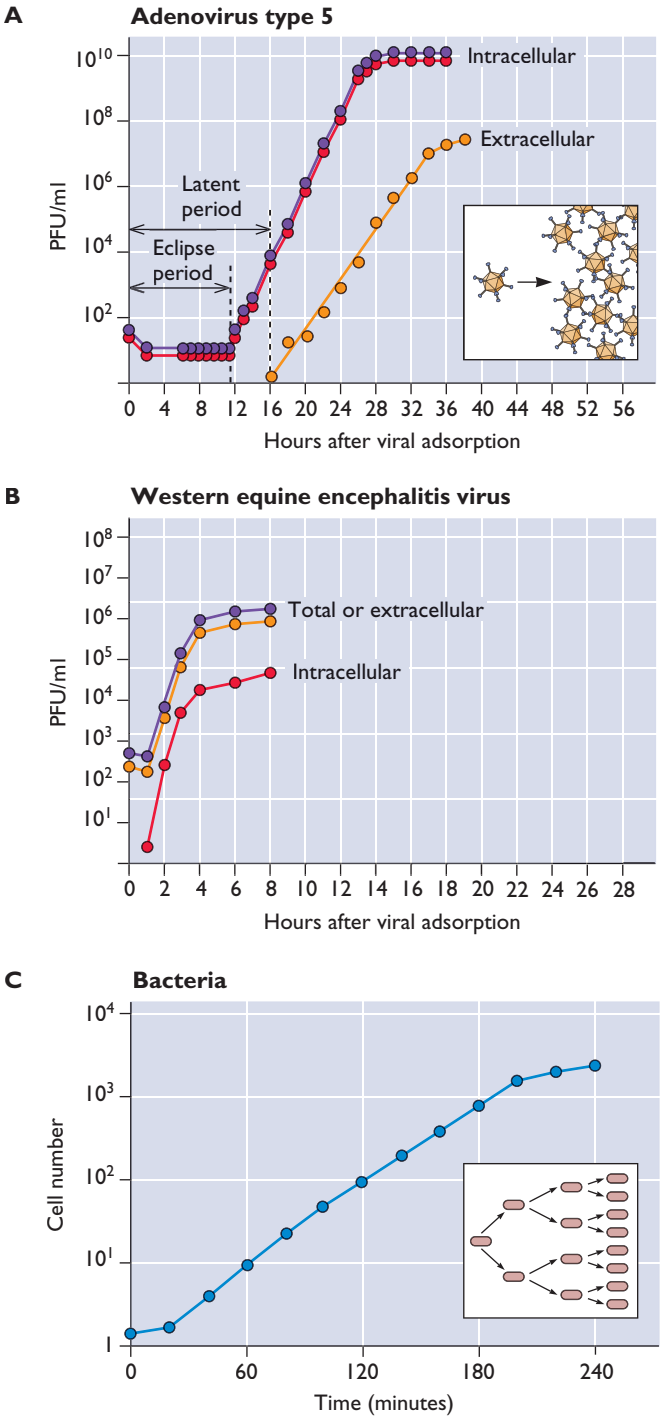


Figure 2.17 One-step growth curves of animal viruses. **(A)** Growth of a nonenveloped virus, adenovirus type 5. The inset illustrates the concept that viruses multiply by assembly of preformed components into particles. **(B)** Growth of an enveloped virus, Western equine encephalitis virus, a member of the *Togaviridae*. This virus acquires infectivity after maturation at the plasma membrane, and therefore little intracellular virus can be detected. The small amounts observed at each time point probably represent released virus contaminating the cell extract. **(C)** Growth curve for a bacterium. The number of bacteria is plotted as a function of time. One bacterium is added to the culture at time zero; after a brief lag, the bacterium begins to divide. The number of bacteria doubles every 20 min until nutrients in the medium are depleted and the growth rate decreases. The inset illustrates the growth of bacteria by binary fission. (A and B) Adapted from B. D. Davis et al., *Microbiology* (J. B. Lippincott Co., Philadelphia, PA, 1980), with permission. (C) Adapted from B. Voyles, *The Biology of Viruses* (McGraw-Hill, New York, NY, 1993), with permission.

The results of a one-step growth experiment establish a number of important features about viral replication. In the example shown in Fig. 2.17A, the first 11 h after infection constitutes the eclipse period, during which the viral nucleic acid is uncoated from its protective shell and no infectious virus can be detected inside cells. The low level of infectivity detected during this period probably results from adsorbed virus that was not uncoated. Beginning at 12 h after adsorption, the quantity of intracellular infectious virus begins to increase, marking the onset of the synthetic phase, during which new virus particles are assembled. During the latent period no extracellular virus can be detected. At 18 h after adsorption, virions are released from cells and found in the extracellular medium. Ultimately, virus production plateaus as the cells become metabolically and structurally incapable of supporting additional replication.

The yield of infectious virus per cell can be calculated from the data collected during a one-step growth experiment (Fig. 2.17). This value varies widely among different viruses and with different virus-host cell combinations. For many viruses, increasing the **multiplicity of infection** (Box 2.8) above a certain point does not increase the yield: cells have a finite capacity to produce new virus particles. In fact, infecting at a very high multiplicity of infection can cause premature cell lysis and decrease virus yields.

The nature of the one-step growth curve can vary dramatically among different viruses. For example, enveloped viruses that mature by budding from the plasma membrane, as discussed in Chapter 13, generally become infectious only as they leave the cell, and therefore little intracellular infectious virus can be detected (Fig. 2.17B). The first one-step growth curves of viruses were prepared for bacteriophages, and the results surprised scientists who had expected that they would resemble the growth curves of bacteria or cultured cells. After a short lag, bacterial cell growth becomes exponential (i.e., each progeny cell is capable of dividing) and follows a straight line (Fig. 2.17C). Exponential growth continues until the nutrients in the medium are exhausted. The one-step growth curves of animal viruses are very different from one another. The curve

BOX 2.8 DISCUSSION

Multiplicity of infection (MOI)

Infection depends on the random collision of cells and virus particles. When susceptible cells are mixed with a suspension of virus, some cells are uninfected and other cells receive one, two, three, etc., particles. The distribution of virus particles per cell is best described by the Poisson distribution:

$$P(k) = e^{-m}m^k/k!$$

In this equation, *P(k)* is the fraction of cells infected by k virus particles. The multiplicity of infection, m, is calculated from the proportion of uninfected cells, $P(0)$, which can be determined experimentally. If k is made 0 in the above equation, then

$$P(0) = e^{-m} \text{ and } m = -\ln P(0)$$

The fraction of cells receiving 0, 1, and more than one virus particle in a culture of 10^6 cells infected with an MOI of 10 can be determined as follows.

The fraction of cells that receive 0 particles is

$$P(0) = e^{-10} = 4.5 \times 10^{-5}$$

and in a culture of 10^6 cells this equals 45 uninfected cells.

The fraction of cells that receive 1 particle is

$$P(1) = 10 \times 4.5 \times 10^{-5} = 4.5 \times 10^{-4}$$

and in a culture of 10^6 cells, 450 cells receive 1 particle.

The fraction of cells that receive >1 particle is

$$P(>1) = 1 - e^{-m}(m + 1) = 0.9995$$

and in a culture of 10^6 cells, 999,500 cells receive more than 1 particle. [The value in this equation is obtained by subtracting from 1 (the sum of all probabilities for any value of k) the probabilities $P(0)$ and $P(1)$.]

The fraction of cells receiving 0, 1, and more than one virus particle in a culture of 10^6 cells infected with an MOI of 0.001 is

$P(0) = 99.99\%$
$P(1) = 0.0999\%$ (for 10^6 cells, 10^4 are infected)
$P(>1) = 10^{-6}$

The MOI required to infect 99% of the cells in a cell culture dish is

$P(0) = 1\% = 0.01$
$m = -\ln (0.01) = 4.6$ PFU per cell

shown in Fig. 2.17A illustrates the pattern observed for a DNA virus with the long latent and synthetic phases typical of many DNA viruses, some retroviruses, and reovirus. For small RNA viruses, the entire growth curve is complete within 6 to 8 h, and the latent and synthetic phases are correspondingly shorter. Counterintuitively, polyomavirus, with one of the smallest genomes of the DNA viruses, has a very long latent period. The basis for these differences is related to the various strategies of gene expression and genome replication (discussed in Chapter 3).

One-step growth curve analysis can provide quantitative information about different virus-host systems. It is frequently employed to study mutant viruses to determine what parts of the replication cycle are affected by a particular genetic lesion. It is also valuable for studying the multiplication of a new virus or viral replication in a new virus-host cell combination.

When cells are infected at a low multiplicity of infection, several cycles of viral replication may occur (Figure 2.16). Growth curves established under these conditions can also provide useful information. For example, if a mutation fails to have an obvious effect on viral replication when infection is done at high multiplicity of infection, the defect may become obvious following a low-multiplicity infection. Because the effect of a mutation in each cycle is multiplied over several cycles, a small effect can be amplified. Defects in the ability of viruses to spread from cell to cell may also be revealed when multiple cycles of replication occur.

Perspectives

The one-step growth analysis is nearly universally used to study virus replication. When millions of cells are infected at a high multiplicity of infection, enough viral nucleic acid or protein can be isolated to allow a study of events during the replication cycle. Synchronous infection is the key to this approach, because under this condition, the same steps of the replication cycle occur in all cells at the same time. Many of the experimental results discussed in subsequent chapters of this book were obtained using one-step growth analysis. The power of this analysis is such that it reports on all stages of the replication cycle in a simple and quantitative fashion. With modest expenditure of time and reagents, virologists can deduce a great deal about viral translation, replication, or assembly. This universal utility is a cardinal feature of a fundamentally important method.

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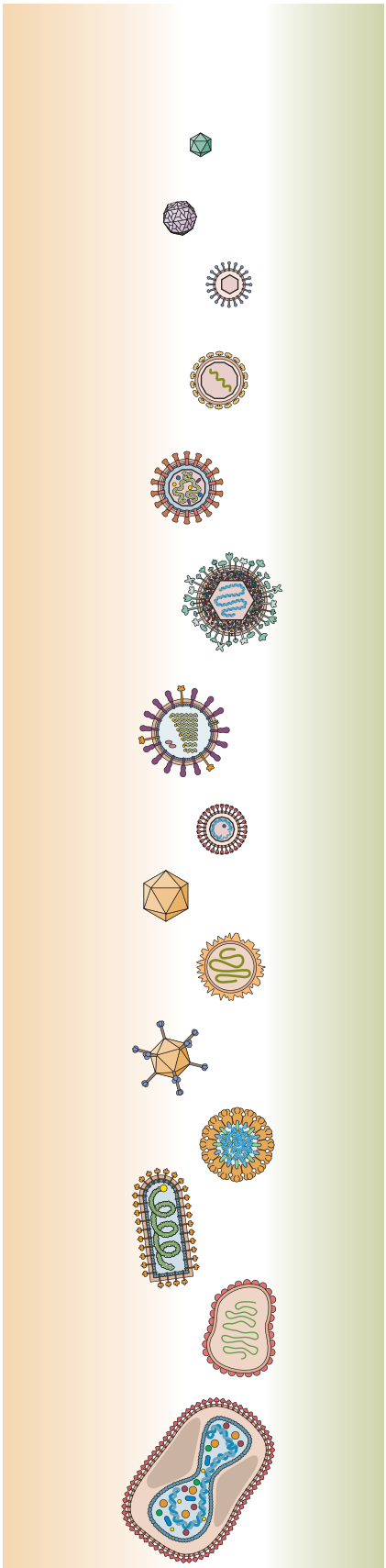
II Molecular Biology



3

Genomes and Genetics

… everywhere an interplay between nucleic acids and proteins; a spinning wheel in which the thread makes the spindle and the spindle the thread.

ERWIN CHARGAFF
as quoted in A. N. H. Creager, The Life of a Virus, University of Chicago Press, 2002

Introduction

The world abounds with uncountable numbers of viruses of apparently overwhelming diversity. Fortunately, as discussed in Chapter 1, taxonomists have devised methods of classifying viruses. As a result, the number of identifiable groups becomes rather manageable (Table 3.1). One of the contributions of molecular biology has been a detailed analysis of the genetic material of representatives of these main virus families. What emerged from these studies was the principle that the **viral genome** is the nucleic acid-based repository of the information needed to build, replicate, and transmit a virus (Box 3.1). These analyses also revealed that the structures of viral genomes are far less complex than is implied by the existence of thousands of distinct entities defined by classical taxonomic methods. In fact, it is possible to organize the known viruses into seven groups, based on the structures of their genomes.

Genome Principles and the Baltimore System

A universal function of viral genomes is to specify proteins. However, viral genomes do not encode the machinery needed to carry out protein synthesis. Consequently, one important principle is that all viral genomes must be copied to produce messenger RNAs (mRNAs) that can be read by host ribosomes. Literally, all viruses are parasites of their host cells' mRNA translation system.

A second principle is that there is unity in diversity; even with immeasurable time, evolution has led to the formation of only seven major types of viral genome. The Baltimore classification system integrates these two principles to construct an elegant molecular algorithm for virologists (see Fig. 1.11). When the bewildering array of viruses is classified by this system, we find fewer than 10 pathways to mRNA. The elegance of the Baltimore system is that by knowing only the nature of the viral genome, one can deduce the basic steps that must take place to produce mRNA. Perhaps more pragmatically, the system simplifies comprehension of the extraordinary life cycles of viruses.

Table 3.1 DNA and RNA viruses according to the report of the International Committee on Taxonomy of Viruses (2005)[a]

			Morphology			
			Isometric[c]		Other[d]	
Type	Genome	No. of families[b]	Enveloped	Naked	Enveloped	Naked
DNA	ds	24	2	9	11	2
	ss	5	0	4	0	1
RNA	ds	8	0	5	1	2
	(+)ss	27	0	14	6	7
	(–)ss	7	0	0	7	0
Subtotal			2	32	25	12
Total		71	34		37	

[a]The known viruses have been grouped into >1,950 species (probably 30,000 to 40,000 total virus isolates), 3 orders, 73 families, 9 subfamilies, 287 genera, subviral agents (satellites, viroids, and prions), and unclassified viruses. These numbers are certainly underestimates as new viruses are discovered regularly.

[b]Not included are the many genera of viruses that have not been assigned to families.

[c]Particles likely to be built with icosahedral symmetry; the latter can be identified only by structural studies, which have not been done for all viruses.

[d]This category comprises nonisometric forms including tailed bacteriophages, pleomorphic, rod shaped, lemon shaped, droplet shaped, spherical, bacilliform, reniform, and filamentous.

The Baltimore system omits the second universal function of viral genomes, i.e., to serve as a template for synthesis of progeny genomes. There is a finite number of nucleic acid copying strategies, each with unique primer, template, and termination requirements. We shall combine this principle with that embodied in the Baltimore system to define seven strategies based on mRNA synthesis **and** genome replication.

For most viruses with DNA genomes, replication and mRNA synthesis present no obvious challenges, as all cells use DNA-based mechanisms. In contrast, animal cells possess no known mechanisms to copy viral RNA templates and to produce mRNA from them. For RNA viruses to survive, their RNA genomes must, by definition, encode a nucleic acid polymerase for genome replication and mRNA synthesis.

BOX 3.1 BACKGROUND

What information is encoded in a viral genome?

Gene products and regulatory signals required for

- replication of the genome
- assembly and packaging of the genome
- regulation and kinetics of the replication cycle
- modulation of host defenses
- spread to other cells and hosts

Information **not** contained in viral genomes:

- no genes or evolutionary relics of genes encoding protein synthesis machinery (e.g., no ribosomal RNA and no ribosomal or translation proteins); note: the genomes of some large DNA viruses contain genes for transfer RNAs (tRNAs), aminoacyl-tRNA synthetases, and genes involved in sugar and lipid metabolism
- no genes encoding proteins of energy metabolism or membrane biosynthesis
- no telomeres (to maintain genomes) or centromeres (to ensure segregation of genomes)

Structure and Complexity of Viral Genomes

By definition, the nucleic acid in the virion is the viral genome. Despite the simplicity of expression strategies, the composition and structures of viral genomes are more varied than those seen in the entire archeal, bacterial, or eukaryotic kingdoms. Nearly every possible method for encoding information in nucleic acid can be found in viruses. Viral genomes can be

- DNA or RNA
- DNA with short segments of RNA
- DNA or RNA with covalently attached protein
- single stranded (+) strand, (–) strand, or ambisense (Box 3.2)
- double stranded
- linear
- circular

BOX 3.2 TERMINOLOGY

Important conventions: plus (+) and minus (–) strands

mRNA is defined as the positive (+) strand, because it contains immediately translatable information. A strand of DNA of the equivalent polarity is also designated as a (+) strand; i.e., if it were mRNA, it would be translated into protein.

The RNA or DNA complement of the (+) strand is the (–) strand. The (–) strand cannot be translated; it must first be copied to make the (+) strand.

- segmented
- gapped

It is clear that the structure of the genome determines the method of replication and packaging. What is the significance of such diversity in genome composition, structure, and replication? Obviously, viral genomes are survivors of constant selective pressure, but can we deduce something from their existence? Is one configuration more advantageous? These are difficult questions to answer. Virus evolution is discussed further in Volume II, Chapter 10.

Figures 3.1 through 3.7 illustrate the seven strategies for expression and replication of viral genomes. In some cases, genomes can enter the replication cycle directly. In others, genomes must first be modified and other viral nucleic acids must participate in the replication cycle. For each strategy, a panel contains the following essential information:

- genome structure
- degree of dependence on host for replication
- gene expression strategies
- noteworthy features of the interaction with the host

Examples of specific viruses in each class are provided.

DNA Genomes

The strategy of having DNA as a viral genome appears at first glance to be simplicity itself: the host genetic system is based on DNA, so viral genome replication and expression could simply emulate the host system. Many surprises await those who believe that this is all such a strategy entails.

Double-Stranded DNA (dsDNA) (Fig. 3.1)

Viral genomes may consist of double-stranded or partially double-stranded nucleic acids. There are 24 families of viruses with such DNA genomes; those that include mammalian viruses are the *Adenoviridae*, *Herpesviridae*, *Papillomaviridae*, *Polyomaviridae*, and *Poxviridae*. These genomes may be linear or circular. mRNA is produced by copying of the genome by host or viral DNA-dependent RNA polymerase. The strategies are shown in Fig. 3.1, with the exception of the *Papillomaviridae*, which have mRNA and replication mechanisms similar to the *Polyomaviridae*.

Gapped DNA (Fig. 3.2)

As the gapped DNA genome is partially double-stranded, the gaps must be filled to produce perfect duplexes. This repair process must precede mRNA synthesis because the host RNA polymerase can transcribe only fully dsDNA. The unusual gapped DNA genome is produced from an RNA template by a virus-encoded enzyme that synthesizes DNA from an RNA template (reverse transcriptase).

Single-Stranded DNA (ssDNA) (Fig. 3.3)

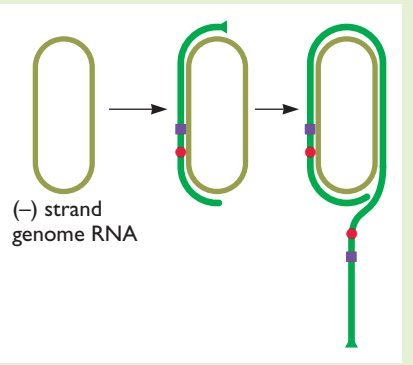
Five families of viruses containing single-stranded DNA genomes have been recognized; the families *Circoviridae*

BOX 3.3 BACKGROUND

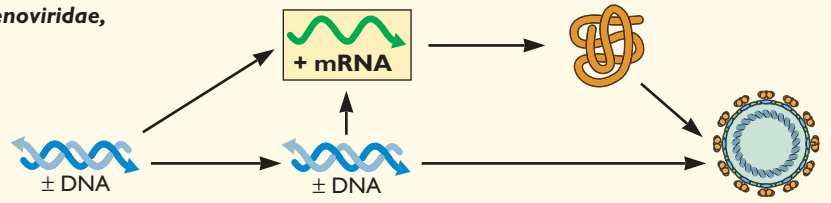
RNA synthesis in cells

There are no known enzymes in mammalian cells that can copy the genomes of RNA viruses. However, at least one enzyme, RNA polymerase II, can copy an RNA template. The 1.7-kb circular ssRNA genome of hepatitis delta satellite virus is copied by RNA polymerase II to form multimeric RNAs (see figure). How RNA polymerase II, an enzyme that produces mRNAs from DNA templates, is reprogrammed to copy a circular RNA template is not known.

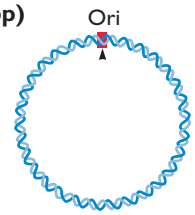
Hepatitis delta satellite (–) strand genome RNA is copied by RNA polymerase II at the indicated position. The polymerase passes the poly(A) signal (purple box) and the self-cleavage domain (red circle). For more information, see Fig. 6.14. Redrawn from J. M. Taylor, *Curr. Top. Microbiol. Immunol.* **239:**107–122, 1999, with permission.



A dsDNA genome: *Polyomaviridae, Adenoviridae, Herpesviridae, Poxviridae*



B *Polyomaviridae* (5 kbp)



Genome configuration

- Circular DNA genomes, <10 open reading frames

Degree of dependence on host cell

- Dependent on cell for both replication and gene expression
- No viral DNA polymerase
- RNA primers and replication fork to replicate DNA, analog of the cellular mechanism

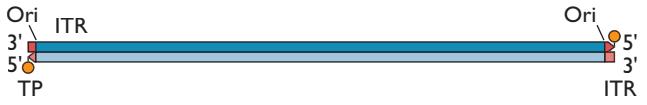
Gene expression strategies

- Temporal control of early and late gene expression
- Splicing is a major regulatory process
- Other decoding tactics: overlapping genes, leaky scanning

Noteworthy features of the interaction with the host

- Polyomavirus and simian virus 40 encode tumor (T) antigens that modulate cell cycle control

C *Adenoviridae* (36–48 kbp)



Genome configuration

- Linear DNA, short inverted terminal repeat sequence, protein bound to 5' termini

Degree of dependence on host cell

- Encodes DNA replication system including DNA polymerase
- Protein priming with unusual strand displacement mechanism

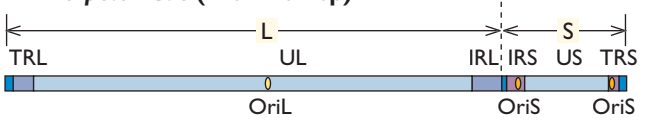
Gene expression strategies

- Temporal control (immediate-early, early, intermediate, and late) of gene expression
- 8 pre-mRNAs that are differentially polyadenylated and/or spliced give rise to many mRNAs translated into viral proteins
- Other decoding tactics: overlapping coding and/or control sequences
- Small viral RNAs produced by host RNA polymerase III

Noteworthy features of the interaction with the host

- E1A proteins are powerful transcriptional activators and regulators of the cell cycle

D *Herpesviridae* (120–220 kbp)



Genome configuration

- Linear DNA
- Genome isomers

Degree of dependence on host cell

- Genomes encode a complete replication system, including a viral DNA polymerase, and enzymes that synthesize DNA precursors
- Genome replication with RNA primers and replication fork

Gene expression strategies

- Temporal regulation; extent of splicing variable among different subfamilies
- Encode a variety of transcriptional and posttranscriptional regulators
- Package activators of transcription and other regulatory proteins in the virion
- Production of many mRNAs from multiple promoters

Noteworthy features of the interaction with the host

- Latent infection

E *Poxviridae* (130–375 kbp)

Genome configuration

- Large DNA genome, cross-linked ends
- Long terminal repeats

Degree of dependence on host cell

- Replicates in cytoplasm
- Genome encodes DNA replication system
- Strand displacement DNA synthesis

Gene expression strategies

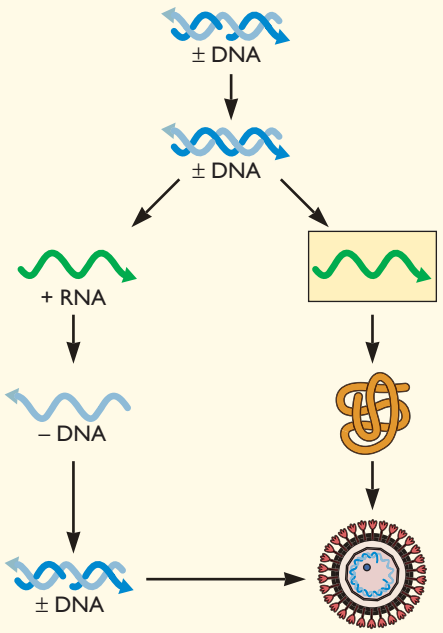
- Genome encodes a complete mRNA synthesis system including RNA polymerase, capping, and polyadenylation enzymes
- Temporal regulation
- Many viral enzymes packaged in virions

Noteworthy features of the interaction with the host

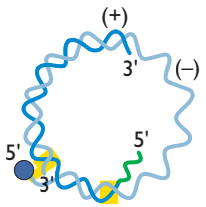
- Encode many immune modulators

Figure 3.1

A Gapped, circular, dsDNA genome: *Hepadnaviridae*



B *Hepadnaviridae* (3.4 kbp)



Genome configuration

- Circular DNA genome; full-length but nicked (–) and partial (+) strand
- Reverse transcriptase bound to 5' end of (–) strand; RNA bound to 5' end of (+) strand

Degree of dependence on host cell

- Gapped DNA genome is repaired in nucleus by host enzymes to form covalently closed circular DNA
- Longer-than-genome-length (+) strand mRNA is produced by host RNA polymerase II, and the viral reverse transcriptase copies it to make the gapped dsDNA genome

Gene expression strategies

- Alternative splicing for synthesis of multiple mRNAs
- Overlapping reading frames

Noteworthy features of the interaction with the host

- The X gene product of mammalian members contributes to tumorigenesis

Figure 3.2

and *Parvoviridae* include viruses that infect mammals. ssDNA must be copied into mRNA before proteins can be produced. However, RNA can be made only from a dsDNA template, whatever the sense of the ssDNA. DNA synthesis **must** precede mRNA production in the replication cycles of these viruses. The single-stranded genome is produced by cellular DNA polymerases.

RNA Genomes

Cells have no RNA-dependent RNA polymerases that can replicate the genomes of RNA viruses or make mRNA from RNA (Box 3.3). Two strategies have evolved to solve this problem. One solution is that RNA virus genomes encode RNA-dependent RNA polymerases that produce RNA (both genomes and mRNA) from RNA templates. The other, exemplified by retrovirus genomes, is reverse transcription of the genome to dsDNA, which can be transcribed by host RNA polymerase.

dsRNA (Fig. 3.4)

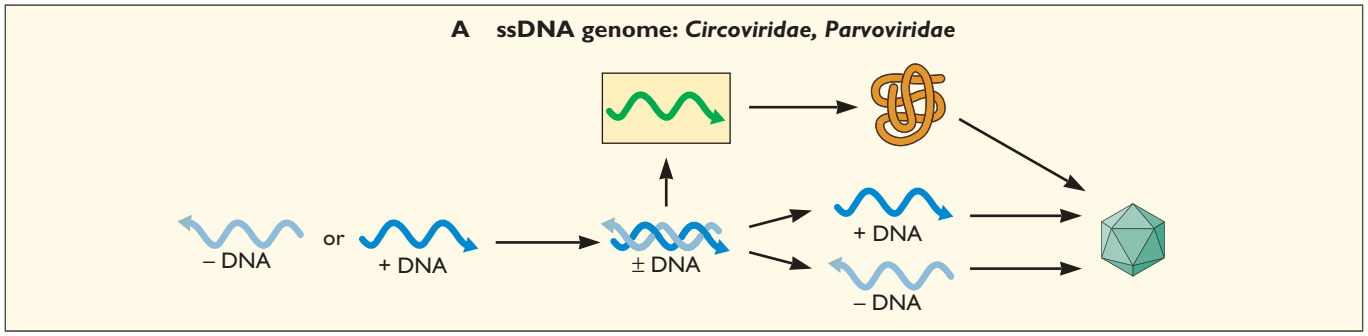
There are eight families of viruses with dsRNA genomes. The number of dsRNA segments ranges from 1 (*Totiviridae* and *Hypoviridae*, viruses of fungi, protozoa, and plants) to 10 to 12 (*Reoviridae*, viruses of mammals, fish, and plants). While dsRNA contains a (+) strand, it cannot be translated as part of a duplex. The (–) strand of the genomic dsRNA is first copied into mRNAs by a viral RNA-dependent RNA polymerase to produce viral proteins. Newly synthesized mRNAs are encapsidated and then copied to produce dsRNAs.

(+) Strand RNA (Fig. 3.5)

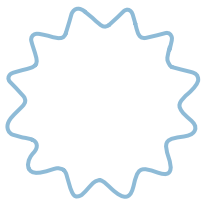
The (+) strand RNA viruses are the most plentiful on this planet; 27 families have been recognized. The families *Arteriviridae*, *Astroviridae*, *Caliciviridae*, *Coronaviridae*, *Flaviviridae*, *Picornaviridae*, and *Togaviridae* include viruses that infect mammals. (+) strand RNA genomes usually can be translated directly into protein by host ribosomes. The genome is replicated in two steps. First, the (+) strand genome is copied into a full-length (–) strand. The (–) strand is then copied into full-length (+) strand genomes. In some cases, a subgenomic mRNA is produced.

(+) Strand RNA with DNA Intermediate (Fig. 3.6)

In contrast to other (+) strand RNA viruses, the (+) strand RNA genome of retroviruses is converted to a dsDNA intermediate by viral RNA-dependent DNA polymerase (reverse transcriptase). This DNA then serves as the template for viral mRNA and genome RNA synthesis by cellular enzymes.



B ***Circoviridae*** **(1.7–2.2 kb)**



Genome configuration

- Circular ssDNA

Degree of dependence on host cell

- Genome replication by host DNA replication system

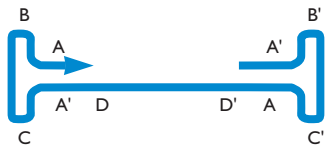
Gene expression strategies

- Two or three open reading frames
- Alternative mRNA splicing

Noteworthy features of the interaction with the host

- Smallest known nondefective genome

C ***Parvoviridae*** **(4–6 kb)**



Genome configuration

- Linear with 3' and 5' hairpins; can be both (–) and (+) strands
- 3' hairpin primes DNA synthesis
- NS1 protein covalently attached to 5' end (autonomous parvoviruses)

Degree of dependence on host cell

- Genome replication by host DNA replication system

Gene expression strategies

- Two open reading frames
- Alternative mRNA splicing

Noteworthy features of the interaction with the host

- Replicate only in cells in S phase (autonomous parvoviruses) or cells infected by a helper virus (dependoviruses)

Figure 3.3

(–) Strand RNA (Fig. 3.7)

Viruses with (–) strand RNA genomes are found in seven families. Viruses of this type that can infect mammals are found in the *Bornaviridae, Filoviridae, Orthomyxoviridae, Paramyxoviridae,* and *Rhabdoviridae* families. Unlike (+) strand RNA, (–) strand RNA genomes cannot be translated directly into protein but first must be copied to make (+) strand mRNA by a viral RNA-dependent RNA polymerase. There are no enzymes in the cell that can produce mRNAs from the RNA genomes of (–) strand RNA viruses. These virions therefore contain virus-encoded RNA-dependent RNA polymerases that produce mRNAs from the (–) strand genome. This strand is also the template for the synthesis of full-length (+) strands, which in turn are copied to produce (–) strand genomes. (–) strand RNA viral genomes can be either single molecules (nonsegmented) or segmented.

The genomes of certain (–) strand RNA viruses (e.g., members of the *Arenaviridae* and *Bunyaviridae*) are ambisense: they contain both (+) and (–) strand information on a single strand of RNA (Fig. 3.7C). The (+) sense information in the genome is translated upon entry of the viral RNA into cells. Then, after replication of the RNA genome, new (+) sense information is translated.

What Do Viral Genomes Look Like?

As we have seen, viral genomes exhibit considerable diversity in form and function. Some small RNA and DNA

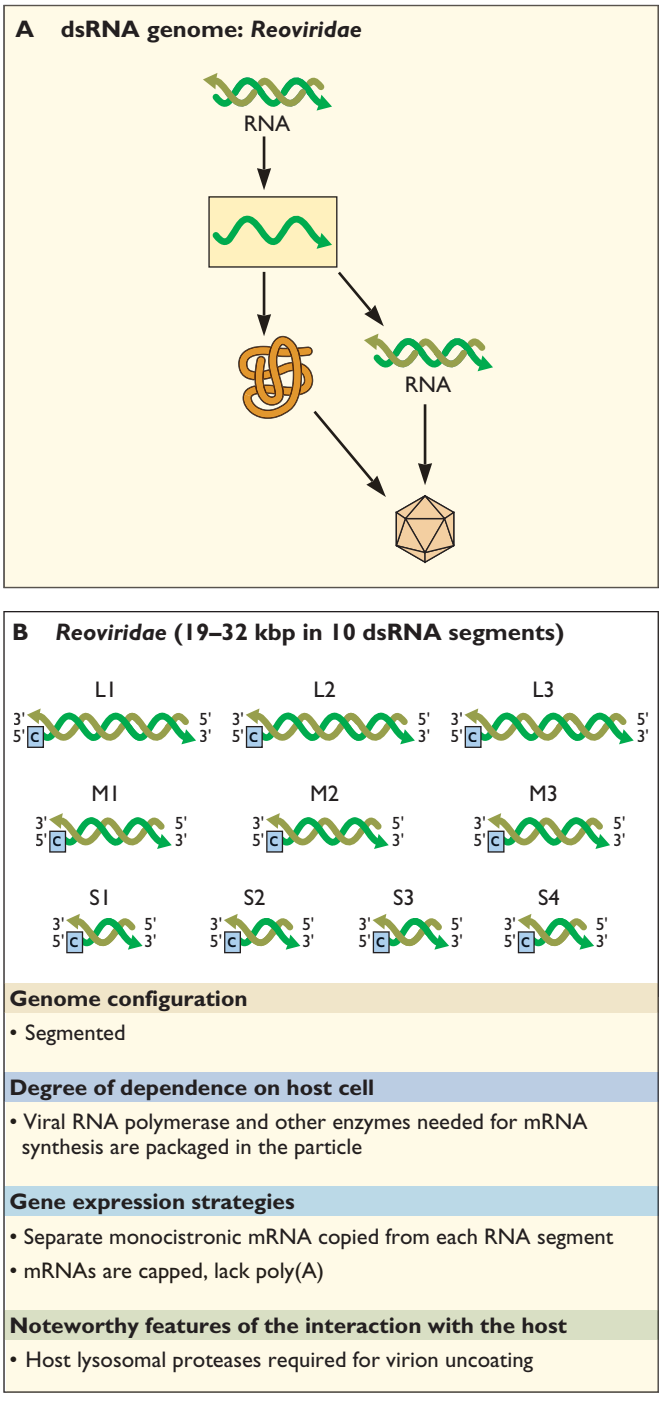


Figure 3.4

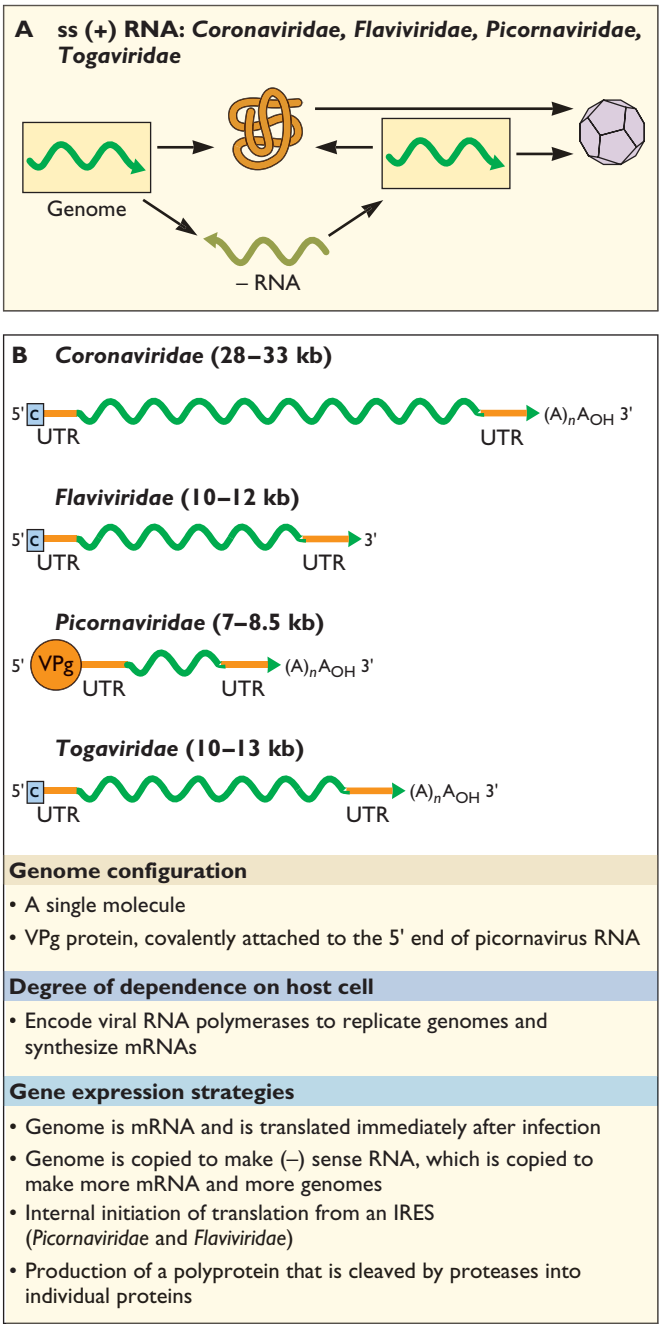


Figure 3.5

genomes enter cells from virus particles as naked molecules of nucleic acid, whereas others are always associated with specialized nucleic acid-binding proteins. A fundamental difference between the genomes of viruses and those of hosts is that while viral genomes often are covered with proteins, they are not bound by histones (polyomaviral genomes are a remarkable exception).

While viral genomes are all nucleic acids, they should **not** be thought of as one-dimensional structures. Virology textbooks (this one included) often draw genomes as straight, one-dimensional lines, but this notation is for illustrative purposes only; physical reality is certain to

be dramatically different. Genomes have the potential to adopt amazing secondary and tertiary structures (Fig. 3.8), some of which have been tested experimentally.

The sequences and structures near the ends of viral genomes are often indispensable for viral replication (Fig. 3.9). The DNA sequences at the ends of parvovirus genomes form T structures that are required for priming during DNA synthesis. Other structures needed for replication of RNA and DNA genomes include proteins covalently attached to 5′ ends, inverted and tandem repeats, and tRNAs. Secondary RNA structures may facilitate translation (the internal ribosome entry site (IRES) of picornavirus genomes) and genome packaging (the structured packaging signal of retrovirus genomes).

Coding Strategies

In general, the compact genome of most viruses renders the "one gene, one mRNA" dogma inaccurate. Extraordinary tactics have evolved for information retrieval from viral genomes (Fig. 3.10). Strategies including the production of multiple subgenomic mRNAs, mRNA splicing, RNA editing, and nested transcription units allow the production of multiple proteins from a single viral genome. Further expansion of the coding capacity of the viral genome is achieved by posttranscriptional mechanisms such as polyprotein synthesis, leaky scanning, suppression of termination, and ribosomal frameshifting. In general, the smaller the genome, the greater the compression of genetic information.

What Can Viral Sequences Tell Us?

Knowledge about the physical nature of genomes and coding strategies was first obtained by study of the nucleic acids of viruses. Indeed, DNA sequencing technology was perfected on viral genomes. The first genome of any kind to be sequenced was that of the *Escherichia coli* bacteriophage MS2, a linear ssRNA of 3,569 nucleotides. dsDNA genomes of the largest viruses, such as herpesviruses and poxviruses (vaccinia virus), were sequenced completely by the 1990s. Since then, the sequences of over 1,861 different viral genomes have been determined. Published viral genome sequences can be found at the website http://www.ncbi.nlm.nih.gov/sites/entrez.

Knowing the sequence of viral genomes has many uses, including classification of viruses. Sequence analysis has identified many relationships among diverse viral genomes, providing considerable insight into the origin of viruses. In outbreaks or epidemics of viral infection, even

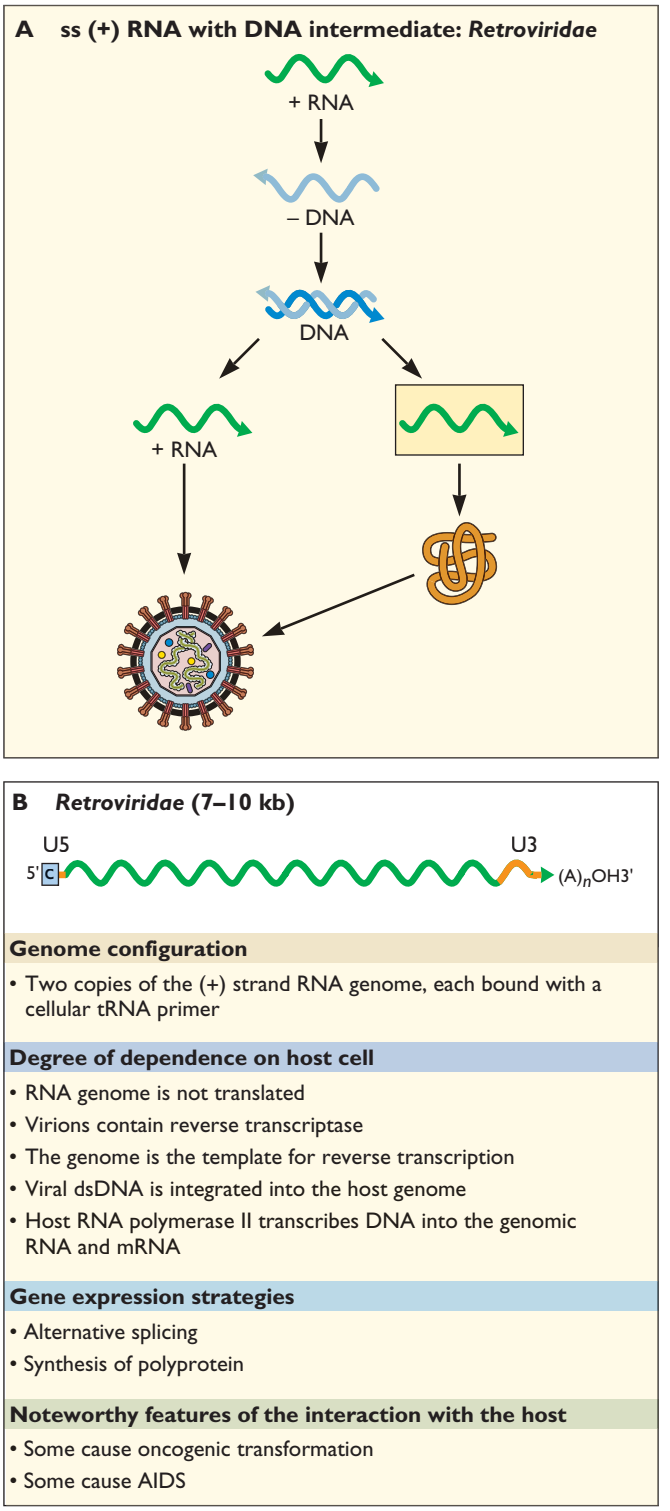


Figure 3.6

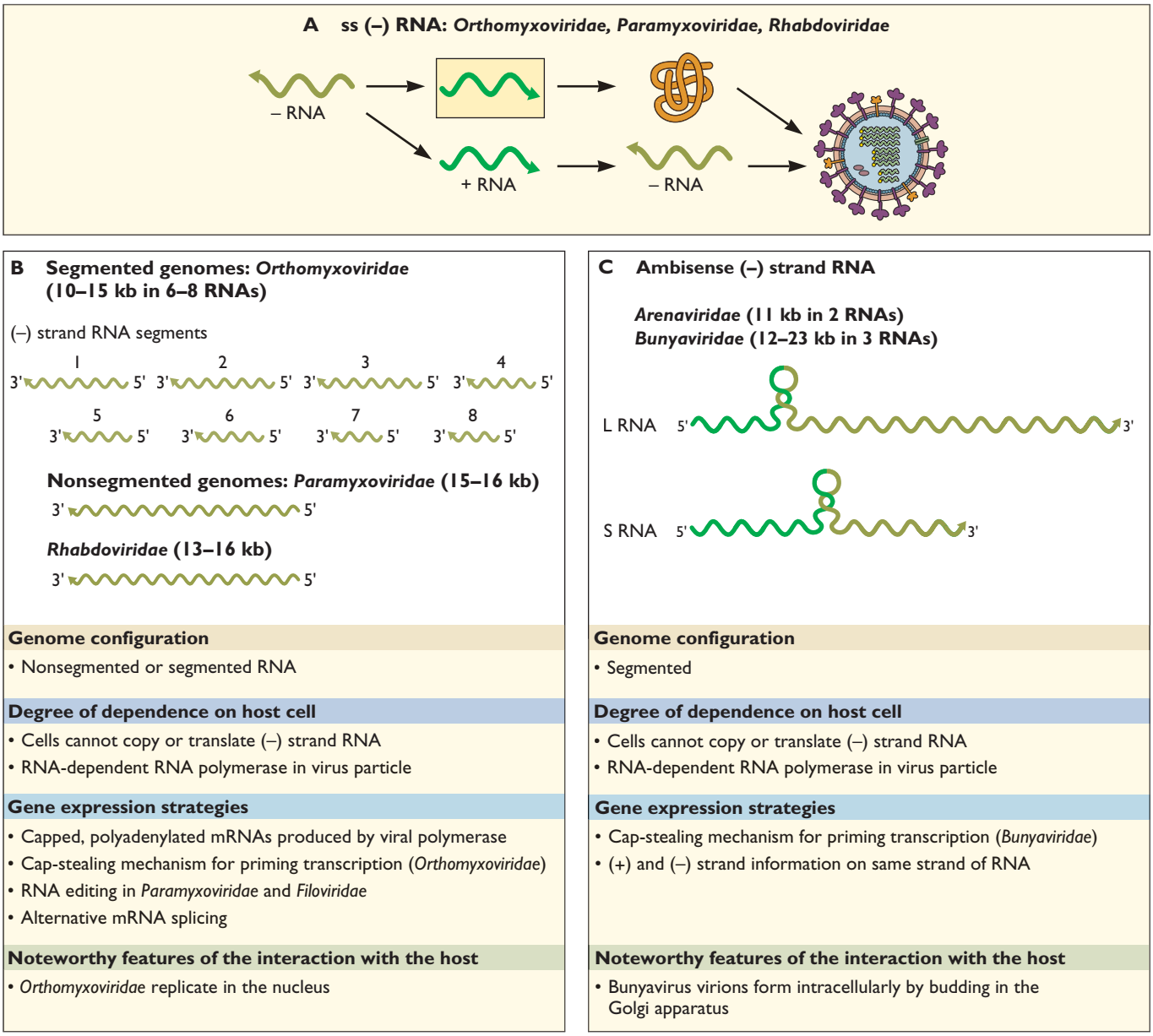


Figure 3.7

partial genome sequences can provide information on the identity of the virus and its movement in different populations. New viral nucleic acid sequences can be associated with disease and characterized in the absence of standard virological techniques (Volume II, Chapter 10). For example, a new herpesvirus called human herpesvirus 8 was identified by comparing sequences present in diseased and nondiseased tissues.

However, it quickly became clear that a complete understanding of how viruses reproduce cannot be obtained solely from the genome sequence or structure. In retrospect, this fact should have been anticipated. The genome sequence of a virus is at best a biological parts list: it provides some information about the intrinsic properties of a virus (e.g., viral proteins and their composition) but little or nothing about how the virus interacts with cells,

Figure 3.8 Genome structures in cartoons and in real life. (Top) Linear representation of the poliovirus genome. **(Bottom)** Model of the genome predicted by folding algorithms. The arrow points to the 5′ end of the RNA. Adapted from A. C. Palmenberg and J.-Y. Sgro, *Semin. Virol*. **8:**231–241, 1997, with permission. Courtesy of J.-Y. Sgro and A. C. Palmenberg, University of Wisconsin, Madison.

hosts, and populations. Because genomes are so complex, understanding them requires a reductionist analysis, the study of individual components in isolation. Although the reductionist approach is often experimentally the simplest, it is also important to understand how the genome behaves among others (population biology) and how the genome changes with time (evolution). Nevertheless, the reductionists have provided much-needed detailed information for tractable virus-host systems. These systems allow genetic and biochemical analyses and provide models of infection *in vivo* and *in vitro*. Unfortunately, viruses and hosts that are difficult or impossible to manipulate in the laboratory are understudied or ignored.

The Origin of Viral Genomes

Some people think that the origin of the viral genome is an impenetrable mystery, because no fossils are available for study. Others feel that by comparing genomes from diverse viruses, an evolutionary history of viruses may be constructed. This question is discussed further in Volume II, Chapter 10. Another question related to the origin of viruses is why there are both viruses with DNA genomes and viruses with RNA genomes. One possibility is that RNA viruses are relics of the "RNA world," a period during which RNA was **the** information **and** catalytic molecule (no proteins yet existed). During this time, possibly billions of years ago, life could have evolved from RNA, and the earliest organisms might have had RNA genomes. Viruses with RNA genomes might have evolved during this time. Later, DNA replaced RNA as the genetic material, perhaps through the action of reverse transcriptases, enzymes that produce DNA from RNA templates. With the emergence of DNA genomes came the evolution of DNA viruses. However, those with RNA genomes were still evolutionarily competitive, and hence they continue to survive to this day. Although the origins of RNA and DNA viruses will probably never be known, we do know that viruses with either type of nucleic acid are very successful.

The "Big and Small" of Viral Genomes: Does Size Matter?

Currently, the prize for the smallest nondefective animal virus genome goes to the circoviruses, which possess circular, ssDNA genomes of 1.7 to 2.3 kb (Fig. 3.3B). Members of the *Circoviridae* include chicken anemia virus, psittacine beak and feather disease virus, and TT virus, a ubiquitous human virus of no known consequence. The consolation prize goes to the *Hepadnaviridae*, such as hepatitis B virus, which causes hepatitis and liver cancer in millions of people. Its genome comprises 3.2 kb of gapped DNA: one strand is full length, but the complementary strand is

Figure 3.9 Genome structures critical for function. Abbreviations: ITR, inverted terminal repeat; TP, terminal protein; IRES, internal ribosome entry site; pbs and PBS, primer-binding site; DIS, dimerization initiation site; DLS, dimer linkage structure.

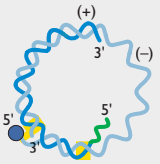
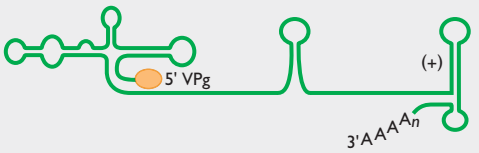
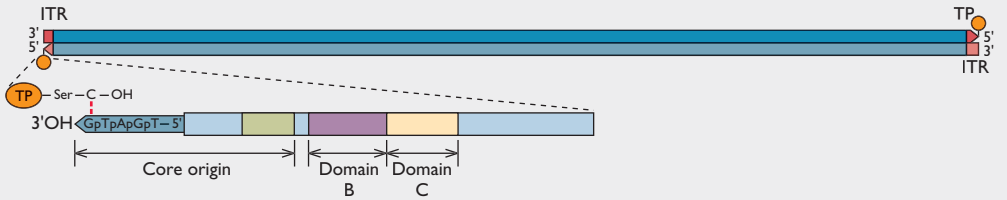
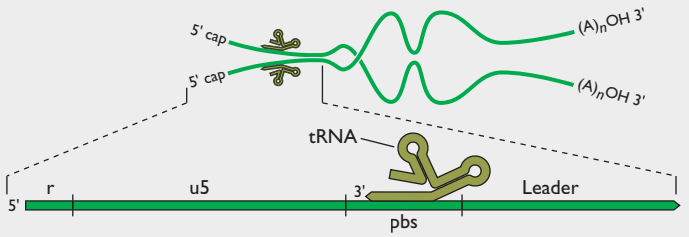
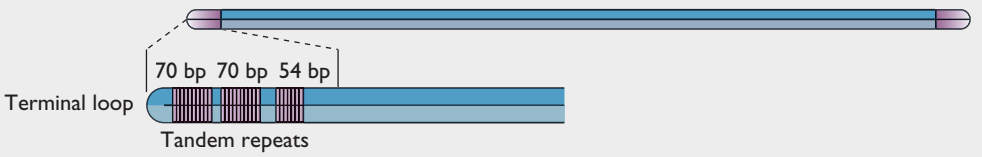
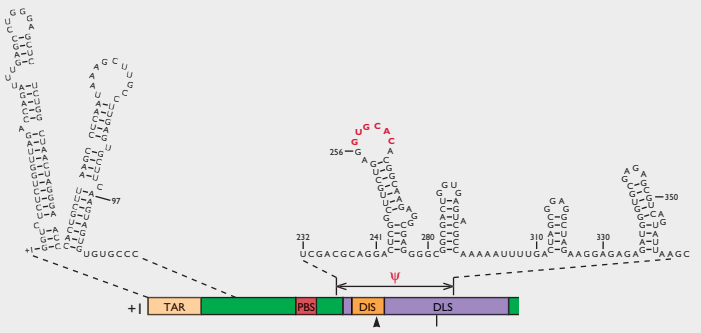
Function	Genome structure
Genome replication	
Parvovirus • T structure for priming DNA synthesis	
Hepatitis B virus • Primers for reverse transcription: 5'-linked P protein 5'-linked capped RNA	
Poliovirus • 5'-linked VPg primer • 5' cloverleaf • 3' pseudoknot • *cis*-acting replication element in coding region	
Adenovirus • Terminal protein • Inverted terminal repeat • Packaging sequences	
Retrovirus • (+) strand RNA dimer • tRNA primer • Inverted repeat sequences	
Poxvirus • Terminal loop • Tandem repeats	
Translation	
Poliovirus • IRES	
Assembly	
Retrovirus • Packaging signal	

Figure 3.9

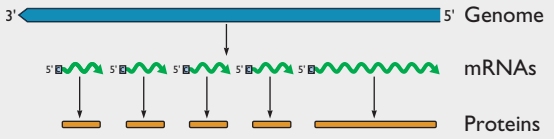
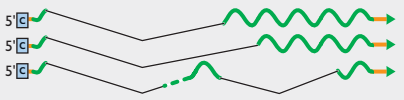
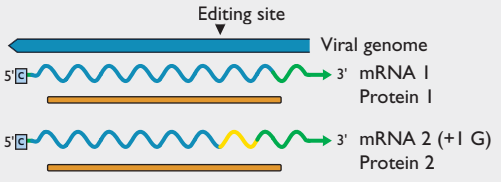
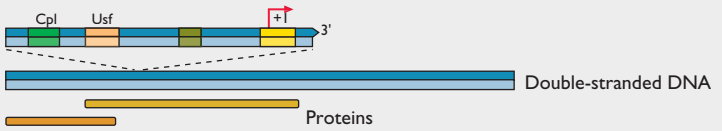
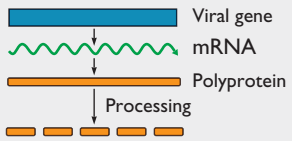
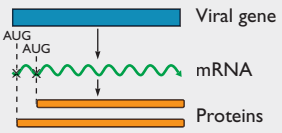
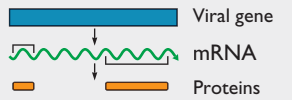
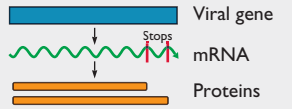
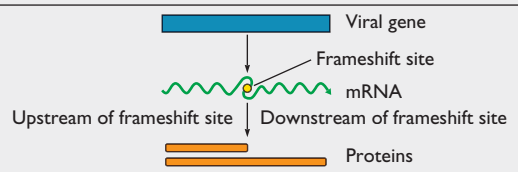
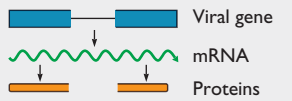
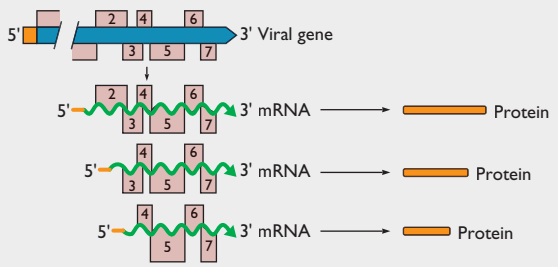
Mechanism	Diagram	Virus	Chapter(s)	Figures in appendix
Multiple subgenomic mRNAs		*Adenoviridae* *Hepadnaviridae* *Herpesviridae* *Paramyxoviridae* *Poxviridae* *Rhabdoviridae*	8, 10 7, 8 8 6 8 6	1, 2 3, 4 5–7 17, 18 23, 24
mRNA splicing		*Adenoviridae* *Orthomyxoviridae* *Papillomaviridae* *Polyomaviridae* *Retroviridae*	8, 10 10 8, 10 8, 10 7, 10	1, 2 8, 9 15, 16 21, 22
RNA editing		*Paramyxoviridae* *Filoviridae* Hepatitis delta satellite	6, 10 10 10	
Information on both strands		*Adenoviridae* *Polyomaviridae* *Retroviridae*	8–10 8–10 7	1, 2 15, 16 21, 22
Polyprotein synthesis		Alphaviruses *Flaviviridae* *Picornaviridae* *Retroviridae*	6, 11 6, 11 6, 11 6, 11	25, 26 13, 14 21, 22
Leaky scanning		*Orthomyxoviridae* *Paramyxoviridae* *Polyomaviridae* *Retroviridae*	11 11 11 11	8, 9 15, 16 21, 22
Reinitiation		*Orthomyxoviridae* *Herpesviridae*	11 11	8, 9 5–7
Suppression of termination		Alphaviruses *Retroviridae*	11 11	25, 26 21, 22
Ribosomal frameshifting		*Astroviridae* *Coronaviridae* *Retroviridae*	11 11 11	 21, 22
IRES		*Flaviviridae* *Picornaviridae*	11 11	 13, 14
Nested mRNAs		*Coronaviridae* *Arteriviridae*	6 6	

Figure 3.10

Table 3.2 Some viral proteins encoded by almost all large DNA viruses

Enzymes
DNA polymerase plus accessory replication proteins
Thymidine kinase
Ribonucleotide reductase
dUTPase
Exonucleases
Proteins that facilitate survival
Many "virulence" gene products to modulate the host's immune response or to foster invasion and spread
Cytokine homologs and receptors
Apoptosis inhibitors
Immune defense system modulators

BOX 3.4 BACKGROUND

Some curious observations

- Most RNA genomes in the world are in capsids (no cells have RNA genomes).
- There are no known animal viruses with "tailed" capsids like those of the bacteriophages.
- Most plant viruses have RNA genomes. DNA viruses of plants exist, of course (e.g., curious geminiviruses and the strange cauliflower mosaic virus).
- Most RNA viruses can cross species barriers; multiple hosts are common. For example, hosts of West Nile virus include birds, horses, and humans.
- DNA viruses tend to be more species specific than RNA viruses.
- Circular RNA viral genomes are rare, but circular DNA genomes abound.

incomplete (Fig. 3.2). Although it is not really a virus, honorable mention goes to hepatitis delta satellite virus, with a 1.7-kb single-stranded but highly base-paired circular RNA genome. This agent depends on hepatitis B virus to provide an envelope for transmission of its genome. Hepatitis B and delta satellite viruses infect hundreds of millions of people around the world.

The largest known virus genome, a DNA molecule of some 1,200 kbp, is that of mimivirus, a virus that infects amoebae. The largest RNA virus genome, 31 kb, is characteristic of some coronaviruses (Fig. 3.5). Despite detailed analyses, there is no evidence that one size is more advantageous than another. All viral genomes have evolved under relentless selection, so extremes of size must provide advantages. One feature distinguishing large genomes from smaller ones is the presence of many genes encoding proteins for viral replication, nucleic acid metabolism, and evasion of the host defense systems (Table 3.2). In other words, these large viruses have sufficient coding capacity to escape some restrictions of host cell biochemistry. The smallest viable cell genome is thought to contain less than 300 genes (predicted from bacterial genome sequences). Remarkably, this number of genes is smaller than the genetic content of large viral DNA genomes. Nevertheless, the big viruses are **not** cells; their replication absolutely requires host translation machinery, as well as host cell systems to make membranes and generate energy.

The factors that limit the size of viral genomes are largely unknown. There are cellular DNA and RNA molecules which are much longer than those found in virus particles. Consequently, the rate of nucleic acid synthesis is not likely to be limiting. For some viruses, the capsid volume (Box 3.4) might limit the size of viral genomes. There is a penalty inherent in having a large genome: a huge particle must be provided, and this is not a simple matter. The 150-kb herpes simplex virus genome resides in a $T = 16$ icosahedral nucleocapsid built from multiple copies of four proteins. In addition, the virion carries multiple copies of 20 or 30 tegument proteins and more than 15 membrane proteins in the viral envelope. Not evident in the inventory of the particle itself are the gene products required to assemble large, complicated capsids. In the case of herpes simplex virus, 50 to 60 gene products are needed to build the final particle to house the genome, yet there are only 84 known open reading frames. In other words, 75% of the viral genetic information is required to build the capsid. The largest known DNA viral genomes, those of mimivirus (1,200 kbp) and phycodnaviruses (330 kbp), are housed in the biggest capsids constructed with icosahedral symmetry. Although the principles of icosahedral symmetry are quite flexible in allowing a wide range of capsid sizes, it is possible that building a very large and stable capsid that can also come apart to release the viral genome is beyond the intrinsic properties of macromolecules.

One solution to the capsid size problem is to exploit helical symmetry. Particles built with helical symmetry can

Figure 3.10 Information retrieval from viral genomes. Different strategies for decoding the information in viral genomes are depicted. IRES, internal ribosome entry site.

in principle accommodate very large genomes, e.g., baculoviruses with DNA genomes up to 180 kbp.

In cells, DNA molecules are much longer than RNA molecules. RNA molecules are less stable than DNA, but in the cell much of the RNA is meant to be used for the synthesis of proteins and therefore need not exceed the size needed to specify the largest polypeptide. However, this constraint does not apply to viral genomes. Yet, the largest viral single-molecule RNA genomes, the 27- to 31-kb (+) strand RNAs of the coronaviruses, are dwarfed by the largest (1,200-kbp) DNA virus genomes. Susceptibility of RNA to nuclease attack might limit the size of viral RNA genomes, but there is little direct support for this hypothesis. The most likely explanation is that, as far as we know, there are no enzymes that can correct errors introduced during RNA synthesis. RNA polymerases, like their DNA counterparts, make mistakes. DNA polymerases can eliminate errors during polymerization, a process known as proofreading, and the errors can also be corrected after synthesis is complete. Such processes are not available during RNA synthesis. The average error frequencies for RNA genomes are about 1 misincorporation in 10^4 or 10^5 nucleotides polymerized. In an RNA viral genome of 10 kb, a mutation frequency of 1 in 10^4 would produce about one mutation in every replicated genome. Hence, very long viral RNA genomes, perhaps longer than 32 kb, would sustain too many lethal mutations. Even the 7.5-kb genome of poliovirus exists at the edge of viability: treatment of the virus with the RNA mutagen ribavirin causes a >99% loss in infectivity after a single round of replication.

Genetic Analysis of Viruses

The application of genetic methods to study the structure and function of animal viral genes and proteins began with development of the plaque assay by Dulbecco in 1952. This assay permitted the preparation of clonal stocks of virus, the measurement of virus titers, and a convenient system for studying viruses with conditional lethal mutations. Although a limited repertoire of classical genetic methods was available, the mutants that were isolated were invaluable in elucidating many aspects of infectious cycles and of cell transformation. Contemporary methods of genetic analysis based on recombinant DNA technology confer an essentially unlimited scope for genetic manipulation; in principle, any viral gene of interest can be mutated, and the precise nature of the mutation can be predetermined by the investigator. Much of the large body of information about viruses and their lifestyles that we now possess can be attributed to the power of these methods.

Classical Genetic Methods

Spontaneous and Induced Mutations

In the early days of experimental virology, mutant viruses could be isolated only by screening stocks for interesting phenotypes, for none of the tools that we now take for granted, such as restriction endonucleases, efficient DNA sequencing methods, or molecular cloning procedures, were developed until the mid- to late 1970s. RNA virus stocks usually contain a high proportion of mutants, and it is only a matter of devising the appropriate selection conditions (e.g., high or low temperature or exposure to drugs that inhibit viral growth) to select mutants with the desired phenotype from the total population. For example, the live attenuated poliovirus vaccine strains developed by Albert Sabin are mutants that were selected from a virulent virus stock (Volume II, Fig. 8.7). RNA virus mutants resistant to neutralization with monoclonal antibodies are often isolated from stocks at a frequency of 1 in 10^5 PFU. Mutation frequencies of RNA virus genomes are on the order of 1 misincorporation in 10^4 to 10^5 nucleotides polymerized, compared with 1 misincorporation in 10^8 to 10^{11} nucleotides incorporated for DNA genomes. This difference has been attributed to a lack of proofreading and error-correcting abilities in enzymes that replicate RNA.

The low spontaneous mutation rate of DNA viruses necessitated random mutagenesis by exposure to a chemical **mutagen**. Mutagens such as nitrous acid, hydroxylamine, and alkylating agents chemically modify the nucleic acid in preparations of virus particles, resulting in changes in base pairing during subsequent replication, and the substitution of an incorrect nucleotide. Mutagens such as base analogs, intercalating agents, or ultraviolet (UV) light are applied to the infected cell and cause changes in the viral genome during replication. Such agents introduce mutations more or less at random. Some mutations are lethal under all conditions, while others have no effect and are said to be silent. To increase the chances of obtaining viruses with a single genetic change, selective screens were applied after exposure to the lowest concentration of mutagen that produced a useful number of mutants. Under such conditions, many of the virus particles in the mutagenized population contain **wild-type** genomes (Box 3.5), making identification of mutants laborious. Some investigators have used chemicals to mutagenize RNA genomes, despite their high mutation rate.

To facilitate identification of mutants, the population must be screened for a phenotype that can be identified easily in a plaque assay. One such phenotype is temperature-sensitive growth of the virus. Virus mutants with this phenotype reproduce well at low temperatures but poorly or not at all at high temperatures. The permissive and nonpermissive temperatures are typically 39 and 33°C,

BOX 3.5 TERMINOLOGY

What is wild type?

Terminology can be confusing. Virologists often use terms such as strains, variants, and mutants to designate a virus that differs in some heritable way from a parental or wild-type virus. In conventional usage, the wild type is defined as the original (often laboratory-adapted) virus from which mutants are selected and which is used as the basis for comparison. A wild-type virus may **not** be identical to a virus isolated from nature. In fact, the genome of a wild-type virus may include numerous mutations accumulated during propagation in the laboratory. For example, the first isolate of poliovirus obtained in 1909 probably is very different from the virus we call wild type today.

We distinguish carefully between laboratory wild types and new virus isolates from the natural host. The latter are called field isolates or clinical isolates.

respectively, for viruses that replicate in mammalian cells. Other commonly sought phenotypes are changes in plaque size or morphology, drug resistance, antibody resistance, and host range (that is, loss of the ability to multiply in certain hosts or host cells). The nomenclature used to identify viral and cellular genes, essential for describing mutations, is explained in Box 3.6.

Mapping Mutations

Before the advent of recombinant DNA technology, it was extremely difficult for investigators to determine the locations of mutations in viral genomes. The **marker rescue** technique (described in "Introducing Mutations into the Viral Genome" below) was a solution to this problem, but before it was developed, other, less satisfactory approaches were exploited.

Recombination mapping can be applied to both DNA and RNA viruses. Recombination results in genetic exchange between genomes within the infected cell. For viruses with unimolecular genomes, the frequency of recombination between two mutations increases with the physical distance separating them. In practice, cells are coinfected with two mutants, and the frequency of recombination is calculated by dividing the titer of phenotypically wild-type virus obtained under restrictive conditions (e.g., high temperature) by the titer obtained under permissive conditions (e.g., low temperature). The recombination frequency between pairs of mutants is determined, allowing the mutations to be placed on contiguous maps. Although a location can be assigned for each mutation relative to others, this approach does not result in a physical map of the actual location of the base change in the genome.

In the case of RNA viruses with segmented genomes, the technique of **reassortment** allowed the assignment of mutations to specific genome segments. When cells are coinfected with both mutant and wild-type viruses, the progeny includes **reassortants** that inherit RNA segments from either parental virus. The origins of the RNA segments can be deduced from their migration patterns during gel electrophoresis (Fig. 3.11) or by nucleic acid

BOX 3.6 TERMINOLOGY

Genetic nomenclature

From the earliest days of genetic analysis, it has been customary to use abbreviations to designate gene names. Unfortunately, no standard nomenclature has been established for the genes of mammalian cells and their viruses. To facilitate our discussion of genes and mutations, we use the following conventions. Abbreviations for cellular genes are given in lowercase, italicized letters (e.g., *pvr* for poliovirus receptor gene). In abbreviations for cellular proteins, the first letter is capitalized and the remainder are lowercase (e.g., Pvr for poliovirus receptor protein). We use the names of some cellular proteins, and all viral genes and proteins, that do not conform to these conventions: they have become firmly entrenched in the scientific literature, and have come to have a life of their own. This nomenclature is not consistent among viruses, and in some cases the gene and protein names do not relate to one another (e.g., the membrane glycoprotein gD of herpes simplex virus is encoded by the US6 gene). The reader should consult the appendix in this volume to avoid confusion about such names.

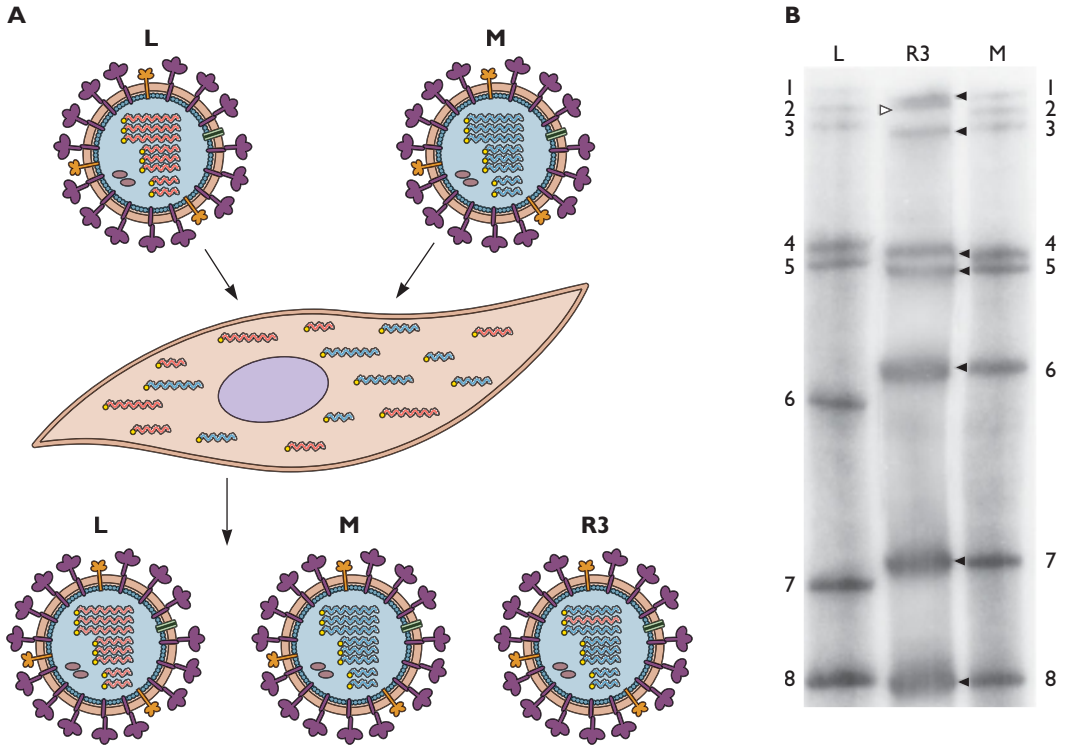


Figure 3.11 Reassortment of influenza virus RNA segments. (A) Progeny viruses of cells that are coinfected with two influenza virus strains, L and M, include both parents and viruses that derive RNA segments from them. Recombinant R3 has inherited segment 2 from the L strain and the remaining seven segments from the M strain. **(B)** ^{32}P-labeled influenza virus RNAs were fractionated in a polyacrylamide gel and detected by autoradiography. Migration differences of parental viral RNAs (M and L) permitted identification of the origin of RNA segments in the progeny virus R3. Panel B is reprinted from V. R. Racaniello and P. Palese, *J. Virol*. **29:**361–373, 1979, with permission.

hybridization. By analyzing a panel of such reassortants, the segment responsible for the phenotype can be identified. When the protein products of each RNA segment are later identified, the mutation can be assigned unambiguously.

Functional Analysis

The term **complementation** describes the ability of gene products from two different nonreplicating mutant viruses to interact functionally in the same cell, permitting viral replication. If the mutations are in separate genes, each virus is able to supply a functional gene product, allowing both viruses to replicate. If the two viruses carry mutations in the same gene, no replication will occur. In this way, the members of collections of mutants obtained by chemical mutagenesis were initially organized into complementation groups defining separate viral functions. In theory, there can be as many complementation groups as genes.

Complementation can be distinguished from recombination or reassortment by examining the progeny produced by coinfected cells. True complementation yields only the two parental mutants, while wild-type genomes also result from recombination or reassortment.

Engineering Mutations into Viral Genomes

Infectious DNA Clones

Recombinant DNA techniques have made it possible to introduce any kind of mutation anywhere in the genome of most animal viruses, whether that genome comprises DNA or RNA. The holy grail of virology today is the **infectious DNA clone,** a dsDNA copy of the viral genome that is carried on a bacterial plasmid. Infectious DNA clones, or *in vitro* transcripts derived from them, can be introduced into cultured cells by **transfection** (Box 3.7) to recover infectious virus. This approach is a modern validation of the Hershey-Chase experiment described in Chapter 1.

BOX 3.7 **TERMINOLOGY**

DNA-mediated transformation and transfection

The introduction of foreign DNA into cells is called DNA-mediated transformation to distinguish it from the oncogenic transformation of cells caused by tumor viruses and other insults. The term "transfection" (transformation-infection) was coined to describe the production of infectious virus after transformation of cells by viral DNA, first demonstrated with bacteriophage lambda. Unfortunately, the term "transfection" is now routinely used to describe the introduction of any DNA or RNA into cells. In this textbook, we use the correct nomenclature: the term "transfection" is restricted to the introduction of viral DNA or RNA into cells with the goal of obtaining virus replication.

The availability of site-specific bacterial restriction endonucleases, DNA ligases, and an array of methods for mutagenesis has made it possible to manipulate these infectious clones at will. Infectious DNA clones also provide a stable repository of the viral genome, which is particularly important for vaccine strains.

DNA viruses. Current genetic methods for the study of most viruses with DNA genomes are based on the infectivity of viral DNA. When deproteinized viral DNA molecules are introduced into permissive cells by transfection, they generally initiate a complete infectious cycle, although the infectivity (i.e., number of plaques per microgram of DNA) may be low. For example, the infectivity of deproteinized human adenoviral DNA is between 10 and 100 plaque-forming units (PFU) per μg. When the genome is isolated by procedures that do not degrade the covalently attached terminal protein, infectivity is increased by 2 orders of magnitude, probably because this protein participates in the assembly of initiation complexes on the viral origins of replication.

The complete genomes of polyomaviruses, papillomaviruses, and adenoviruses can be cloned in plasmid vectors, and such DNA is infectious under appropriate conditions. The DNA genomes of herpesviruses and poxviruses are too large to insert into conventional bacterial plasmid vectors, but they can be cloned in vectors that accept larger insertions (e.g., cosmids and bacterial artificial chromosomes). The plasmids containing these cloned herpesvirus genomes are infectious. Poxvirus DNA is not infectious, because the viral promoters cannot be recognized by cellular DNA-dependent RNA polymerase. Poxvirus DNA is infectious when early functions (viral DNA-dependent RNA polymerase and transcription proteins) are provided by a helper virus.

RNA viruses. *(+) strand RNA viruses.* The genomic RNA of retroviruses is copied into a dsDNA form by reverse transcriptase early during infection, a process described in Chapter 7. Such DNA is infectious when introduced into cells, as are molecularly cloned forms inserted into bacterial plasmids.

Introduction of a plasmid containing cloned poliovirus DNA into cultured mammalian cells results in the production of progeny virus (Fig. 3.12A). The mechanism by which cloned poliovirus DNA initiates infection is not known, but it has been suggested that the DNA enters the nucleus, where it is transcribed by cellular DNA-dependent RNA polymerase from cryptic, promoter-like sequences on the plasmid. The resulting (+) strand RNA transcripts initiate an infectious cycle. During replication, the extra terminal nucleotide sequences must be removed or ignored, because the viruses that are produced contain RNA with the authentic 5′ and 3′ termini.

The genomic RNA of poliovirus has a higher specific infectivity (10^6 PFU per μg) than does cloned DNA (10^3 PFU per μg). By incorporating promoters for bacteriophage T7 DNA-dependent RNA polymerase in plasmids containing poliovirus DNA, full-length (+) strand RNA transcripts can be synthesized *in vitro*. The specific infectivity of such RNA transcripts resembles that of genomic RNA. Infectious DNA clones have been constructed for many (+) strand RNA viruses, including members of the *Arteriviridae*, *Caliciviridae*, *Coronaviridae*, *Flaviviridae*, *Picornaviridae*, and *Togaviridae*.

(–) strand RNA viruses. Genomic RNA of (–) strand RNA viruses is not infectious, because it can be neither translated nor copied into (+) strand RNA by host cell RNA polymerases, as discussed in Chapter 6. Two different experimental approaches have been used to develop infectious DNA clones of these viral genomes (Fig. 3.12B and C).

The recovery of influenza virus from cloned DNA is achieved by an expression system in which cloned DNA copies of the eight RNA segments of the viral genome are inserted between two promoters (Fig. 3.12B). When eight plasmids carrying DNA for each viral RNA segment

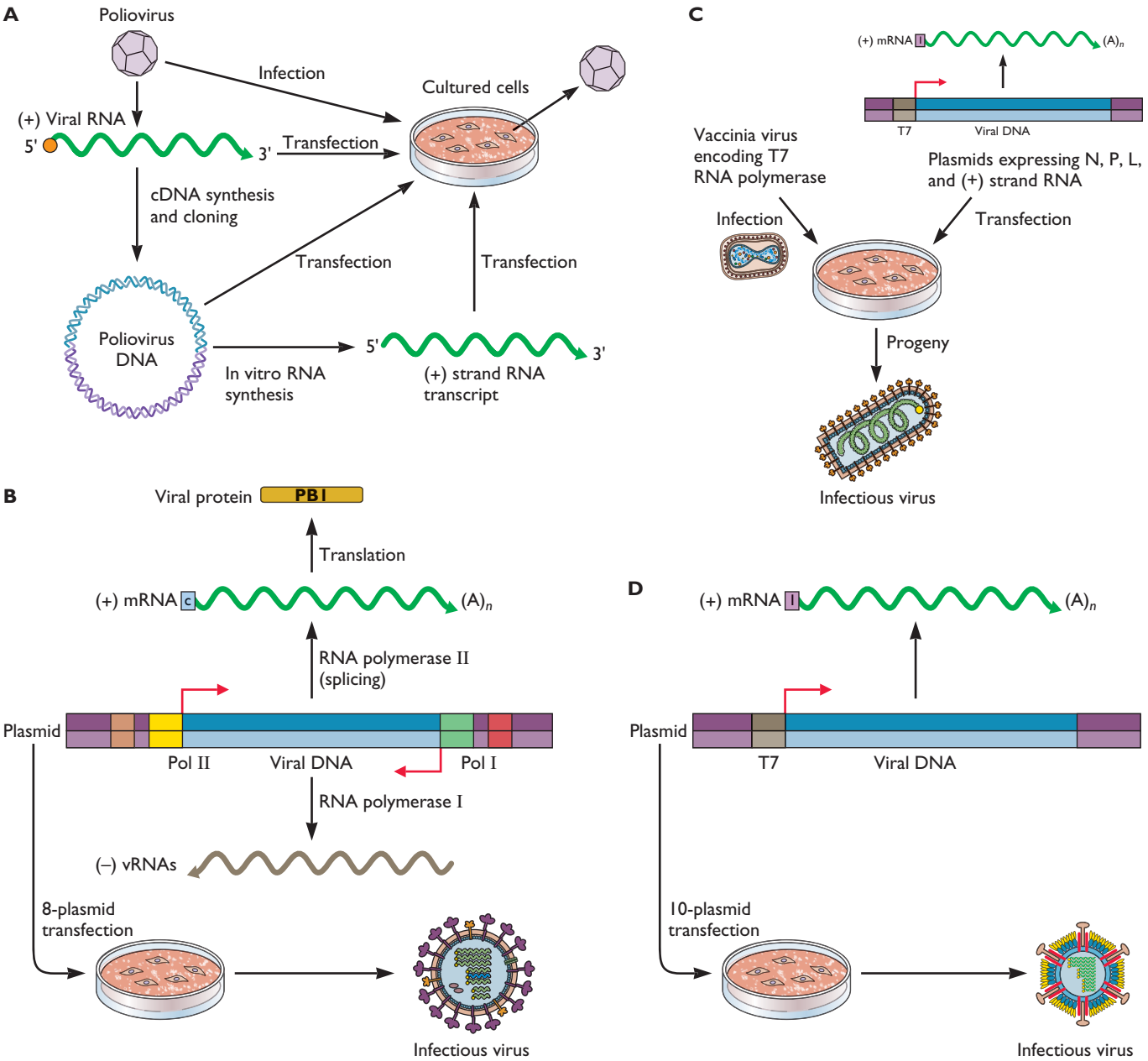


Figure 3.12 Genetic manipulation of RNA viruses. (A) Recovery of infectivity from cloned DNA of (+) strand RNA genomes as exemplified by genomic RNA of poliovirus, which is infectious when introduced into cultured cells by transfection. A complete DNA clone of the viral RNA, carried in a plasmid, is also infectious, as are RNAs derived by *in vitro* transcription of the full-length DNA. **(B)** Recovery of influenza viruses by transfection of cells with eight plasmids. Cloned DNA of each of the eight influenza virus RNA segments is inserted between an RNA polymerase I promoter (Pol I, green) and terminator (brown), and an RNA polymerase II promoter (Pol II, yellow) and a polyadenylation signal (red). When the plasmids are introduced into mammalian cells, (–) strand viral RNA (vRNA) molecules are synthesized from the RNA polymerase I promoter, and mRNAs are produced by transcription from the RNA polymerase II promoter. The mRNAs are translated into viral proteins, and infectious virus is produced from the transfected cells. For clarity, only one cloned viral RNA segment is shown. Adapted from E. G. Hoffmann et al., *Proc. Natl. Acad. Sci. USA* **97:**6108–6113, 2000, with permission. **(C)** Recovery of infectious virus from cloned DNA of viruses with a (–) strand RNA genome. Cells are infected with a vaccinia virus recombinant that synthesizes T7 RNA polymerase and transformed with plasmids that encode a full length (+) strand copy of the viral

(continued on next page)

BOX 3.8 **TERMINOLOGY**

Operations on DNA and on protein

A mutation is a change in DNA or RNA comprising base changes and nucleotide additions, deletions, and rearrangements. When mutations occur in open reading frames, they can be manifested as changes in the synthesized proteins. For example, one or more base changes in a specific codon may produce a single amino acid substitution, a truncated protein, or no protein. The terms "mutation" and "deletion" are often used incorrectly, or ambiguously to describe alterations in proteins. In this textbook, these terms are used to describe genetic changes, and the terms "amino acid substitution" and "truncation" are used to describe protein alterations.

are introduced into cells, infectious influenza virus is produced.

For (–) strand RNA viruses with a nonsegmented genome, such as vesicular stomatitis virus (a rhabdovirus), the full-length (–) strand genomic RNA is not infectious, because it cannot be translated into protein or copied into mRNA by the host cell. When the full-length (–) strand is introduced into cells that produce viral proteins required for production of mRNA, no infectious virus is recovered. However, when a full-length (+) strand RNA is transfected into cells that synthesize the vesicular stomatitis virus nucleocapsid protein, phosphoprotein, and polymerase, the (+) strand RNA is copied into (–) strand RNAs. These RNAs initiate an infectious cycle, leading to the production of new virus particles.

dsRNA viruses. Genomic RNA of dsRNA viruses is not infectious because the (+) strand cannot be translated. The recovery of reovirus from cloned DNA is achieved by an expression system in which cloned DNA copies of the 10 RNA segments of the viral genome are inserted under the control of an RNA polymerase promoter (Fig. 3.12D). When 10 plasmids carrying DNA for each viral dsRNA segment are introduced into cells, infectious reovirus is produced.

Types of Mutation

Recombinant DNA techniques allow the introduction of many kinds of mutation at any desired site in cloned DNA (Box 3.8). Indeed, provided that the sequence of the segment of the viral genome to be mutated is known, there is little restriction on the type of mutation that can be introduced. **Deletion mutations** can be used to remove an entire gene to assess its role in replication, to produce truncated gene products, or to assess the functions of specific segments of a coding sequence. Noncoding regions can be deleted to identify and characterize regulatory sequences such as promoters. **Insertion mutations** can be made by the addition of unrelated sequences or sequences derived from a closely related virus. **Substitution mutations**, which can correspond to one or more nucleotides, are often made in coding or noncoding regions. Included in the latter class are **nonsense mutations**, in which a termination codon is introduced, and **missense mutations**, in which a single nucleotide or a codon is changed, resulting in the production of a protein with a single amino acid substitution. The introduction of a termination codon is frequently exploited to cause truncation of a membrane protein so that it is secreted or to eliminate the synthesis of a protein without changing the size of the viral genome or mRNA. Substitutions are used to assess the roles of specific

Figure 3.12 ***(continued)*** genome RNA and proteins required for viral RNA synthesis (N, P, and L proteins). Production of RNA from these plasmids is under the control of the bacteriophage T7 RNA polymerse promoter (brown). Because bacteriophage T7 RNA transcripts are uncapped, an internal ribosome entry site (I) is included so the mRNAs will be translated. After the plasmids are transfected into cells, the (+) strand RNA is copied into (–) strands, which in turn are used as templates for mRNA synthesis and genome replication. The example shown is for viruses with a single (–) strand RNA genome (e.g., rhabdoviruses and paramyxoviruses). A similar approach has been demonstrated for Bunyamwera virus, with a genome comprising three (–) strand RNAs. **(D)** Recovery of infectious virus from cloned DNA of dsRNA viruses. Cloned DNA of each of the 10 reovirus dsRNA segments is inserted under the control of a bacteriophage T7 RNA polymerase promoter (brown). Because bacteriophage T7 RNA transcripts are uncapped, an internal ribosome entry site (I) is included so the mRNAs will be translated. Cells are infected with a vaccinia virus recombinant that synthesizes T7 RNA polymerase and transformed with all 10 plasmids. For clarity, only one cloned viral RNA segment is shown.

nucleotides in regulatory sequences or of amino acids in protein function, such as polymerase activity or binding of a viral protein to a cell receptor. An example of a longer substitution is the exchange of nucleic acid sequences encoding a specific protein sequence among virus strains that differ in host range. The construction of such hybrid viruses can be used to assign a biological function to a specific gene product and to determine whether it functions independently of the viral genetic background.

Introducing Mutations into the Viral Genome

Mutations can be introduced rapidly into a viral genome when it is cloned in its entirety. Mutagenesis is usually carried out on cloned subfragments of the viral genome, which are then substituted into full-length cloned DNA. The final step is introduction of the mutagenized DNA into cultured cells by transfection. This approach has been applied to cloned DNA copies of RNA and DNA viral genomes.

For the large DNA viruses such as adenoviruses, herpesviruses, and poxviruses, it is not always practical to replace cloned and mutagenized subfragments of the viral DNA because of the lack of convenient, unique restriction enzyme sites. A less direct, but effective, method for introducing mutations into these viral DNA genomes is **marker transfer** (where a mutation is marked or traced by its phenotype). The marker transfer method is applicable to any DNA virus provided that the target sequence is available as a DNA fragment in a vector that allows cloning and amplification in *E. coli*. Cells are transfected with the viral genome together with a DNA fragment containing the desired mutation. Recombination between the DNAs produces viral genomes containing the desired mutation. The use of marker transfer to introduce mutations into the genome of a DNA virus is shown in Fig. 3.13.

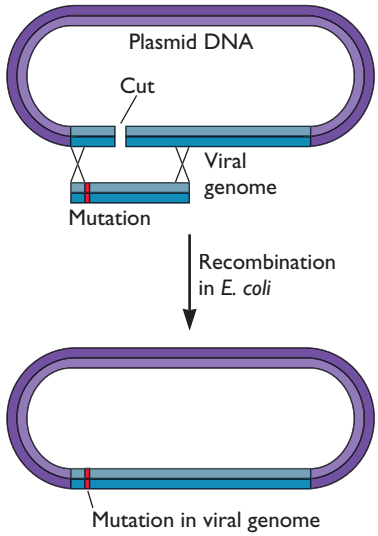


Figure 3.13 Introduction of mutations into a DNA viral genome. A plasmid carrying the entire viral DNA genome is cleaved with a suitable restriction endonuclease to produce a single cut in the area of the desired mutation. This cleaved plasmid is introduced into *E. coli* cells together with a shorter DNA containing the desired mutation. The cleaved plasmid is unable to replicate in *E. coli*. Only plasmids produced by homologous recombination between the adenoviral sequences in the cleaved plasmid and on the DNA fragment will be propagated in *E. coli*. The resulting recombinant plasmids contain a full-length copy of the viral DNA genome with the desired mutation.

Introduction of mutagenized viral nucleic acid into cultured cells by transfection may produce one of several possible results. The mutation may have no effect on viral replication; it may have a subtle effect that is discovered only on subsequent study; it may impart a readily detectable phenotype; or it may prevent the production of infectious virus. In the last-mentioned case, it is necessary to complement the defect in cell lines that have been engineered to produce specific viral proteins.

When the sequences of interest control viral gene expression or replication, complementation is not possible. This limitation is not as prohibitive as it might appear. One approach that can be used to study control regions is to introduce mutations that impair but do not eliminate virus replication. For example, certain mutations introduced into the major late promoter of human adenoviruses reduce virus yield more than 30-fold. An alternative approach is to study the effects of lethal mutations in cloned copies of the viral genome carried in plasmids or bacterial artificial chromosomes.

Mutations introduced into a specific gene might have unanticipated **polar** effects on other genes that are not altered in sequence. Insertions of the *lacZ* gene into the coding sequence for the gG gene of pseudorabies virus impaired cell-to-cell spread of the virus. It was initially concluded that this phenotype is the result of loss of gG gene expression. However, introduction of a nonsense mutation that prevents gG synthesis produced viruses with no defects in cell-to-cell spread. It was then discovered that large DNA insertions in US4 cause dramatic reductions in proteins encoded in an upstream gene, US3. These findings emphasize the risk of unanticipated changes in gene expression when inserting new sequences into the viral genome.

Reversion Analysis

The phenotypes caused by mutation can **revert** in one of two ways: by change of the original mutation to the wild-type sequence or by acquisition of a mutation at a second site, either in the same gene or in a different gene.

BOX 3.9 DISCUSSION

The raison d'être of reversion analysis: is the observed phenotype due to the mutation?

The Problem

The requirement for construction and analysis of revertants is well understood in genetic analysis. The assertion that a phenotype arises from the mutation and the conclusion that the mutated gene mediates the function cannot stand without careful reversion analysis.

In genetic analysis of viruses, mutations are made *in vitro* by a variety of techniques, all of which can introduce unexpected mutations. Errors can be introduced during cloning, during polymerase chain reactions (pcr), and during sequencing. Unexpected mutations often arise during the introduction of viral DNA and plasmid DNA into the eukaryotic cell. The process of recombination when viral DNA meets plasmid DNA in the eukaryotic cell is not error-proof. Tens of thousands of DNA copies are forced into a cell (transfection), and only a tiny portion give rise to the desired genome. Viral DNA isolated from cells or virions contains nicks, gaps, and even RNA. Isolation of large, linear viral DNA by standard micropipetting techniques usually shears the DNA into a population of subgenomic fragments that must be repaired and rejoined in the eukaryotic cells. Less than perfect repair fidelity is a potential source of undesired, second-site mutations. Indeed, linear DNA in eukaryotic cells can be attacked at the ends, and the ssDNA that results from such an attack can invade at nicked sites in linear DNA. In addition, the transfection process itself induces a variety of stress responses that might activate repair pathways.

Some Solutions

- Construct more than one mutant by using totally different sources of DNA. It is unlikely that an unlinked mutation with the same phenotype would occur twice.
- Marker rescue. All local DNA around the mutation is replaced with parental DNA. If the mutation indeed causes the phenotype, the wild-type phenotype should be restored in the rescued virus.
- Ectopic expression of the wild-type protein in the mutant background. If the wild-type phenotype is restored (complemented), then the probability is high that the phenotype arises from the mutation. The merit of this method over marker rescue is that the latter shows only that unlinked mutations are unlikely to be the cause of the phenotype.

All of these approaches have limitations, and it is prudent to use more than one.

Reversion analysis is an integral part of genetics: when confidence is high that the phenotype observed is due to the known mutation, research progresses rapidly.

Phenotypic reversion caused by second-site mutation is known as **suppression**, or **pseudoreversion**, to distinguish it from reversion at the original site of mutation. Reversion has been studied since the beginnings of classical genetic analysis (Box 3.9). When suspected pseudorevertants are crossed with wild-type viruses, the mutant phenotype should be observed in the progeny as a result of segregation of the original mutation from the suppressor mutation. In the modern era of genetics, cloning and sequencing techniques can be used to demonstrate suppression and to identify the nature of the suppressor mutation (see below). The identification of suppressor mutations is a powerful tool for studying protein-protein and protein-nucleic acid interactions. Some suppressor mutations complement changes made at several sites, whereas **allele-specific** suppressors complement only a specific change. The allele specificity of second-site mutations provides evidence for physical interactions among proteins and nucleic acids.

Phenotypic revertants can be isolated either by propagating the mutant virus under restrictive conditions or, with mutants exhibiting nonconditional phenotypes, by searching for wild-type properties. For DNA viruses, chemical mutagenesis may be required to produce revertants, but this is not necessary for RNA viruses, which spawn mutants at a higher frequency. Next, nucleotide sequence analysis is used to determine if the original mutation is still present in the genome of the revertant. The presence of the original mutation indicates that reversion has occurred by second-site mutation. The objective is to identify a fragment that contains the suppressor mutation and is small enough (~1 kb) that its nucleotide sequence can be determined. A similar analysis can be carried out for RNA viruses, except that DNA clones must first be derived from the genomes of phenotypic suppressors. The final step is introduction of the suspected suppressor mutation into the genome of the original mutant virus to confirm its effect. Several specific examples of suppressor analysis are provided below.

Some mutations within the origin of replication (Ori) of simian virus 40 reduce viral DNA replication and induce the formation of small plaques. Pseudorevertants of Ori mutants were isolated by random mutagenesis of mutant viral DNA followed by introduction into cultured cells and selection of viruses that form large plaques. The second-site mutations that suppressed the replication defects were localized to a specific region within the gene for large T antigen. These results indicated that a specific domain of large T antigen interacts with the Ori sequence during viral replication.

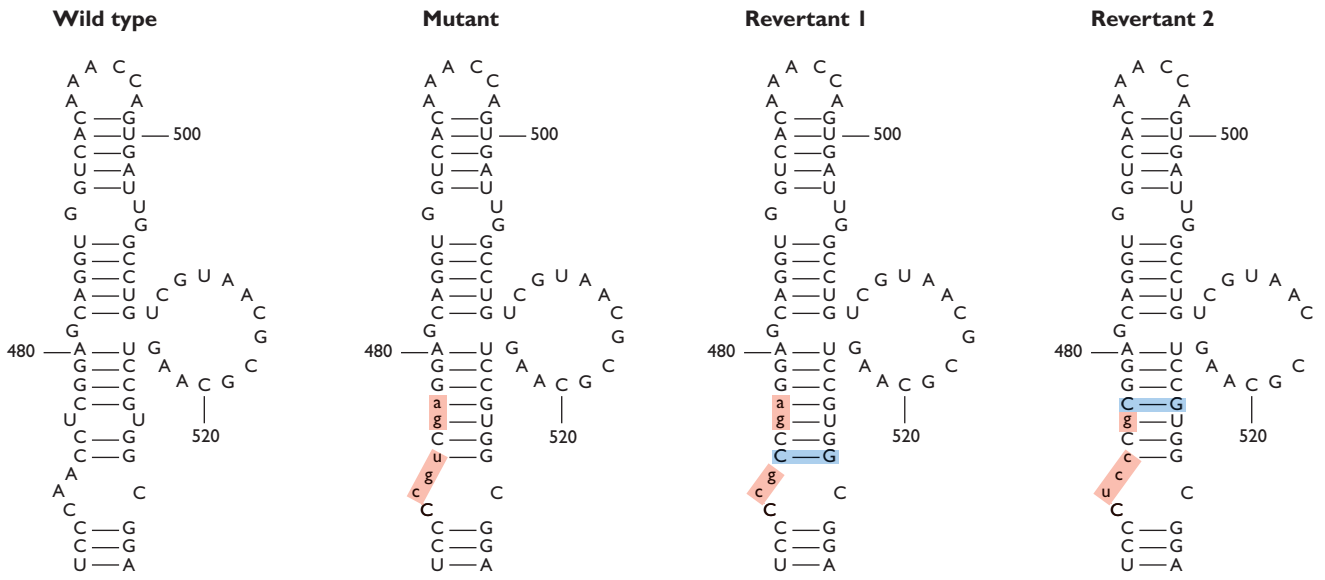


Figure 3.14 Effect of second-site suppressor mutations on predicted secondary structure in the 5' untranslated region of poliovirus (+) strand RNA. Diagrams of the region between nucleotides 468 and 534, which corresponds to stem-loop V (Chapter 11), are shown. These include, from left to right, sequences of wild-type poliovirus type 1, a mutant containing the nucleotide changes highlighted in orange, and two phenotypic revertants. Two C:G base pairs present in the wild-type parent and destroyed by the mutation are restored by second-site reversion (blue shading). Adapted from A. A. Haller et al., *J. Virol.* **70:**1467–1474, 1996, with permission.

The 5′ untranslated region of the poliovirus genome contains extensive RNA secondary-structure features that are important for RNA translation and replication, as discussed in Chapters 6 and 11. Disruption of such secondary structure by substitution of an 8-nucleotide sequence produces a virus that replicates poorly and readily gives rise to pseudorevertants that replicate more efficiently (Fig. 3.14). Nucleotide sequence analysis of two pseudorevertants demonstrated that they contain base changes that restore the disrupted secondary structure. These results confirm that the RNA secondary structure is important for the biological activity of this untranslated region.

Genetic Interference by Double-Stranded RNA

RNA interference (RNAi) has become a powerful and widely used tool for analyzing gene function. In this technique, duplexes of 21-nucleotide RNA molecules, called **small interfering RNAs (siRNAs)**, corresponding to the gene to be silenced are synthesized chemically or by transcription reactions. siRNAs or plasmids which encode them are introduced into cultured cells by transformation, and they efficiently inhibit the production of specific proteins by causing sequence-specific mRNA degradation. The functions of specific viral or cellular proteins during infection can therefore be studied by using this technique (Fig. 3.15). The mechanism of mRNA degradation mediated by siRNA is discussed in Chapter 10.

Engineering Viral Genomes: Viral Vectors

Naked DNA can be introduced into cultured animal cells as complexes with calcium phosphate or lipid-based reagents or by electroporation. Such DNA can support expression of its gene products transiently or stably from an integrated or episomal copy. Introduction of DNA into cells is a routine method in virological research and is also employed for certain clinical applications, such as the production of a therapeutic protein or a vaccine. However, this approach is not suitable for certain applications. For example, one goal of gene therapy is to deliver a gene to patients who either lack the gene or carry defective versions of it (Table 3.3). An approach to this problem is to create a cell line that synthesizes the gene product. After infusion into patients, the cells can become permanently established in tissues. If the primary cells to be used are limiting in a culture (e.g., stem cells), it is not practical to select and amplify the rare cells that receive naked DNA. Recombinant viruses carrying foreign genes can infect a greater percentage of cells and thus facilitate generation of the desired cell lines. These viral vectors have also found widespread use in the research laboratory. A complete understanding of the

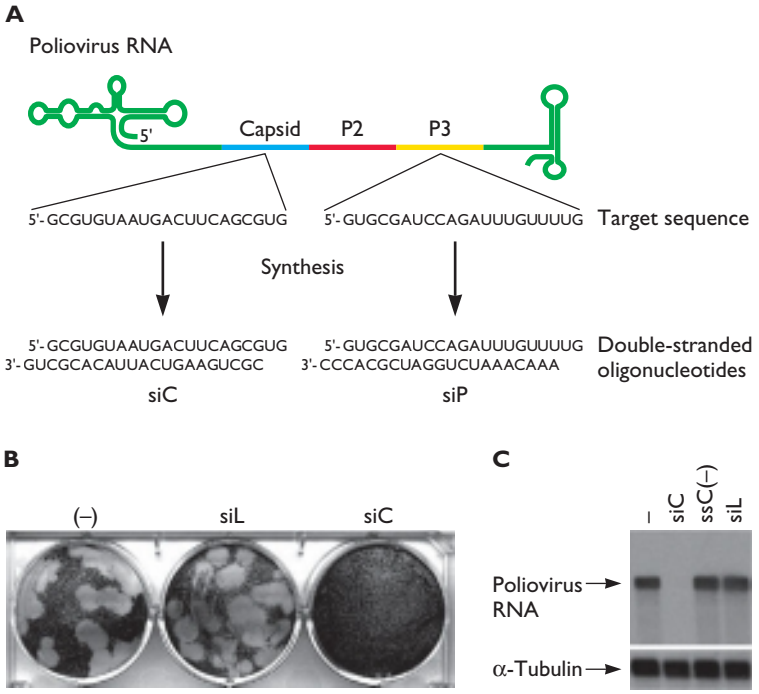


Figure 3.15 Inhibition of poliovirus replication by siRNA. siRNAs were introduced into cells by transformation, and the cells were then subjected to poliovirus infection. **(A)** Location of siRNAs siC and siP on a map of the poliovirus RNA genome. **(B)** Inhibition of plaque formation by siRNA siC. The number of plaques is not reduced in untreated cells (–) or when siRNA from *Renilla* luciferase is used (siL). Plaque formation was also inhibited with siP (not shown). **(C)** Northern blot analysis of RNA from poliovirus-infected cells 6 h after infection. Poliovirus RNA replication is blocked by siC but not by the (–) strand of siC RNA, ssC(–), or siL. The blot was rehybridized with a DNA probe directed against α-tubulin to ensure that all lanes contained equal amounts of RNA. Adapted from L. Gitlin et al., *Nature* **418:**430–434, 2002, with permission.

Table 3.3 Some genetic diseases that might be treated using viral vectors containing human genes

Disease	Defect	Incidence	Target cell
Severe combined immunodeficiency	Adenosine deaminase (25% of patients)	Rare	Bone marrow cells or T lymphocytes
Hemophilia A	Factor VII deficiency	1 in 10,000 males	Liver, muscle, fibroblasts, bone marrow cells
Hemophilia B	Factor IX deficiency	1 in 30,000 males	Liver, muscle, fibroblasts, bone marrow cells
Familial hypercholesterolemia	Deficiency of low-density lipoprotein receptor	1 in 1,000,000	Liver
Cystic fibrosis	Defective salt transport in lung epithelium	1 in 3,000 whites	Lung airways
Hemoglobinopathies and thalassemias	Defects in α- or β globin gene	1 in 600 in specific ethnic groups	Bone marrow precursors of red blood cells
Gaucher's disease	Defect in glucocerebrosidase	1 in 450 Ashkenazi Jews	Bone marrow cells, macrophages
α_1-Antitrypsin deficiency, inherited emphysema	α_1-Antitrypsin not produced	1 in 3,500	Lung or liver cells
Duchenne muscular dystrophy	Dystrophin not produced	1 in 3,000 males	Muscle cells

structure and function of viral vectors requires knowledge of viral genome replication, which is discussed in subsequent chapters for selected viruses and is summarized in the appendix in this volume.

The design requirements for viral vectors include the inclusion of an appropriate promoter, maintenance of genome size within the packaging limit of the particle, and elimination of viral virulence, the capacity of the virus to cause disease. Expression of foreign genes from viral vectors may be controlled by homologous or heterologous promoters and enhancers chosen to support efficient transcription (e.g., the human cytomegalovirus immediate-early transcriptional control region), depending on the goals of the experiment. Such genes can be built directly into the viral genome or introduced by recombination in cells, as described above (see "Introducing Mutations into the Viral Genome"). The recipient viral genome generally carries deletions and sometimes additional mutations. Deletion of some viral sequences is often required to overcome the limitations on the size of viral genomes that can be packaged in virions. For example, adenoviral DNA molecules more than 105% of the normal length are packaged very poorly. As this limitation would allow only 1.8 kbp of exogenous DNA to be inserted into the genome, adenovirus vectors often include deletions of the E3 gene (which is not essential for growth in cells in culture) and of the E1A and E1B transcription units, which encode proteins that can be provided by complementing cell lines.

When viral vectors are designed for therapeutic purposes, it is essential to prevent replication and destruction of target host cells. The deletions necessary to accommodate a foreign gene may contribute to such disabling of the vector. For example, the E1A protein-coding sequences that are invariably deleted from adenovirus vectors are necessary for efficient transcription of viral early genes; in their absence, viral yields from cells in culture are reduced by about 3 to 6 orders of magnitude (depending on the cell type). Removal of E1A coding sequences from adenovirus vectors is therefore doubly beneficial, although it is not sufficient to ensure that the vector cannot replicate or induce damage in a host animal. As discussed in detail in Volume II, Chapter 8, production of viruses that do not cause disease can be more difficult to achieve.

A summary of viral vectors is presented in Table 3.4, and examples are discussed below.

Table 3.4 Some viral vectors

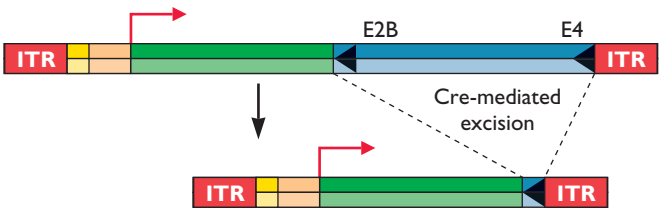
Virus	Insert size	Integration	Duration of expression	Advantages	Potential disadvantages
Adeno-associated virus	~4.5–9 (?) kb	Low efficiency	Long	Nonpathogenic, episomal, infects nondividing cells	Immunogenic, toxicity, small packaging limit
Adenovirus	2–38 kb	No	Short	Efficient gene delivery, infects nondividing cells	Transient, immunogenic
Alphavirus	~5 kb	No	Short	Broad host range, high level expression	Virulence
Epstein-Barr virus	~120 kb	No; episomal	Long	High capacity, episomal, long-term expression	
Gammaretrovirus	1–7.5 kb	Yes	Shorter than formerly	Stable integration	May rearrange genome, insertional mutagenesis, require cell division
Herpes simplex virus	~30 kb	No	Long in central nervous system, short elsewhere	Infects nondividing cells; neurotropic, large capacity	Virulence, persistence in neurons, immunogenic
Lentivirus	7–18 kb	Yes	Long	Stable integration; infects nondividing and terminally differentiated mammalian cells	Insertional mutagenesis
Poliovirus	~300 bp for helper-free virus; ~3 kb for defective virus	No	Short	Excellent mucosal immunity	Limited capacity; reversion to neurovirulence
Rhabdovirus	Unknown	No	Short	High-level expression, rapid cell killing	Virulence, highly cytopathic
Vaccinia virus	At least ~25 kb, probably ~75–100 kb	No	Short	Wide host range, ease of isolation, large capacity, high-level expression	Transient, immunogenic

DNA Virus Vectors

One goal of gene therapy is to introduce genes into terminally differentiated cells. Such cells normally do not divide, they cannot be cultured *in vitro*, and the organs of which they are a part cannot be infused with virus-infected cells. DNA virus vectors have been developed to overcome some of these problems.

Adenovirus vectors were originally developed for the treatment of cystic fibrosis because of the tropism of the virus for the respiratory epithelium. Adenovirus can infect terminally differentiated cells, but only transient expression is provided, as this viral DNA is not integrated into host cell DNA. Adenoviruses carrying the cystic fibrosis transmembrane conductance regulator gene, which is defective in patients with this disease, have been used in clinical trials. Many other gene products with therapeutic potential have been produced from adenovirus vectors in a wide variety of cell types. In the earliest adenovirus vectors that were designed, foreign genes were inserted into the E1 and/or E3 regions. As these vectors had limited capacity, vectors that contain minimal adenovirus sequences were designed (Fig. 3.16). This strategy allows 27 to 38 kb of foreign sequence to be introduced into the vector. In addition, elimination of most viral genes reduces the host immune response to viral proteins, simplifying multiple immunizations. Considerable efforts have been made to modify the adenovirus capsid to target the vectors to different cell types. The fiber protein, which mediates adenovirus binding to cells, has been altered by insertion of ligands that bind particular cell surface receptors. Such alterations could increase the cell specificity of adenovirus attachment and the efficiency of gene transfer and could decrease the amount of virus that is administered.

Figure 3.16 Adenovirus vectors. High-capacity adenovirus vectors are produced by inserting a foreign gene and promoter into the viral E1 region, which has been deleted. The E3 region also has been deleted. Two *loxP* sites for cleavage by the Cre recombinase have been introduced into the adenoviral genome (black arrowheads). Infection of cells that produce Cre leads to excision of sequences flanked by the *loxP* sites. The result is a "gutless" vector that contains only the origin-of-replication-containing inverted terminal repeats (ITR), the packaging signal (yellow), the viral E4 transcription unit (orange), and the transgene with its promoter (green). Additional DNA flanking the foreign gene must be inserted to allow packaging of the viral genome (not shown). Adapted from A. Pfeifer and I. M. Verma, *in* D. M. Knipe et al. (ed.), *Fields Virology,* 4th ed. (Lippincott Williams & Wilkins, Philadelphia, PA, 2001), with permission.



Adeno-associated virus has attracted much attention as a vector for gene therapy, because the virus integrates into the host genome. Genomes packaged into recombinant viruses can integrate at numerous sites in the human genome or replicate as an episome. The vectors persist, in some cases with high levels of expression, in many different tissues. There has been increasing interest in these vectors to target therapeutic genes to smooth muscle, a tissue that is highly susceptible and supports sustained high-level expression of foreign genes. Although the first-generation adeno-associated virus vectors were limited in the size of inserts that could be transferred, other systems have been developed to overcome the limited genetic capacity (Fig. 3.17). The cell specificity of adeno-associated virus vectors has been altered by inserting receptor-specific ligands into the capsid. In addition, many new viral serotypes have been identified that vary in their tropism and ability to trigger immune responses.

Vectors based on the genome of Epstein-Barr virus, a gammaherpesvirus, replicate as episomes in the cell. This property confers the advantage of long-term expression of foreign genes. Episomal replication of the vector is maintained by the origin for plasmid maintenance, OriP, whose activity is dependent on Epstein-Barr virus nuclear antigen 1 (EBNA-1) (see Chapter 10). Vectors containing OriP and encoding EBNA-1 can accept at least 120 kbp of foreign DNA.

Vaccinia virus and other animal poxvirus vectors offer the advantages of a wide host range, a genome that accepts very large fragments, high expression of foreign genes, and relative ease of preparation. Foreign DNA is usually inserted into the viral genome by homologous recombination, using an approach similar to that described for marker transfer. Because of the relatively low pathogenicity of the virus, vaccinia virus recombinants have been considered candidates for human and animal vaccines (Volume II, Fig. 8.11).

RNA Virus Vectors

A number of RNA viruses have also been developed as vectors for foreign gene expression (Table 3.4). Because poliovirus is such an excellent mucosal immunogen, it has been an attractive candidate for the delivery of antigens to these surfaces. Alphavirus vectors have been shown to be capable of expressing foreign genes. These (+) strand RNA viruses produce subgenomic mRNAs from which efficient gene expression can be obtained. In one type of vector,

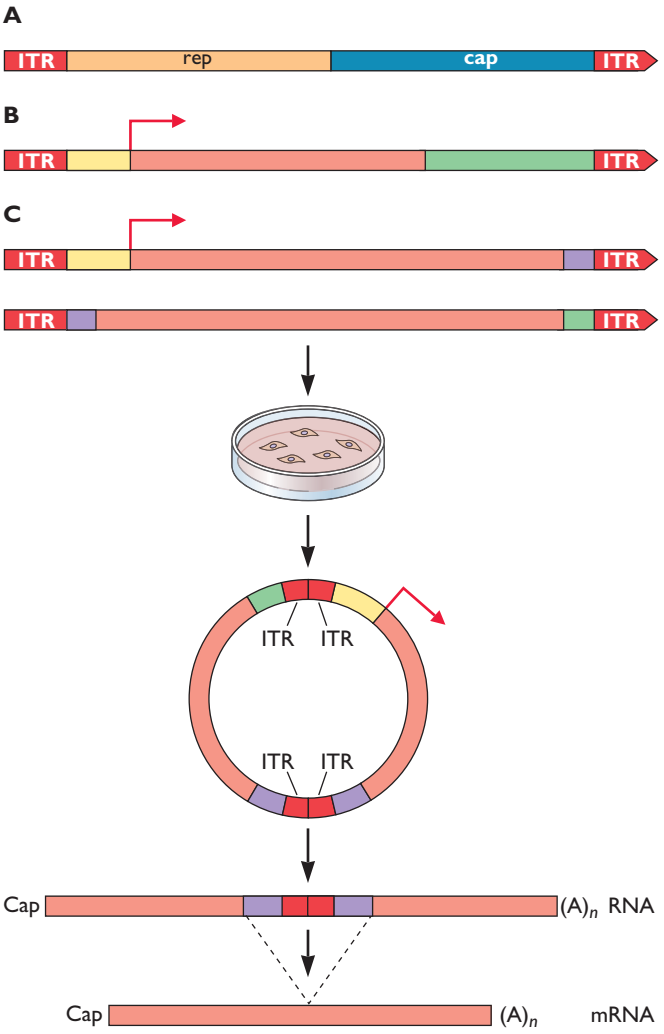


Figure 3.17 Adeno-associated virus vectors. (A) Map of the genome of wild-type adeno-associated virus. The viral DNA is single stranded and flanked by two inverted terminal repeats (ITR); it encodes capsid (blue) and nonstructural (orange) proteins. **(B)** In one type of vector, the viral genes are replaced with the transgene (pink) and its promoter (yellow) and a poly(A) addition signal (green). These DNAs are introduced into cells which have been engineered to produce capsid proteins, and the vector genome is encapsidated into virus particles. A limitation of this vector structure is that only 4.1 to 4.9 kb of foreign DNA can be packaged efficiently. To overcome this limitation, a two-plasmid expression system was developed. **(C)** The transgene of interest (pink) is divided between two adeno-associated virus vectors. One plasmid contains the promoter, the 5′ part of the transgene, and a 5′ splice site (purple), and the second plasmid contains a 3′ splice site, the remainder of the transgene, and the polyadenylation site. In cells, the recombinant viral genomes form circular multimers that bring the divided transgene together in a head-to-tail orientation. A long RNA is then produced that undergoes splicing to form a functional mRNA that can be translated into protein. Adapted from A. Pfeifer and I. M. Verma, *in* D. M. Knipe et al. (ed.), *Fields Virology*, 4th ed. (Lippincott Williams & Wilkins, Philadelphia, PA, 2001), and Z. Yan et al., *Proc. Natl. Acad. Sci. USA* **97:**6716–6721, 2000, with permission.

the foreign gene replaces those encoding the viral structural proteins. As a result, this vector requires the presence of a helper virus to provide the missing proteins, so that the vector genome can be packaged into virus particles. An alternative arrangement is to place the foreign gene under the control of a second, subgenomic RNA promoter, leaving intact the promoter that controls synthesis of the structural proteins. This vector replicates without a helper and produces infectious particles.

The development of an infectious DNA clone of vesicular stomatitis virus, a (–) strand RNA virus, has led to an approach to therapy for human immunodeficiency virus type 1 infection. Recombinant vesicular stomatitis virus particles that lack the viral glycoprotein G, required for attachment to host cells, have been produced. The G gene is replaced by genes encoding two cellular proteins required for the attachment of human immunodeficiency virus type 1 to cells (CD4 and CXCr4) (see Chapter 5). The recombinant vesicular stomatitis viruses cannot infect normal cells. However, they can infect and lyse cells infected with human immunodeficiency virus type 1, because these cells synthesize viral glycoproteins on the surface. This approach has the potential of limiting the number of infected cells in the host.

Retroviruses have enjoyed great popularity as vectors (Fig. 3.18) because their replication cycles include the integration of a dsDNA copy of viral RNA into the cell genome, a topic of Chapter 7. The integrated provirus remains permanently in the cell's genome and is passed on to progeny during cell division. This feature of retroviral vectors results in permanent modification of the genome of the infected cell. The choice of the envelope glycoprotein carried by retroviral vectors has a significant influence on their tropism. The vesicular stomatitis virus G glycoprotein is often used because it confers a wide tissue tropism. Retrovirus vectors can be targeted to specific cell types by using other viral envelope proteins.

One problem with the use of retroviruses in correcting genetic deficiencies is that only a few cell types can be infected by the commonly used murine retroviral vectors, and these can integrate their DNA efficiently only in actively dividing cells. Often the cells that are targets of gene therapy, hepatocytes, and muscle cells, do not divide. This problem can be circumvented if ways can be found to induce such cells to divide before being infected with the retrovirus. Another important limitation of the murine retrovirus vectors is the phenomenon of gene silencing, which represses foreign gene expression in many cells. An alternative approach is to use viral vectors that contain sequences from human immunodeficiency virus type 1, which can infect nondividing cells and is less severely affected by gene silencing.

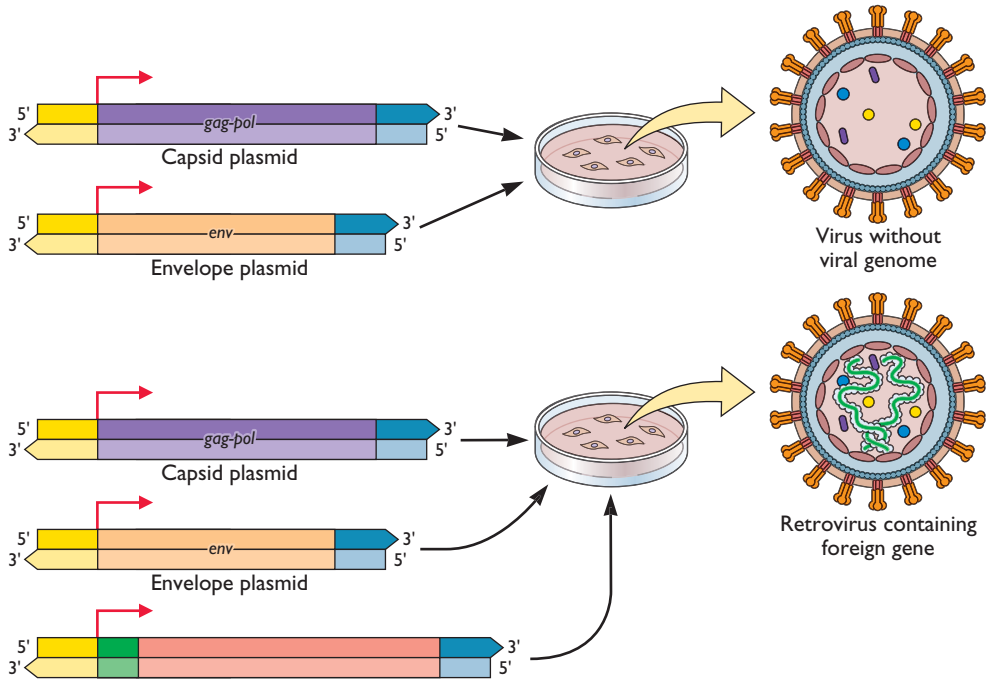


Figure 3.18 Retroviral vectors. The minimal viral sequences required for retroviral vectors are 5′- and 3′-terminal sequences (yellow and blue, respectively) that control gene expression and packaging of the RNA genome. The foreign gene (pink) and promoter (green) are inserted between the viral sequences. To package this DNA into viral particles, it is introduced into cultured cells with plasmids that encode viral proteins required for encapsidation. No wild-type viral RNA is present in these cells. If these plasmids alone are introduced into cells, virus particles that do not contain viral genomes are produced. When all three plasmids are introduced into cells, retrovirus particles are formed that contain only the recombinant vector genome and no wild-type particles. The host range of the recombinant vector can be controlled by the type of envelope protein. Envelope protein from amphotropic retroviruses allows the recombinant virus to infect human and mouse cells. The vesicular stomatitis virus glycoprotein G allows infection of a broad range of cell types in many species and also permits concentration with simple methods. Adapted from A. Pfeifer and I. M. Verma, *in* D. M. Knipe et al. (ed.), *Fields Virology,* 4th ed. (Lippincott Williams & Wilkins, Philadelphia, PA, 2001), with permission.

Perspectives

The information in this chapter can be used as a "road map" for negotiating this book and for planning a virology course. Figures 3.1 through 3.7 serve as the points of departure for detailed analyses of the principles of virology. These figures illustrate seven strategies based on viral mRNA synthesis and genome replication, with references to chapters containing information about the replication and pathogenesis of members of each class. The material in this chapter can be used to structure individual reading or to design a virology course based on specific viruses or groups of viruses while adhering to the overall organization of this textbook by function. Refer to this chapter and the figures to find answers to questions about specific virus families or individual species. For example, Fig. 3.5 provides information about (+) strand RNA viruses and Fig. 3.10 indicates specific chapters in which these viruses are discussed.

Since the earliest days of experimental virology, genetic analysis has proven invaluable for studying the function of the viral genome. Initially, methods were developed to produce viral mutants by chemical or UV mutagenesis followed by screening for readily identifiable phenotypes. Because it was not possible to identify the genetic changes in such mutants, it was difficult to associate proteins with virus-specific processes. This limitation vanished with the development of infectious DNA clones of viral genomes: it suddenly was possible to introduce defined mutations into any region of the viral genome. This complete genetic toolbox provides countless possibilities for studying the function of the viral genome, limited only by the creativity and enthusiasm of the investigator.

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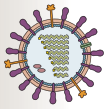
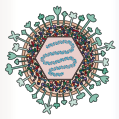
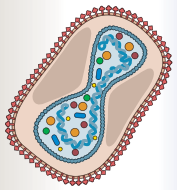




4

Structure

In order to create something that functions properly—a container, a chair, a house—its essence has to be explored, for it should serve its purpose to perfection, i.e. it should fulfill its function practically and should be durable, inexpensive and beautiful.

WALTER GROPIUS
Neue Arbeiten der Bauhaus Werk Stäten, Bauhaus Books, no. 7

Introduction

Virus particles are elegant assemblies of viral, and occasionally cellular, macromolecules. They are marvelous examples of architecture on the molecular scale, with forms beautifully adapted to their functions. Virus particles come in many sizes and shapes (Fig. 4.1; also see Fig. 1.7) and vary enormously in the number and nature of the molecules from which they are built. Nevertheless, they fulfill common functions and are built according to general principles that apply to them all. These properties are described in subsequent sections, in which we also discuss some examples of the architectural detail characteristic of members of different virus families.

Functions of the Virion

Virus particles are designed for effective transmission of the nucleic acid genome from one host cell to another within a single animal or among host organisms. A primary function of the **virion**, an infectious virus particle, is therefore protection of the genome, which can be damaged irreversibly by a break in the nucleic acid or by mutation during passage through hostile environments. During its travels, a virus particle may encounter a variety of potentially lethal chemical and physical agents, including proteolytic and nucleolytic enzymes, extremes of pH or temperature, and various forms of natural radiation. In all virus particles, the nucleic acid is sequestered within a sturdy barrier formed by extensive interactions among the viral proteins that comprise the protein coat. Such protein-protein interactions maintain surprisingly stable capsids: many virus particles composed of only protein and nucleic acid survive exposure to large variations in the temperature, pH, or chemical composition of their environment. Some, such as certain picornaviruses, are even resistant to strong detergents such as sodium dodecyl sulfate. The highly folded nature of coat proteins and their dense packing within virions render them largely inaccessible to proteolytic enzymes. Some viruses also possess an **envelope**, derived from cellular membranes, into which viral glycoproteins have been inserted.

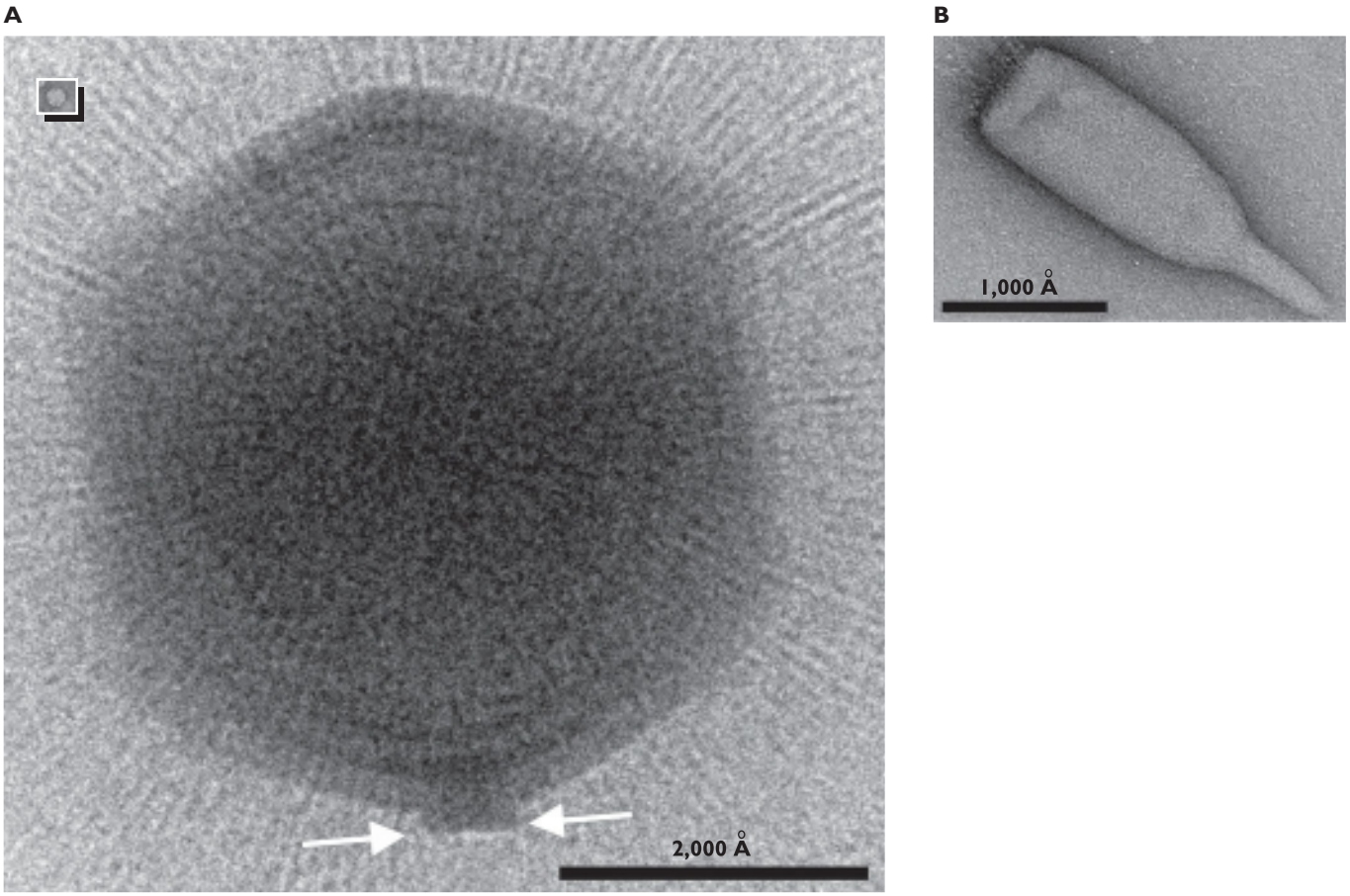


Figure 4.1 Variation in the size and shape of virus particles. (A) Cryo-electron micrographs of mimivirus and, in the inset (upper left), the parvovirus adeno-associated virus type 4 shown to scale relative to one another to illustrate the ~50-fold range in diameter among viruses that appear roughly spherical. Rod-shaped viruses also exhibit considerable variation in size, ranging in length from less than 200 nm to over 2,000 nm. Adapted from C. Xiao et al., *J. Mol. Biol.* **353:**493–496, 2005, and E. Pardon et al., *J. Virol.* **79:**5047–5058, 2005, respectively, with permission. Courtesy of M. G. Rossmann, Purdue University, and M. Agbandje-McKenna, University of Florida, Gainesville. **(B)** Complex shape of acidianus bottle virus isolated from a hot spring in Italy. The mimivirus particle (A) is also structurally complex: a large number of long, closely packed filaments project from its surface and a unique, protruding structure (white arrows) is present at one vertex. Adapted from M. Häring et al., *J. Virol.* **79:**9904–9911, 2005, with permission. Courtesy of D. Prangishvili, Institut Pasteur.

The envelope adds not only a protective lipid membrane but also an external layer of protein and sugars formed by the glycoproteins. Like the cellular membranes from which they are derived, viral envelopes are impermeable and block entry of chemicals or enzymes in aqueous solution into virus particles.

To protect the nucleic acid genome, virus particles must be stable structures. However, virions must also attach to an appropriate host cell and deliver the genome to the interior of that cell, where the particle is at least partially disassembled. The protective function of virus particles depends on very stable intermolecular interactions among their components during assembly, egress from the virus-producing cell, and transmission. On the other hand, these interactions must be reversed readily during entry and uncoating in a new host cell. In only a few cases do we understand the molecular mechanisms by which these apparently paradoxical requirements are met. Nevertheless, it is clear that contact of a virion with the appropriate cell surface receptor, or exposure to a specific intracellular environment, can trigger substantial conformational changes. Virus particles are therefore **metastable structures** that have not yet attained the minimum free energy conformation. The latter state can be attained only when an unfavorable energy barrier is surmounted, following induction of the irreversible conformational transitions associated with attachment and

Table 4.1 Functions of virion proteins

Protection of the genome
Assembly of a stable protective protein shell
Specific recognition and packaging of the nucleic acid genome
In many virions, interaction with host cell membranes to form the envelope
Delivery of the genome
Specific binding to external receptors of the host cell
Transmission of specific signals that induce uncoating of the genome
Induction of fusion with host cell membranes
Interaction with internal components of the infected cell to direct transport of the genome to the appropriate site
Other interactions with the host
With cellular components for transport to intracellular sites of assembly
With cellular components to ensure an efficient infectious cycle
With the host immune system

entry. Virions are **not** simply inert structures. Rather, they are molecular machines that play an active role in delivery of the nucleic acid genome to the appropriate host cell and initiation of the reproductive cycle.

Specific functions of virion proteins associated with protection and delivery of the genome are summarized in Table 4.1.

Nomenclature

Virus architecture is described in terms of **structural units** of increasing complexity, from the smallest biochemical unit (the polypeptide chain) to the infectious particle (or virion). These terms, which are used throughout this text, are defined in Table 4.2. Although virus particles are complex assemblies of macromolecules exquisitely suited for protection and delivery of viral genomes, they are constructed according to the general principles of biochemistry and protein structure.

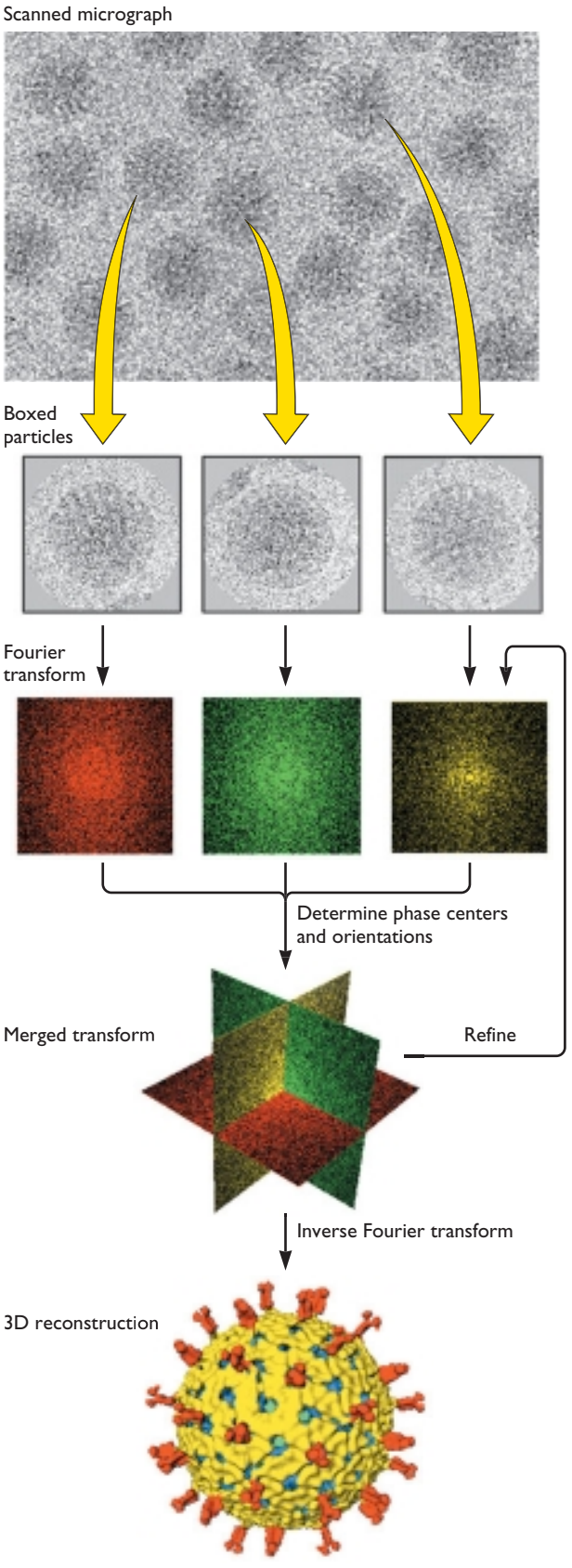
Methods for Studying Virus Structure

The method most widely used for the examination of virus structure and morphology is electron microscopy. This technique, which has been applied to viruses since the middle decades of the last century, traditionally relied on negative staining of purified virus particles (or of sections of infected cells) with an electron-dense material, such as uranyl acetate or phosphotungstate. It can yield quite detailed and often beautiful images (Fig. 1.7; see the appendix) and provided the first rational basis for the classification of viruses.

The greatest contrast between virus particle and stain occurs where portions of the folded protein chain protrude from the virion surface. Consequently, surface knobs or projections, termed morphological units, are the main features identified by negative-contrast electron microscopy. However, these structures are often formed by multiple proteins and so their organization does not necessarily correspond to that of the individual proteins that form the capsid shell. Even when virus structure is well preserved and a high degree of contrast can be achieved, the minimal size of an object that can be distinguished by classical electron microscopy, its **resolution**, is limited to 50 to 75 Å. Detailed structural interpretation of images of negatively stained virus particles, which possess dimensions on a scale of a few hundred to a few thousand angstroms, is therefore impossible. Cryo-electron microscopy, in which samples are rapidly frozen and examined at very low temperatures in a hydrated, vitrified (noncrystalline, glasslike) state, preserves native structure. Because samples are not stained, this technique allows direct visualization of the contrast inherent in the virus particle. When combined with computerized mathematical methods of image analysis and three-dimensional reconstruction (Fig. 4.2), cryo-electron

Table 4.2 Nomenclature used in description of virus structure

Term	Synonym	Definition
Subunit (protein subunit)		Single, folded polypeptide chain
Structural unit	Asymmetric unit	Unit from which capsids or nucleocapsids are built; may comprise one protein subunit or multiple, different protein subunits
Morphological unit	Capsomere	Surface structures (e.g., knobs, projections, clusters) seen by electron microscopy; as these structures do not necessarily correspond to structural units (see the text), the term is restricted to descriptions of electron micrographs of viruses
Capsid	Coat	The protein shell surrounding the nucleic acid genome
Nucleocapsid	Core	The nucleic acid-protein assembly packaged within the virion; used when this complex is a discrete substructure of a complex particle
Envelope	Viral membrane	The host cell-derived lipid bilayer carrying viral glycoproteins
Virion		The infectious virus particle



Concentrated preparations of purified virus particles are prepared for cryo-electron microscopy by rapid freezing on an electron microscope grid so that a glasslike, noncrystalline water layer is produced. This procedure avoids sample damage that can be caused by crystallization of the water or by chemical modification or dehydration during conventional negative-contrast electron microscopy. The sample is maintained at or below –160°C during all subsequent operations. Fields containing sufficient numbers of vitrified virus particles are identified by transmission electron microscopy at low magnification (to minimize sample damage from the electron beam) and photographed at high resolution (top).

These electron micrographs can be treated as two-dimensional projections (Fourier transforms) of the particles. Three-dimensional structures can be reconstructed from such two-dimensional projections by mathematically combining the information given by different views of the particles. For the purpose of reconstruction, the images of different particles are treated as different views of the same structure.

For reconstruction, micrographs are digitized for computer processing. Each particle to be analyzed is then centered inside a box, and its orientation is determined by application of programs that orient the particle on the basis of its icosahedral symmetry. In cryo-electron tomography, a series of images are collected with the sample at different angles to the electron beam and combined computationally to reconstruct a three-dimensional structure. The advantage of this approach is that no assumptions about the symmetry of the structure are required. The parameters that define the orientation of the particle must be determined with a high degree of accuracy, for example, to within 1° for even a low-resolution reconstruction (~40 Å). These parameters are improved in accuracy (**refined**) by comparison of different views (particles) to identify common data.

Once the orientations of a number of particles sufficient to represent all parts of the asymmetric unit have been determined, a low-resolution three-dimensional reconstruction is calculated from the initial set of two-dimensional projections by using computerized algorithms.

This reconstruction is refined by including data from additional views (particles). The number of views required depends on the size of the particle and the resolution sought. The reconstruction is initially interpreted in terms of the external features of the virus particle. Various computational and computer graphics procedures have been developed to facilitate interpretation of internal features. Courtesy of B. V. V. Prasad, Baylor College of Medicine.

And is it not true that even the small step of a glimpse through the microscope reveals to us images that we should deem fantastic and over-imaginative if we were to see them somewhere accidentally, and lacked the sense to understand them.
Paul Klee, *On Modern Art,* translated by Paul Findlay (London, United Kingdom, 1948)

Figure 4.2 Cryo-electron microscopy and image reconstruction illustrated with images of rotavirus.

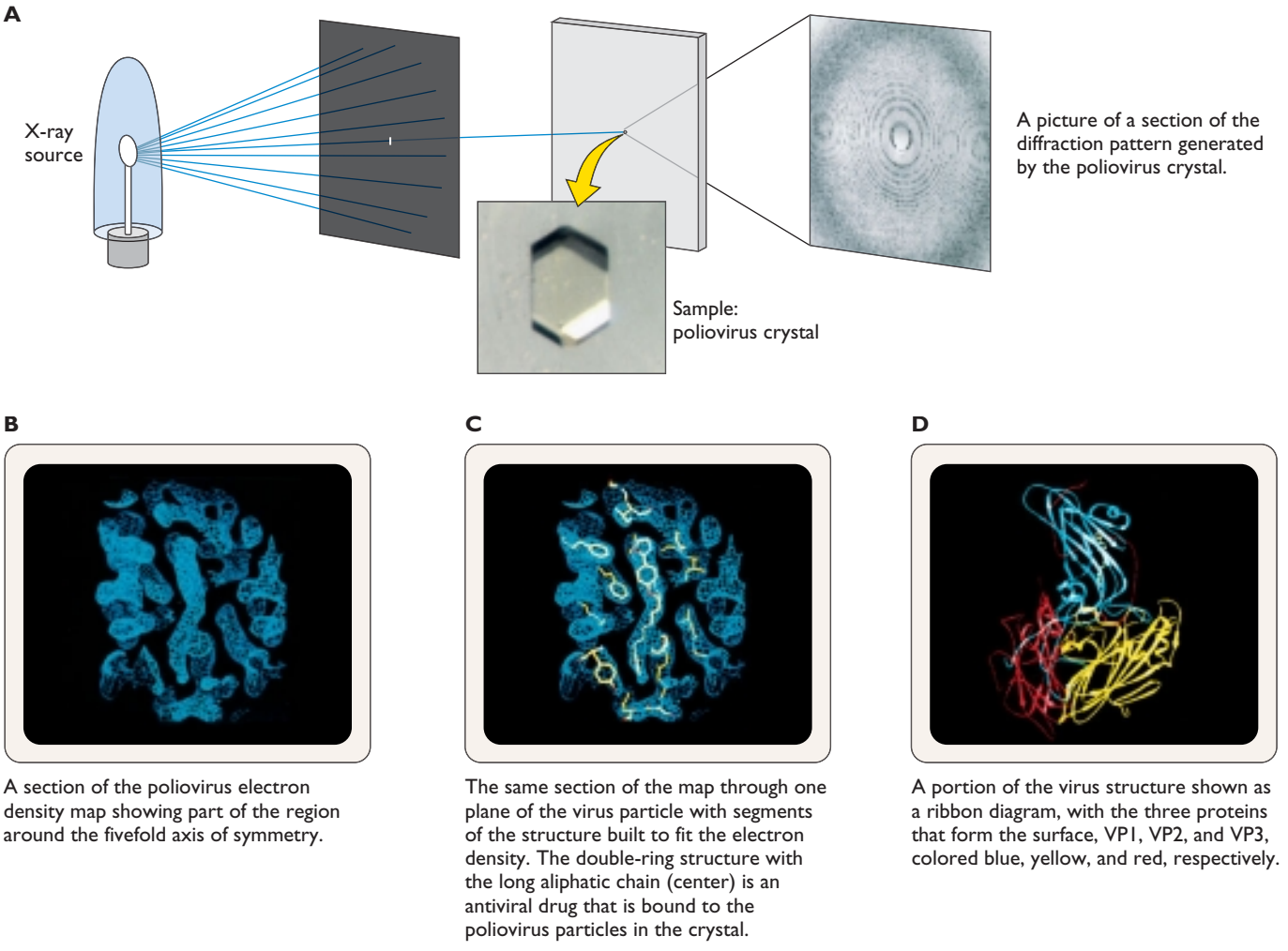


Figure 4.3 Determination of virus structure by X-ray diffraction. (A) A virus crystal is composed of virus particles arranged in a well-ordered three-dimensional lattice. When the crystal is bombarded with a monochromatic X-ray beam, each atom within the virus particle scatters the radiation. Interactions of the scattered rays with one another form a diffraction pattern that is recorded. Each spot contains information about the position and the identity of all atoms in the crystal. The locations and intensities of the spots are stored electronically. Determination of the three-dimensional structure of the virus from the diffraction pattern requires information that is lost in the X-ray diffraction experiment. This missing information (the phases of the diffracted rays) can be retrieved by collecting the diffraction information from otherwise identical (isomorphous) crystals in which the phases have been systematically perturbed by the introduction of heavy metal atoms at known positions. Comparison of the two diffraction patterns yields the phases. This process is called **multiple isomorphous replacement**. Alternatively, if the structure of a related molecule is known, the diffraction pattern collected from the crystal can be interpreted by using the phases from the known structure as a starting point and subsequently using computer algorithms to calculate iteratively the actual values of the phases. This method is known as **molecular replacement**. Once the phases are known, the intensities and spot positions from the diffraction pattern are used to calculate the locations of the atoms within the crystal, again by using computer programs. **(B)** The product of this mathematical analysis is an electron density map, which is a map of the location of electron-dense atoms (C, N, O, and S), as well as of the covalent bonds within and between amino acids in the virus. **(C)** A model of the peptide backbone linking amino acids within proteins is built by tracing the electron density through each section of the map and specifying the location of each atom. The amino acids are identified by the shapes of the electron density of the side chains and by using the primary sequence of the proteins. **(D)** When the entire structure is built, the completed images can be reassembled and visualized in various representations. Courtesy of J. Hogle, Harvard Medical School, and M. Chow, University of Arkansas for Medical Sciences.

microscopy can improve resolution to 10 to 20 Å. Indeed, sufficient resolution (~6 Å) was attained in recent applications of these techniques to identify α-helices in structural proteins of adenovirus particles, in the core protein of hepatitis B virus, and in the transmembrane domains of viral glycoproteins. Within the past decade, cryo-electron microscopy has become a standard tool of structural biology. Its application to virus particles has provided a wealth of previously inaccessible information about the external and internal structures of multiple members of at least 20 virus families.

The diameter of an α-helix in a protein is on the order of 10 Å, so even the highest resolution attained by these sophisticated techniques cannot reveal the molecular interactions that cement structural units in the virion. Such information can be obtained by X-ray crystallography (Fig. 4.3), provided that the virus yields crystals suitable for X-ray diffraction. It has been known for more than 60 years that simple plant viruses can be crystallized, and the first high-resolution virus structure determined was that of tomato bushy stunt virus. Since this feat was accomplished in 1978, high-resolution structures of a number of increasingly larger animal viruses have been determined, placing our understanding of the principles of capsid structure on a firm molecular foundation. Although they cannot capture dynamic processes, atomic-level descriptions of specific viruses, or of individual viral proteins, have also greatly improved our knowledge of mechanisms of attachment and entry of virions and of virus assembly. They have also provided new opportunities for the design of antiviral drugs.

Not all viruses can be examined directly by X-ray crystallography: some do not form suitable crystals, and the larger viruses lie beyond the power of the current procedures by which X-ray diffraction spots are converted into a structural model (Fig. 4.3). Nevertheless, individual viral proteins can be examined by this method and by multidimensional nuclear magnetic resonance (NMR) techniques (Box 4.1). The latter methods, which allow structural models to be constructed from knowledge of the distances between specific atoms in a polypeptide chain (Fig. 4.4), can be applied to proteins in solution, a significant advantage. At present, NMR methods can be applied to only relatively small proteins (20 to 30 kilodaltons [kDa]), but their power is being expanded rapidly.

High-resolution structures of individual proteins have been particularly important in illuminating mechanisms of attachment and entry of enveloped viruses. However, even more valuable is the more recent development of methods in which high-resolution structures of individual viral proteins are combined with cryo-electron microscopy reconstructions of intact virus particles. For example, in difference imaging, the structures of individual proteins are in essence subtracted from the reconstruction of the particle to yield new structural information (Fig. 4.5). In the past few years, this powerful approach has provided fascinating new views of interactions of viral envelope proteins embedded in lipid bilayers, and even of internal surfaces and components of virus particles.

Building a Protective Coat

Regardless of their structural complexity, all virions contain at least one protein coat, the capsid or nucleocapsid (Table 4.2), that encases and protects the nucleic acid genome. As first pointed out by Francis Crick and James Watson in 1956, most viruses appear to be rod shaped or spherical under the electron microscope. Because the coding capacities of viral genomes are limited, these authors proposed that construction of capsids from a small number of subunits by using helical symmetry (rod-shaped viruses) or the symmetry of Platonic polyhedra (e.g., tetrahedron or icosahedron) (spherical viruses) would minimize the genetic cost of encoding structural proteins. Such genetic economy dictates that capsids and nucleocapsids be built from identical copies of a small number of viral proteins with structural properties that permit regular and repetitive interactions among them. These protein molecules are arranged to provide maximal contact and noncovalent bonding among subunits and structural units. The repetition of such interactions among a limited number of proteins results in a regular structure, with symmetry that is determined by the spatial patterns of the interactions. In fact, the protein coats of all but a few viruses display **helical** or **icosahedral symmetry**.

Helical Structures

The **nucleocapsids** of some enveloped animal viruses, as well as certain plant viruses and bacteriophages, are rodlike or filamentous structures with helical symmetry. Helical symmetry is described by the number of structural units per turn of the helix, μ, the axial rise per unit, ρ, and the pitch of the helix, *P*, given by the formula

$$P = \mu \times \rho$$

A characteristic feature of a helical structure is that any volume can be enclosed simply by varying the length of the helix. Such a structure is said to be **open.** In contrast, capsids with icosahedral symmetry (described below) are **closed** structures of fixed internal volume.

From a structural point of view, the best-understood helical nucleocapsid is that of tobacco mosaic virus, the first virus to be identified. The virus particle comprises a single molecule of (+) strand RNA, about 6.4 kb in length,

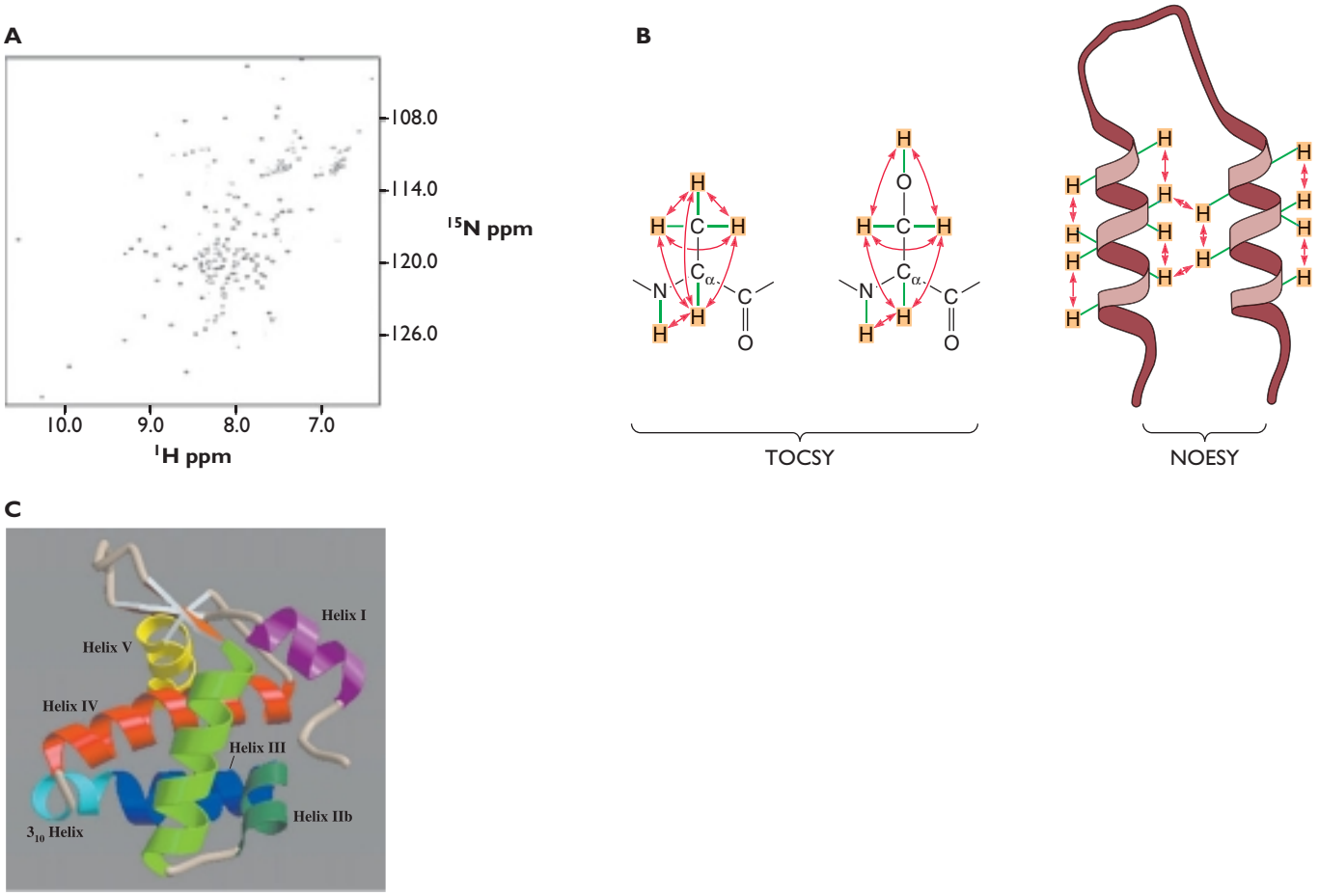


Figure 4.4 Information obtained from multidimensional nuclear magnetic resonance (NMR) spectroscopy. (A) Example showing part of a two-dimensional $^{1}H^{15}N$ heteronuclear, NMR spectrum of the human immunodeficiency virus type 1 matrix (MA) protein. The horizontal and vertical axes show the radiation absorbed by covalently linked protons and nitrogen atoms, respectively, expressed relative to reference signals and termed the **chemical shifts**. These signals are observed when the protein is placed in a strong magnetic field and exposed to radiofrequency pulses. The chemical shift is different for each atom and is determined in part by the molecular environment of its nucleus. The different atoms in a complex molecule like the MA protein therefore exhibit different chemical shifts in this kind of experiment. The initial problem is to resolve and assign the very large number of signals generated by even a small protein (~1,000 for a protein of 15 kDa). The assignment is typically made by using multidimensional experiments, which allow individual protons to be resolved on the basis of several different chemical shift indices, ^{15}N and ^{1}H in the spectrum shown. **(B)** Proton-proton correlations obtained from $^{1}H,^{1}H$ total correlation spectroscopy (TOCSY), and nuclear Overhauser effect spectroscopy (NOESY) NMR spectra. TOCSY reveals correlations between protons that are covalently connected via only one or two additional atoms, and therefore identifies interactions among protons within the same amino acid in a protein; hydrogen atoms in adjacent amino acid are connected by at least three other atoms. A TOCSY experiment therefore generates a characteristic set of linked signals for each amino acid, because each bears a unique side chain. NOESY identifies correlations between protons that are closer than 5 Å in space, regardless of whether they are closely linked in the primary sequence. Secondary-structure elements, such as α-helices, generate characteristic sets of NOE signals. NOE signals also provide information about the tertiary structure of the protein because they place constraints on the distances between specific pairs of hydrogen atoms in the protein. **(C)** When sufficient NOE constraints have been collected and assigned, it is possible to produce structural models of the protein that are consistent with these constraints, as shown for the human immunodeficiency virus type 1 MA protein. (A and C) Courtesy of M. F. Summers, University of Maryland, Baltimore County, and W. I. Sundquist, University of Utah. (B) Adapted from C. Branden and J. Tooze, *An Introduction to Protein Structure* (Garland Publishing, Inc., New York, NY, 1991), with permission.

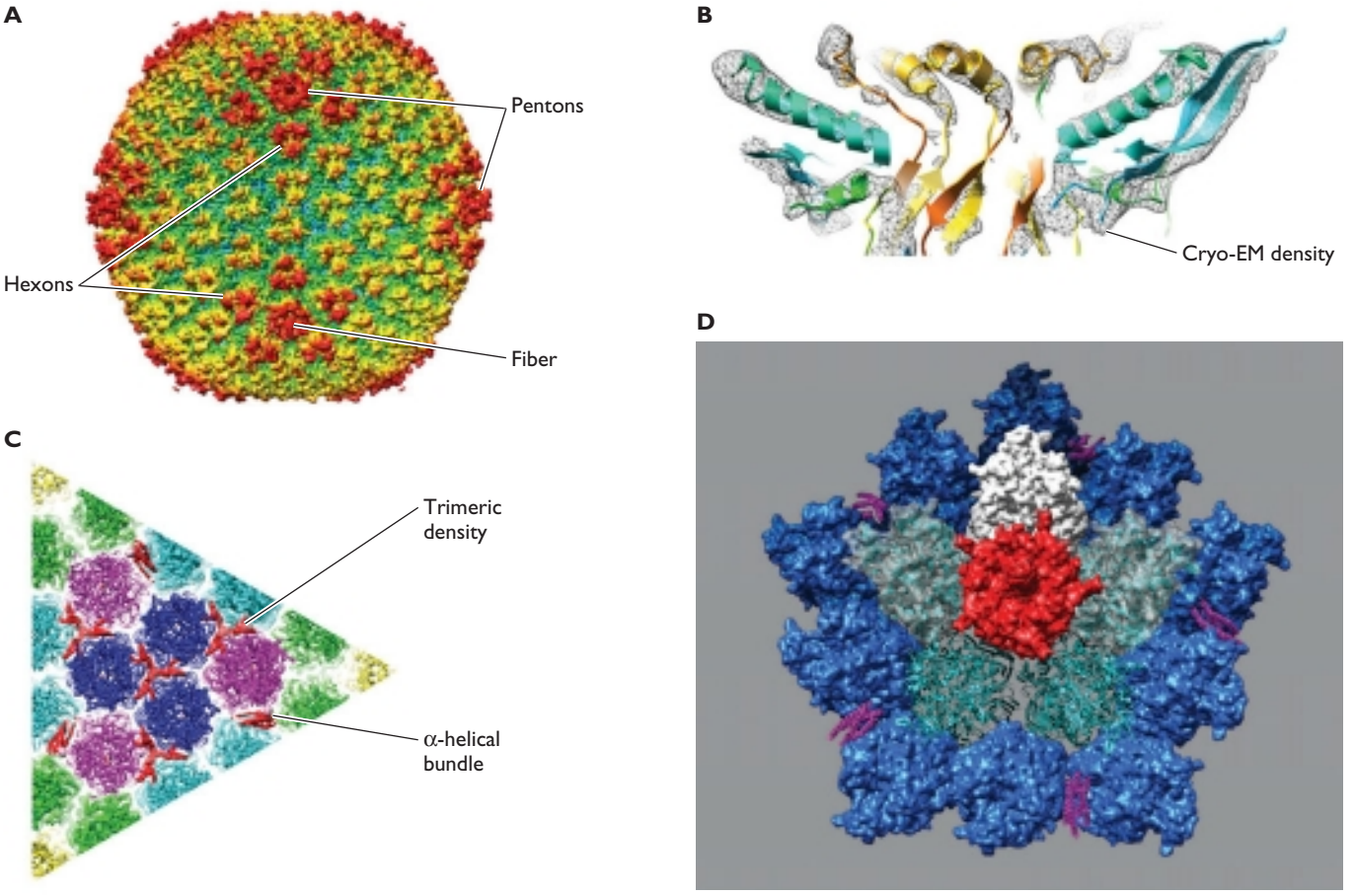


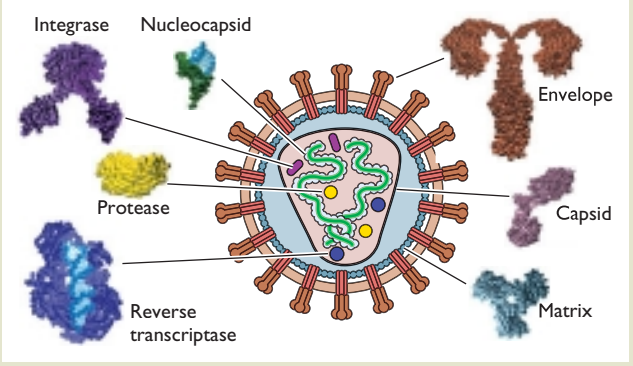
Figure 4.5 Difference mapping illustrated by a 6-Å-resolution reconstruction of adenovirus. **(A)** Surface view along a twofold symmetry axis of the 6-Å-resolution cryo-electron microscopy reconstruction of a derivative of adenovirus type 5 carrying the Ad35 fiber. As this fiber is flexible, only a short portion protruding from the penton base is visible in the reconstruction. The density is radially color coded (red = 596 Å; blue = 316 Å). **(B)** Comparison of α-helices of the penton base in the cryo-electron microscopic density and crystal structure of this protein bound to a fiber peptide (ribbon). The excellent agreement shown established that α-helices could be detected reliably in the 6-Å cryo-electron microscopy reconstruction. **(C)** Portion of the cryo-electron microscopy difference map corresponding to the surface of one icosahedral face of the capsid. The crystal structures of the penton base (yellow) and the hexon (green, cyan, blue, and magenta at different positions) at appropriate resolution were docked within the cryo-electron microscopic density at 6-Å resolution. The cryo-electron microscopic density that does not correspond to these structural units (the difference map) is shown in red. At this resolution, the difference map revealed four trimeric structures located between neighboring hexons and three bundles of coiled-coiled α-helices. The former were previously assigned to protein IX. Predictions of secondary structure and formation of α-helical coiled coils indicated that the helical bundles at the edges of the icosahedral face are formed by the C-terminal half of protein IX. **(D)** One vertex region of the capsid illustrating the α-helical coiled coil assigned to the C-terminal domain of protein IX (magenta). The penton base and fiber are in red; peripentonal hexons are in white, gray, or cyan (depending on how they are represented); and other hexons are in blue. Adapted from S. D. Saban et al., *J. Virol*. **80:**12049–12059, 2006, with permission. Courtesy of Phoebe Stewart, Vanderbilt University Medical Center.

BOX 4.1 DISCUSSION

Human immunodeficiency virus type 1, a virus understood in great structural detail

This causative agent of acquired immunodeficiency syndrome (AIDS) was identified about 25 years ago. As illustrated in the figure, the structures of most of the proteins present in the complex virions have been solved at high resolution by one or more of the methods described in the text. The need to develop effective drugs for the treatment of AIDS has provided an important impetus for structural studies of human immunodeficiency virus type 1 proteins.

Structures of human immunodeficiency virus type 1 proteins. The virion is depicted at the center, with the structures of the component proteins illustrated on either side in surface representation. RNA is green. Adapted from H. Berman et al., *Am. Sci.* **90:**350–359, 2002, with permission.



enclosed within a helical protein coat (Fig. 4.6A; see also Fig. 1.7). The coat is built from a single protein that folds into an extended structure shaped like a Dutch clog. Repetitive interactions among coat protein subunits form disks that have been likened to lock washers, which in turn assemble as a long, rodlike, right-handed helix with 16.3 coat protein molecules per turn. In the interior of the helix, each coat protein molecule binds three nucleotides of the RNA genome. The coat protein molecules therefore engage in **identical**, equivalent interactions with one another and with the genome, allowing the construction of a large, stable structure from multiple copies of a single protein subunit.

The virions of several families of animal viruses with (–) strand RNA genomes, including paramyxoviruses, rhabdoviruses, and orthomyxoviruses, contain internal structures with helical symmetry encased within an envelope. In all cases, these structures contain an RNA molecule, many copies of an RNA-packaging protein (designated NP or N), and the viral RNA polymerase and associated enzymes responsible for synthesis of mRNA. Despite common helical symmetry and similar composition, the internal components of these (–) strand RNA viruses exhibit considerable diversity in morphology and organization. Like tobacco mosaic virus particles, the nucleocapsids of paramyxoviruses and rhabdoviruses contain a single molecule of RNA (of about 15 and 11 kb, respectively) tightly associated with the nucleocapsid protein. Nucleocapsids of paramyxoviruses, such as Sendai virus, are long, filamentous structures in which the RNA and NP protein form a left-handed helix with a hollow core (Fig. 4.6B). In contrast, nucleocapsids of rhabdoviruses such as vesicular stomatitis virus are squat, bullet-shaped structures closed at one end (Fig. 4.6C). Furthermore, an additional virion protein is essential to maintain their organization. Vesicular stomatitis virus nucleocapsids released from within the virion envelope retain the dimensions and morphology observed in intact particles, but become highly extended and filamentous once the matrix (M) protein is also removed (Fig. 12.22). The inherent flexibility of the resulting ribonucleoprotein, which contains the RNA-packaging nucleocapsid (N) protein, as well as the viral polymerase (L) and phosphoprotein (P), has precluded high-resolution structural studies. However, it has been possible to determine the X-ray crystal structure of a ring-like N protein-RNA complex containing 10 molecules of the N protein bound to a 90-nucleotide RNA (Fig 4.7). In this complex, each N protein molecule binds to 9 nucleotides of RNA, which is largely sequestered within cavities within each protein. Furthermore, each N protein makes extensive and regular contacts with neighboring N molecules, exactly as predicted from first considerations by Crick and Watson.

The internal components of influenza A virus particles differ more radically. In the first place, they comprise not a single nucleocapsid but, rather, multiple ribonucleoproteins, one for each molecule of the segmented RNA genome present in the virion (Appendix, Fig. 8). The viral NP protein organizes each RNA into a helical structure analogous to the nucleocapsids of viruses with nonsegmented (–) strand RNA genomes. However, with the exception of terminal sequences, the RNA in these ribonucleoproteins is fully accessible to solvent. This property suggests that the RNA is wound **around** a helical core formed by the NP protein rather than being sequestered within a helical structure. Each ribonucleoprotein is further folded into a compact, circular conformation as a result of the binding of the virion enzyme complex (P proteins) to specific sequences conserved at the 5′ and 3′ ends of each (–) strand RNA molecule.

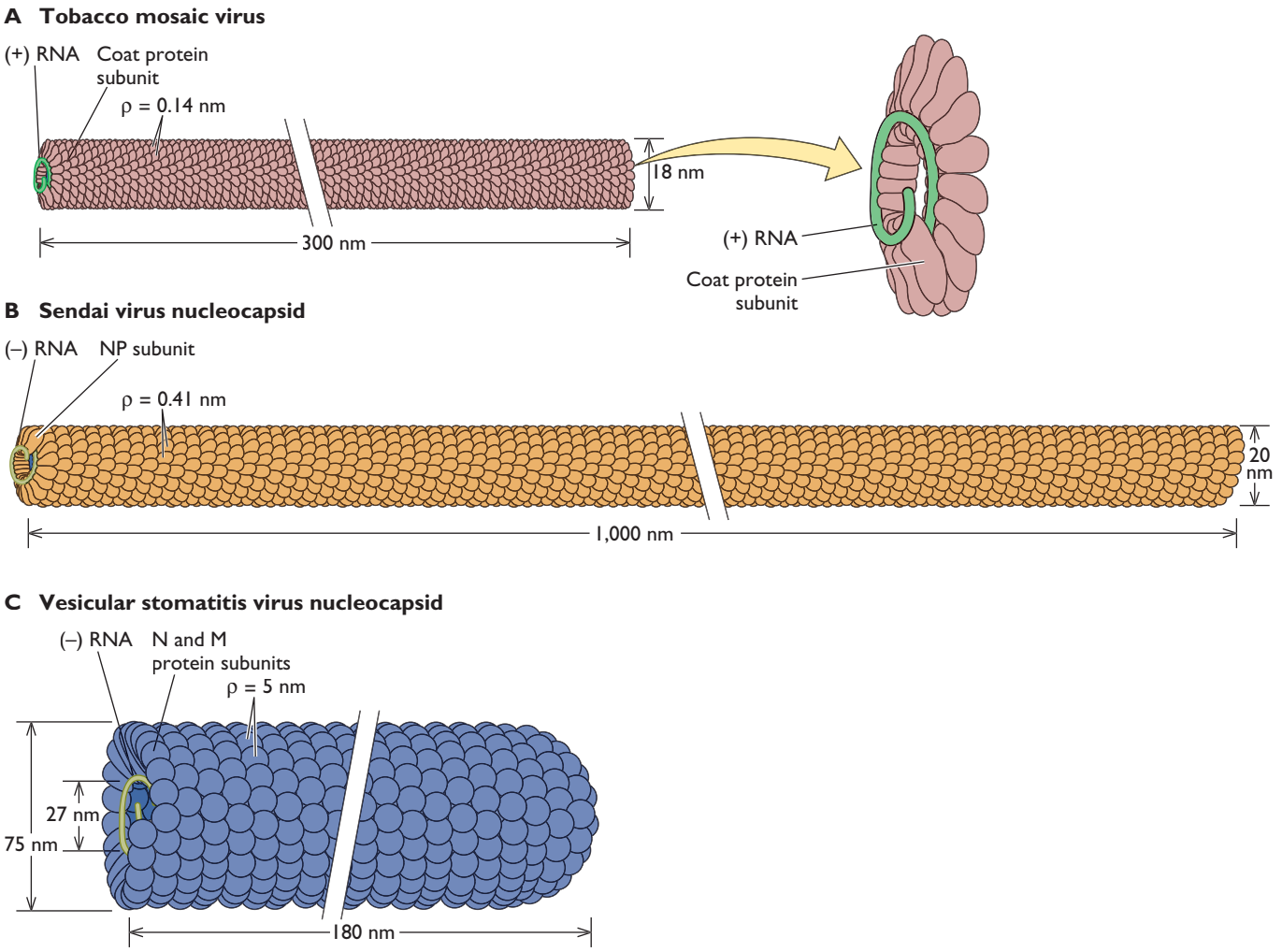


Figure 4.6 Virus structures with helical symmetry. (A) Tobacco mosaic virus. The structure of this virus has been determined at high resolution by X-ray diffraction of fibers or oriented gels of the particles. The single genomic RNA molecule and the single coat protein form an extended right-handed helix with 16.3 protein subunits per turn, μ, and an axial rise per residue, ρ, of 0.14 nm, for a pitch, *P*, of 2.3 nm. The lock-washer-like broken-disk organization of the proteins in a single turn of the helix can be seen in the expanded view. **(B and C)** The structures of Sendai virus (a paramyxovirus) and vesicular stomatitis virus (a rhabdovirus) nucleocapsids are based on electron microscopy of virions or nucleocapsids released from them. The Sendai virus nucleocapsid resembles the tobacco mosaic virus particle in diameter and the presence of a hollow core, but it is in the form of a left-handed helix with distinctive helical parameters (μ = 13, ρ = 0.41 nm, *P* = 5.3 nm). The vesicular stomatitis virus nucleocapsid is also hollow and comprises about 30 turns of uniform diameter followed by 5 or 6 turns of decreasing diameter.

Capsids or Nucleocapsids with Icosahedral Symmetry

General Principles

Icosahedral symmetry. An icosahedron is a solid with 20 triangular faces and 12 vertices related by two-, three-, and fivefold axes of rotational symmetry (Fig. 4.8A). In a few cases, virus particles can be readily seen to be icosahedral (for examples of such particles, see Fig. 4.15 and 4.25). However, most closed capsids and nucleocapsids **look** spherical, and they often possess prominent surface structures or viral glycoproteins in the envelope that do not conform to the underlying icosahedral symmetry of the capsid shell. Nevertheless, the symmetry with which the structural units interact is that of an icosahedron.

In solid geometry, each of the 20 faces of an icosahedron is an equilateral triangle (the triangle is one of the three basic units that can form solid structures approximating

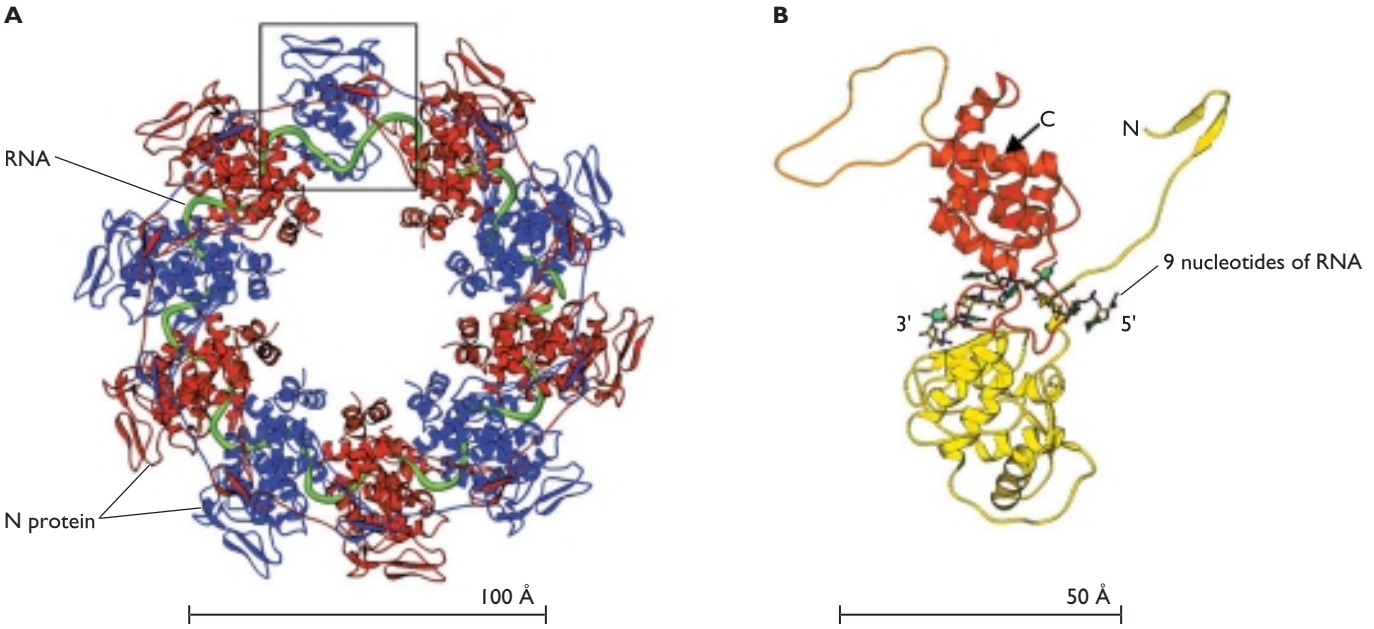


Figure 4.7 Structure of a ribonucleoprotein-like complex of vesicular stomatitis virus. (A) The structure of the decamer of the N protein bound to RNA is shown, with alternating monomers in the ring colored red and blue and the RNA ribose-phosphate backbone depicted as a green tube. To allow visualization of the RNA, the C-terminal domain of the boxed monomer is not shown. The decamer was isolated by dissociation of the viral P protein from RNA-bound oligomers formed when the N and P proteins were synthesized in *Escherichia coli*. **(B)** A single N protein molecule bound to 9 nucleotides of RNA is shown, looking out from the inside of the ring and perpendicular to the view in panel A. The RNA, depicted as a ball-and-stick model, is tightly bound at the interface between the N- and C-terminal lobes of the N protein, which are colored yellow and orange, respectively. The N-terminal extension and the extended loop in the C-terminal lobe contribute to the extensive interactions among neighboring N monomers. Courtesy of M. Luo, University of Alabama at Birmingham. Adapted from T. J. Green et al., *Science* **313:**357–360, 2006, with permission.

a sphere). Five such triangles interact at each of the 12 vertices of the icosahedron (Fig 4.8A). In the simplest protein shells, a trimer of a single viral protein (the **subunit**) corresponds to each triangular face of the icosahedron: as shown in Fig 4.8B, such trimers interact with one another at the five-, three-, and twofold axes of rotational symmetry that define an icosahedron. As an icosahedron has 20 faces, 60 identical subunits (3 per face × 20 faces) is the minimum number needed to build a capsid or nucleocapsid with icosahedral symmetry. Such well-defined symmetry poses a number of interesting questions about the organization and assembly of icosahedral viruses. This property may also prove to have great practical value (Box 4.2).

Large capsids and quasiequivalent bonding. In the simple icosahedral packing arrangement shown in Fig. 4.8B, each of the 60 subunits (**structural** or **asymmetric units**) consists of a single molecule in a structurally identical environment. Consequently, all subunits interact with their neighbors in an identical (or **equivalent**) manner, just like the subunits of a helical nucleocapsid such as that of tobacco mosaic virus. The size of a protein shell constructed according to this simplest icosahedral design is therefore determined by the size of its protein subunits. As the viral proteins that form such closed shells are generally less than ~100 kDa in molecular mass, the size of the viral genome that can be accommodated in this type of particle is restricted severely. In fact, the capsids or nucleocapsids of the majority of animal viruses are built from many more than 60 subunits and can house quite large genomes. In 1962, Donald Caspar and Aaron Klug developed a theoretical framework accounting for the structural properties of larger particles with icosahedral symmetry. This theory has had enormous influence on the way virus structures are described and interpreted.

The triangulation number *T*. A crucial idea introduced by Caspar and Klug was that of **triangulation**, the description of the triangular face of a large icosahedral structure in terms of its subdivision into smaller triangles, termed **facets**. In the example shown in Fig. 4.9, four such

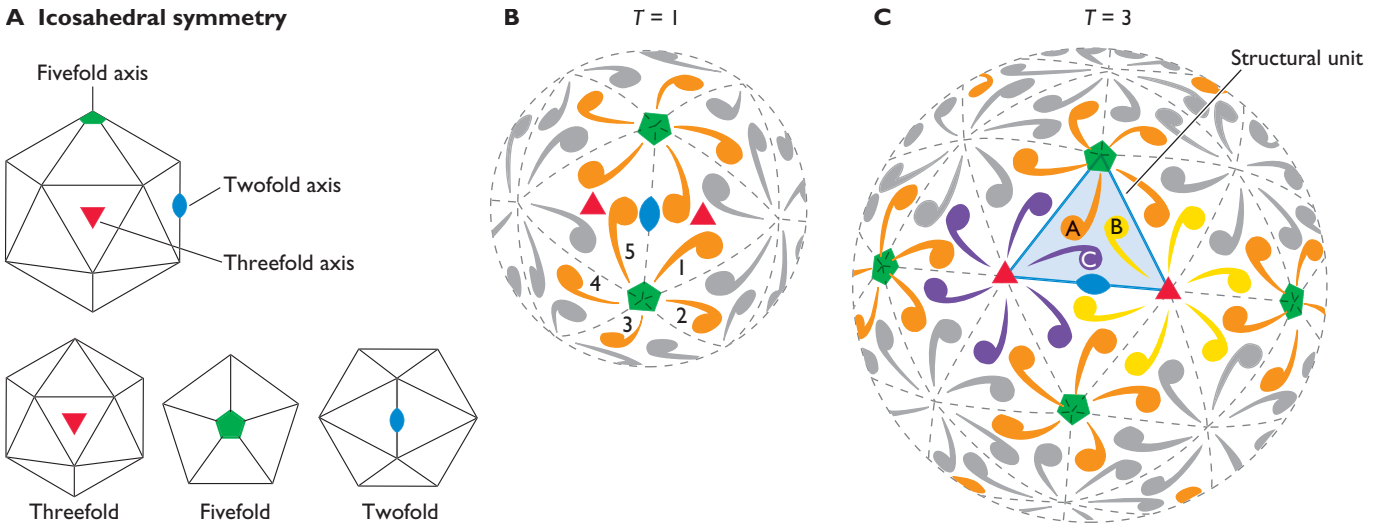


Figure 4.8 Icosahedral packing in simple structures. (A) An icosahedron, which comprises 20 equilateral triangular faces characterized by positions of five-, three-, and twofold rotational symmetry. The three views at the bottom illustrate these positions. **(B and C)** A comma represents a single protein molecule, and axes of rotational symmetry are indicated as in panel A. In the simplest and ideal case, $T = 1$ **(B)**, the protein molecule forms the structural unit, and each of the 60 molecules is related to its neighbors by the two-, three-, and fivefold rotational axes that define a structure with icosahedral symmetry. In such a simple icosahedral structure, the interactions of all molecules with their neighbors are identical. In the $T = 3$ structure **(C)** with 180 identical protein subunits, there are three modes of packing of a subunit (shown in orange, yellow, and purple): the structural unit (outlined in blue) is now the asymmetric unit, which, when replicated according to 60-fold icosahedral symmetry, generates the complete structure. The orange subunits are present in pentamers, formed by tail-to-tail interactions, and interact in rings of three (head to head) with purple and yellow subunits, and in pairs (head to head) with a purple or a yellow subunit. The purple and yellow subunits are arranged in rings of six molecules (by tail-to-tail interactions) that alternate in the particle. Despite these packing differences, the bonding interactions in which each subunit engages are similar, that is, **quasiequivalent:** for example, all engage in tail-to-tail and head-to-head interactions. Adapted from S. C. Harrison et al., *in* B. N. Fields et al. (ed.), *Fundamental Virology* (Lippincott-Raven, New York, NY, 1995), with permission.

facets (each equivalent to one face of the simplest structure shown in Fig. 4.8B) are combined to assemble a larger face of an icosahedrally symmetric structure from the same homotrimer (structural unit). This process is described by the triangulation number, *T*, which gives the number of structural units (small "triangles") per face. Because the minimum number of subunits required is 60, the total number of subunits in the structure is 60*T*.

Quasiequivalence. A second cornerstone of the theory developed by Caspar and Klug was the proposition that when a capsid contains more than 60 subunits, each subunit occupies a **quasiequivalent position**; that is, the noncovalent bonding properties of subunits in different structural environments are **similar** (but not identical, as is the case for the simplest, 60-subunit structure). This property is illustrated in Fig. 4.8C for a particle with 180 identical subunits. In the small, 60-subunit structure, 5 subunits make fivefold symmetric contact at each of the 12 vertices (Fig. 4.8B). In the larger structure with 180 subunits, this arrangement is retained at the 12 vertices, but the additional subunits, arranged with sixfold symmetry, are interposed between the fivefold-symmetric clusters (Fig. 4.8C). In such a structure, each subunit can be present in one of three **different** structural environments (designated A, B, or C in Fig. 4.8C). Nevertheless, all subunits bond to their neighbors in similar (**quasiequivalent**) ways, for example, via head-to-head and tail-to-tail interactions (Fig. 4.8C). Likewise, in a $T = 4$ structure (Fig. 4.9), the packing interactions of the 240 subunits are very similar. It is important to appreciate that there is not a simple and direct relationship between structural (asymmetric) units and icosahedral faces (Box 4.3). However, the *T* number defines the number of subunits in a face.

Icosahedrally symmetric structures can be formed from quasiequivalent subunits for only certain values of *T* (Box 4.4). Virus structures corresponding to various values of *T*, some very large, have been described (Table 4.3). The triangulation number and quasiequivalent bonding among subunits describe the structural properties of many simple

BOX 4.2 METHODS

Nanoconstruction with virus particles

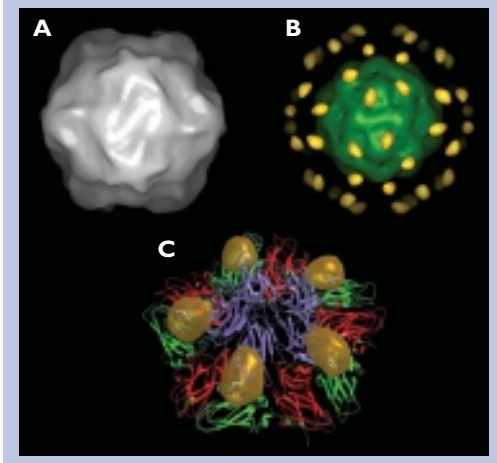
Nanochemistry is the synthesis and study of well-defined structures with dimensions of 1 to 100 nm. Nano-building blocks span the size range between molecules and materials. Molecular biologists study nanochemistry, nanostructures, and molecular machines including the ribosome, the photosynthetic center, and membrane-bound signaling complexes. Icosahedral virus particles are proving to be precision building blocks for nanochemistry. The $T = 1$ icosahedral cowpea mosaic virus particle is 30 nm in diameter, and its atomic structure is known in detail. Grams of virions can be prepared easily from kilograms of infected leaves. Insertional mutagenesis is straightforward, and precise amino acid changes can be introduced. As illustrated in the figure, cysteine residues inserted in the capsid protein provide functional groups for chemical attachment of 60 precisely placed molecules.

High local concentrations of the attached chemical agent, coupled with precise placement, enable rather remarkable nanoconstruction. For example, virus surfaces can be patterned with metal nanoparticles that may function as a conducting "wire" for electronics. In addition, the propensity of virions for self-organization into two- and three-dimensional lattices leads to well-ordered arrays of 10^{13} particles in a 1-mm^3 crystal.

Viruses are not just for infections any more! They will provide a rich source of building blocks for applications spanning the worlds of molecular biology and materials science.

Nam, K. T., D. W. Kim, P. J. Yoo, C. Y. Chiang, N. Meethong, P. T. Hammond, Y. M. Chiang, and A. M. Belcher. 2006. Virus-enabled synthesis and assembly of nanovirus for lithium ion battery electrodes. *Science* **312:**885–888.

Wang, Q., T. Lin, L. Tang, J. E. Johnson, and M. G. Finn. 2002. Icosahedral virus particles as addressable nanoscale building blocks. *Angew. Chem. Int. Ed. Engl.* **41:**459–462.



Gold particles attached to cowpea mosaic virus. Cryo-electron microscopy was performed on derivatized cowpea mosaic virus with a cysteine residue inserted on the surface of each of the 60 subunits and to which nanogold particles with a diameter of 1.4 nm were chemically linked. **(A)** Three-dimensional reconstruction of such derivatized virus particles at 29-Å resolution. **(B)** Difference electron density map obtained by subtracting the density of unaltered cowpea mosaic virus at 29 Å from the density map shown in panel A. This procedure reveals both the genome (shown in green) and the gold nanoparticles. **(C)** A section of the difference map imposed on the atomic model of cowpea mosaic virus. The positions of the gold indicate that it is attached at the sites of the introduced cysteine residues. Courtesy of M. G. Finn and J. Johnson, The Scripps Research Institute.

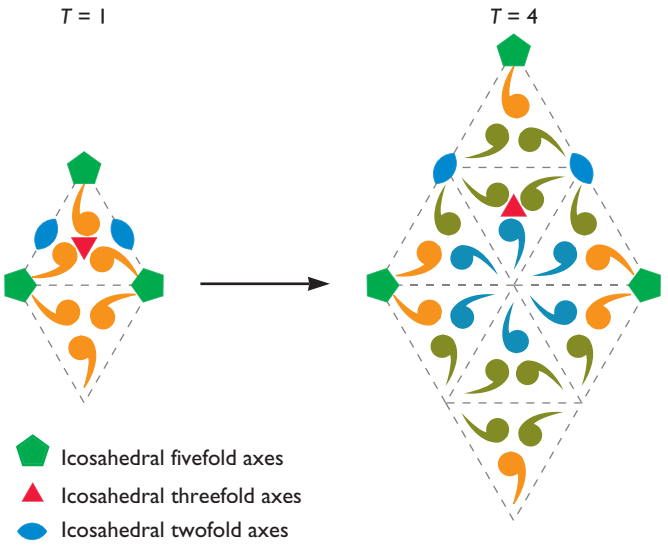


Figure 4.9 *T* = 4 triangulation of an icosahedral face. Two faces of a $T = 1$ icosahedron, each composed of three protein subunits, are shown flattened into a single plane on the left. The two-, three-, and fivefold rotational axes that define icosahedral symmetry are indicated on the top face. The combination of four identical faces (now facets) to form a $T = 4$ face is shown on the right. The icosahedral symmetry axes are in the same relative positions as in the $T = 1$ face. In such a $T = 4$ structure, the size of the face, which is defined by the length of one of its sides, is twice that in the $T = 1$ structure. Adapted from S. Casjens, *in* S. Casjens (ed.), *Virus Structure and Assembly* (Jones and Bartlett Publishers Inc., Boston, MA, 1985), with permission.

BOX 4.3 TERMINOLOGY

Structural (asymmetric) units and faces of virus particles with icosahedral symmetry: not one and the same

The structural (asymmetric) unit of an icosahedral capsid is the **smallest** unit that can give rise to the complete structure when replicated according to the rules of icosahedral symmetry. Therefore

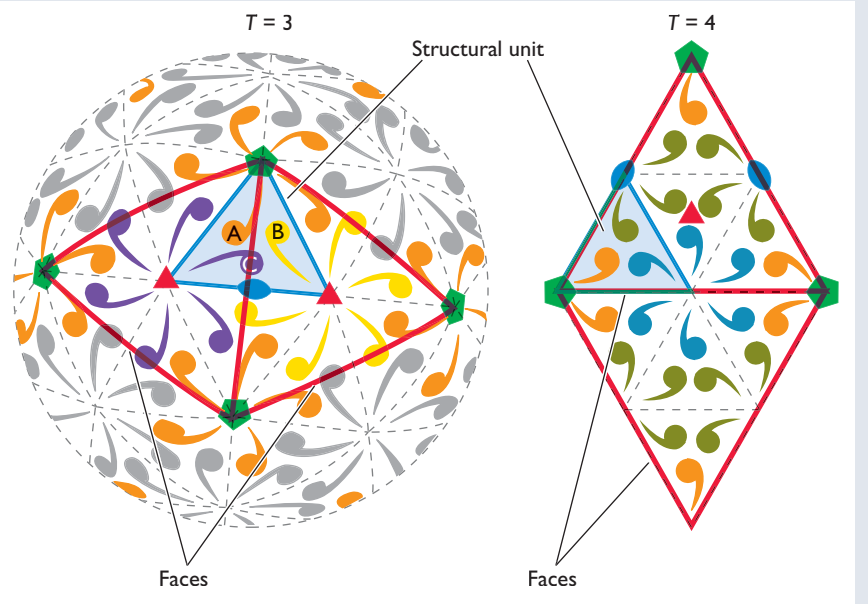
- the capsid must contain 60 copies of the structural unit
- five structural units must interact around each of the axes of fivefold rotational symmetry.

In the $T = 1$ structure shown in Fig. 4.8, the structural unit is a single protein subunit, whereas it is a homotrimer in the $T = 3$ structure.

An icosahedron has 20 faces. Nevertheless, there is **not** a simple relationship between a structural (asymmetric) unit and a face, as shown in the figure.

On the other hand, the ***T*** number defines the number of subunits in each face:

T number	No. of subunits ($60 \times T$)	No. of subunits/face [$(60 \times T)/20$]
1	60	3
3	180	9
4	240	12



Structural units and faces of icosahedral virus particles. A $T = 3$ particle and part of a $T = 4$ particle are depicted as in Fig. 4.8 and 4.9, respectively. Structural asymmetric units are outlined in blue, and faces are shown in red.

viruses with icosahedral symmetry. However, it is now clear that the structures adopted by specific segments of capsid proteins can govern the packing interactions of identical subunits. This property indicates that interactions among chemically identical protein subunits must be regulated during assembly, with appropriate conformational switching of flexible segments. Such large conformational differences between small regions of chemically identical (and otherwise quasiequivalent) subunits were not anticipated in early considerations of virus structure. This omission is not surprising, for these principles were formulated when little was known about the structural properties of proteins. As we discuss in the next sections, the structural properties of both small and more complex viruses can depart radically from the constraints imposed by quasiequivalent bonding. For example, the capsid of the small polyomavirus simian virus 40 is built from 360 subunits, corresponding to the $T = 6$ triangulation number excluded by the rules formulated by Caspar and Klug (Box 4.4). Moreover, a capsid stabilized by covalent joining of subunits to form viral "chain mail" has been described (Box 4.5). Our current view of icosahedrally symmetric virus structures is therefore one that includes greater diversity in the mechanisms by which stable capsids can be formed than was anticipated by the pioneers in this field.

Structurally Simple Capsids

Several nonenveloped animal viruses are small enough to be amenable to high-resolution structural studies by X-ray crystallography. We have chosen four examples, the parvovirus adenovirus-associated virus 2, the nodavirus Nodamura virus, the picornavirus poliovirus, and the polyomavirus simian virus 40, to illustrate the molecular foundations of icosahedral architecture.

Structure of adeno-associated virus 2: classic $T = 1$ icosahedral design. The parvoviruses are very small animal viruses, with particles of ~25 nm in diameter that encase single-stranded DNA genomes of less than 5 kb. These small naked nucleocapsids are built from 60 copies of a

single subunit organized according to $T = 1$ icosahedral symmetry. The structure of adenovirus-associated virus type 2, a member of the dependovirus subgroup of parvoviruses that require a helper virus for replication (Appendix, Fig. 11), has been determined at high resolution by X-ray crystallography. The subunits that form the nucleocapsid contain a three-dimensional motif, the **β-barrel jelly roll** that is conserved in the structural proteins of unrelated viruses. In adenovirus-associated virus particles, the interactions among neighboring subunits are mediated by loops that connect the β-strands in the β-barrel. Some of these loops are long and contain additional β-strands or α-helices (Fig. 4.10A). As illustrated in Fig. 4.10B, the interactions among them result in formation of a particle with classic $T = 1$ icosahedral symmetry. The prominent projections near the threefold axes of rotational symmetry (Fig. 4.10B), which have been implicated in receptor binding of adenovirus-associated virus type 2, are formed by extensive interdigitation among loops from adjacent subunits.

Structure of Nodamura virus: typical $T = 3$ icosahedral design. The nodaviruses are small (290 to 320 Å in diameter), nonenveloped viruses that infect mammals, fish, and insects. The capsid of Nodamura virus (Fig. 4.11A) encases the (+) strand RNA genome, which comprises two molecules of RNA. It is built from 180 copies of a single coat protein, organized according to a $T = 3$ quasiequivalent design: the 60 structural units contain one copy of each of three types of coat protein subunit that are defined by occupancy of structurally distinct environments (A, B, and C in Fig. 4.11B). The A subunits are arranged as pentamers around the 12 axes of fivefold rotational symmetry, whereas the B and C subunits alternate in hexameric rings around the axes of threefold symmetry (Fig. 4.11B; compare with Fig. 4.8B). Despite the differences in subunit packing, interactions among A, B, and C subunits within a structural unit are very similar. However, adjacent subunits in neighboring asymmetric units engage in either flat or bent contacts (Fig. 4.11B). This difference is the result of the presence of a short, ordered protein segment only in the C subunits and of a duplex segment of the RNA genome at the flat contacts. These structural features hold the neighboring subunits farther apart than at the bent contacts, so that the asymmetric units form a flat, diamond-shaped structure (Fig. 4.11B). Whether internal surfaces of adjacent subunits interact with ordered segments of the RNA genome is therefore a crucial determinant of the quasiequivalent, icosahedral architecture of Nodamura virus (and other members of the *Nodaviridae*).

Structure of poliovirus: a pseudo $T = 3$ structure. As their name implies, the picornaviruses are among the smallest of animal viruses. The approximately 300-Å-diameter poliovirus particle is composed of 60 copies of each of four virally encoded structural proteins, VP1, VP2, VP3, and VP4 (VP = virion protein). These proteins form a closed capsid encasing the (+) strand RNA genome of about 7.5 kb and its covalently attached 5′-terminal protein, VPg (Appendix, Fig. 13). Our understanding of the structure of the *Picornaviridae* took a quantum leap in 1985 with the determination of high-resolution structures of human rhinovirus 14 (genus *Rhinovirus*) and poliovirus (genus *Enterovirus*).

The heteromeric structural unit of the poliovirus capsid contains one copy each of VP1, VP2, VP3, and VP4 (Fig. 4.12A). VP4 lies on the inner surface of the protein shell formed by VP1, VP2, and VP3. Although the three latter proteins are not related in amino acid sequence, all contain a central β-sheet structure termed a **β-barrel jelly roll**. The organization of this β-barrel domain is illustrated schematically in Fig. 4.12B, for comparison with the actual structures of VP1, VP2, and VP3. It is a wedge-shaped structure comprising two antiparallel β-sheets. One of the β-sheets forms one wall of the wedge, while the second, sharply twisted β-sheet forms both the second wall and the floor (Fig. 4.12B). The β-barrel domains of VP1, VP2, and VP3 are folded in the same way; that is, they possess the same **topology**. The differences among these proteins are therefore restricted largely to the loops that connect β-strands and to the N- and C-terminal segments that extend from the central β-barrel domains (Fig. 4.12B).

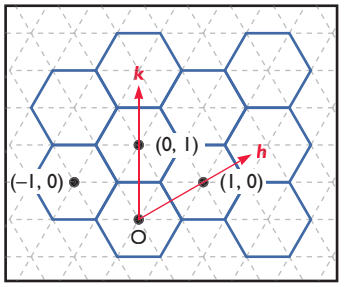
The β-barrel jelly rolls of these picornaviral proteins are similar in structure to the core domains of capsid proteins of a number of plant, insect, and vertebrate (+) strand RNA viruses such as tomato bushy stunt virus and Nodamura virus. This property was entirely unanticipated. Even more remarkably, this relationship is not restricted to small RNA viruses: the major capsid proteins of the DNA-containing parvoviruses, polyomaviruses, and adenoviruses also contain such β-barrel domains. In fact, the β-barrel jelly roll continues to be the structural motif most commonly encountered as the structures of additional capsid proteins are determined. It is well established that the three-dimensional structures of cellular proteins have been highly conserved during evolution, even though there may be very little amino acid sequence identity. For example, all globins possess a common three-dimensional structure based on eight α-helices, even though the only residues conserved are those important for function. One interpretation of the common occurrence of the β-barrel jelly roll domain in viral capsid proteins is therefore that seemingly unrelated modern viruses (e.g., picornaviruses and adenoviruses) share some portion of their evolutionary history. It is also possible that this structural domain represents one of a limited number commensurate with

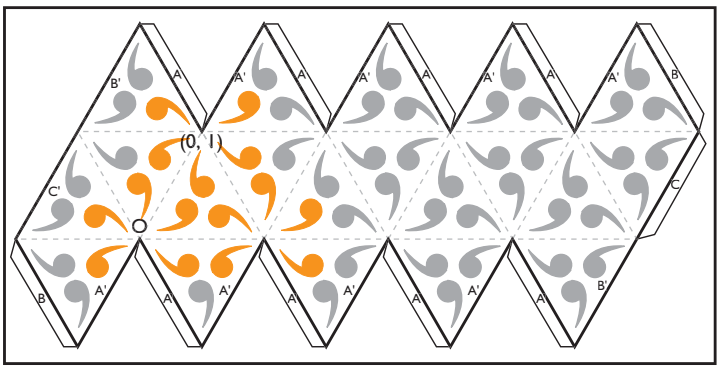
BOX 4.4

BACKGROUND

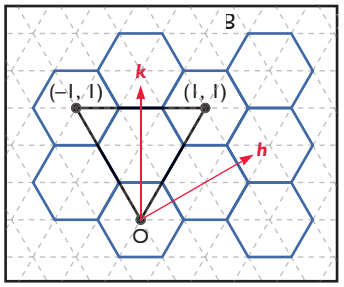
The triangulation number, T, and how it is determined

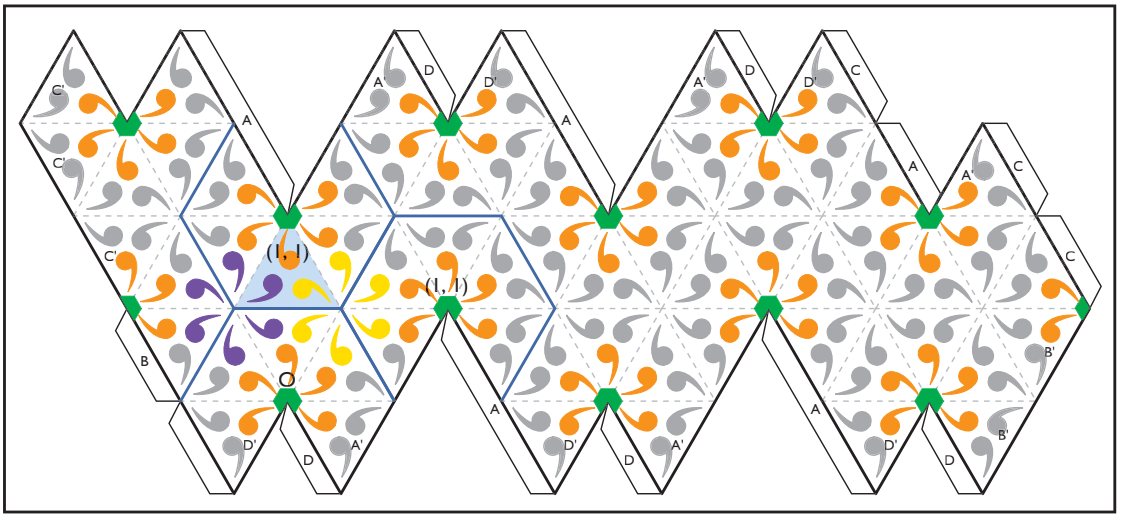
A $T = 1$





B $T = 3$





In developing their theories about virus structure, Caspar and Klug used graphic illustrations of capsid subunits, such as the net of flat hexagons shown at the top of panels **A** and **B**. Each hexagon represents a hexamer, with identical subunits shown as equilateral triangles. When all subunits assemble into such hexamers, the result is a flat sheet, or lattice, which can never form a closed structure. However, curvature can be introduced into the hexagonal net by converting specific hexamers to pentamers. As an icosahedron has 12 axes of fivefold symmetry, 12 pentamers must be introduced to form a closed structure with icosahedral symmetry. If 12 adjacent hexamers are converted to pentamers **(A)**, an icosahedron of the minimal size possible for the net is formed. This structure is built from 60 equilateral-triangle asymmetric units and corresponds to a $T = 1$ icosahedron. Larger structures with icosahedral symmetry are built by converting 12 **nonadjacent** hexamers to pentamers at precisely spaced and regular intervals. To illustrate this, we use nets in which an origin (O) is fixed and the positions of all other hexamers are defined by the coordinates along the axes labeled h and k, where h and k are any integers greater than or equal to zero (that is, positive integers). The hexamer (h, k) is therefore defined as that reached from the origin (O) by h steps in the direction of the h axis and k steps in the direction of the k axis. In the $T = 1$ structure, $h = 1$ and $k = 0$ (or $h = 0$ and $k = 1$), and adjacent hexamers are converted to pentamers (A, top left). To construct a model of an icosahedron when $h = 1$ and $k = 1$ **(B)**, we generate

one of its faces in the net by conversion of the origin (O) and (1, 1) hexamers to pentamers. The lattice point (1, 1) shown is reached by one step in each direction. The third hexamer replaced (–1, 1) is that identified by threefold symmetry to complete the large, marked equilateral triangle (black) representing the face of an icosahedron formed when $h = 1$ and $k = 1$ (**B**, top left). The resulting quasiequivalent lattice (**B**, bottom panel) and the $T = 1$ lattice (**A**, bottom panel) can be folded to form the icosahedra shown, by excision from the sheet and sequential joining of the edges marked A to D with those marked A′ to D′, respectively.

The triangulation number, T, is the number of asymmetric units per face on icosahedron constructed in this way. It can be shown, for example by geometry, that

$$T = h^2 + hk + k^2$$

Therefore, when both h and k are 1 **(B)**, $T = 3$, and each face of the icosahedron contains three asymmetric units. The total number of units, which must be $60T$ (see the text), is 180.

The integers h and k describe the spacing and spatial relationships of pentamers in the lattice and of fivefold vertices in the corresponding icosahedron. For example, when $h = 1$ and $k = 0$, or when $h = 0$ and $k = 1$, pentamers are in direct contact with other pentamers (see, e.g., Fig. 4.8B), but when $h = 1$ and $k = 1$, each pentamer is separated from neighboring pentamers by one hexamer (Fig. 4.8C). The values of h and k are therefore determined by inspection of electron micrographs of virus particles or their constituents **(C)**. For example, in the bacteriophage p22 capsid (top), one pentamer is separated from another by two steps (from one structural unit to the next) along the h axis and one step along the k axis, as illustrated for the bottom left pentamer shown. Hence, $h = 2$, $k = 1$, and $T = h^2 + hk + k^2 = 7$. In contrast, pentamers of the herpes simplex virus type 1 (HSV-1) nucleocapsid (bottom) are separated by four and zero steps along the directions of the h and k axes, respectively. Therefore, $h = 4$, $k = 0$, and $T = 16$. Cryoelectron micrographs of bacteriophage P22 and herpes simplex virus type 1 courtesy of B. V. V. Prasad and W. Chiu (Baylor College of Medicine), respectively. (A and B) Adapted from Fig. 2 of J. E. Johnson and A. J. Fisher, *in* R. G. Webster and A. Granoff (ed.), *Encyclopedia of Virology*, 3rd ed. (Academic Press, London, England, 1994), with permission.

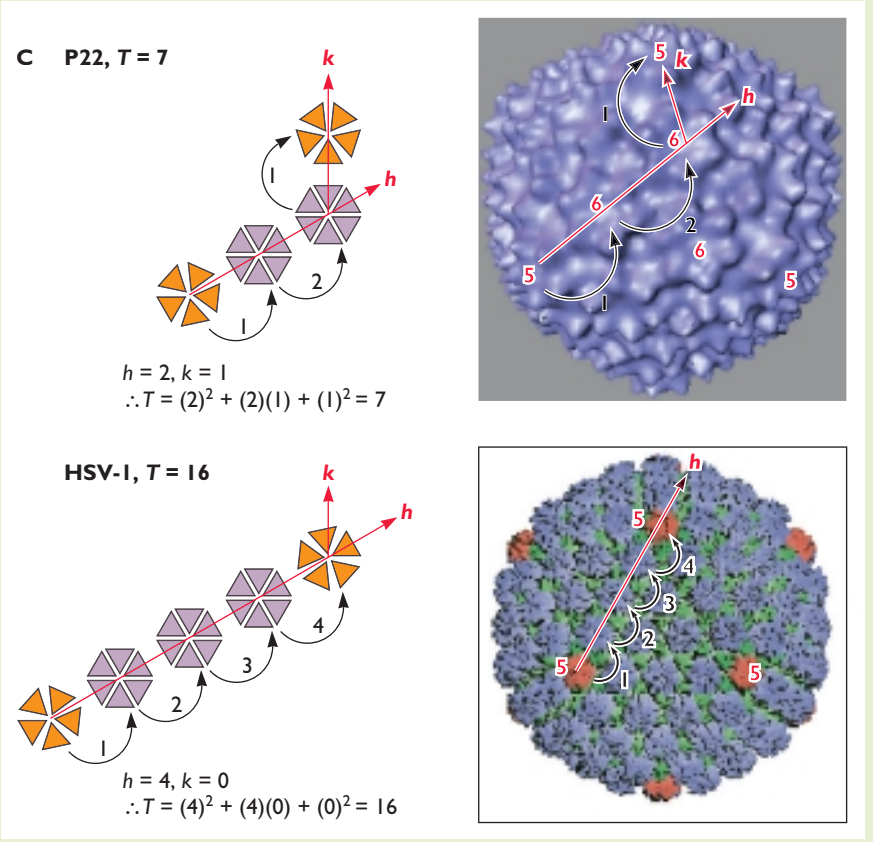


Table 4.3 *T* numbers of representative viruses

Triangulation no. (*T*)[a]	Family	Member(s)	60*T*	Protein(s) that forms the capsid or nucleocapsid shell
1	*Parvoviridae*	Canine parvovirus	60	60 copies of VP2
3	*Nodaviridae*	Black beetle virus	180	180 copies of coat protein
3 (pseudo)	*Picornaviridae*	Poliovirus, human rhinovirus	180	60 copies each of VP1, VP2, and VP3
4	*Alphaviridae*	Ross River virus	240	240 copies of C protein
7	*Polyomaviridae*	Simian virus 40, polyomavirus	420	360 copies of VP1
13	*Reoviridae*	Reovirus (outer shell)	780	60 copies of λ2, 600 copies of μ1
16	*Herpesviridae*	Herpes simplex virus type 1	960	960 copies of VP5
25	*Adenoviridae*	Adenovirus type 2	1,500	720 copies of protein II, 60 copies of protein III
219	Unassigned	*Phaeocystis pouchetii* virus	13,050	Major capsid protein (hexamers)

[a]The triangulation number, *T*, is a description of the face of an icosahedron indicating the number of equilateral triangles into which each face is divided following the laws of solid geometry (Box 4.4). The total number of subunits is equal to 60*T*. The simplest assembly is $T = 1$. A $T = 3$ icosahedron requires 180 subunits in sets of three; a $T = 4$ icosahedron requires 240 subunits; a $T = 7$ icosahedron requires 420 subunits; and so forth! The 60 structural units of picornavirus capsids each contain four different polypeptides, and the capsid is described as a pseudo $T = 3$ structure. Note that papovaviruses, reoviruses, and adenoviruses do not contain the number of subunits predicted.

packing of proteins to form a sphere and is an example of convergent evolution. The remarkable similarities in the detailed structures of the capsid proteins of human adenoviruses and the bacteriophage PRD1 (Box 4.6), provide support for the first hypothesis.

The overall similarity in shape of the β-barrel domains of poliovirus VP1, VP2, and VP3 facilitates both their interaction with one another to form the 60 structural units of the capsid and the packing of these structural units in this structure. How well these interactions are tailored to form a protective shell is illustrated by the space-filling model of the capsid shown in Fig. 4.13A and B: the extensive interactions among the β-barrel domains of adjacent proteins form a dense, rigid protein shell around a central cavity in which the genome resides. The packing of the β-barrel domains is reinforced by a network of protein-protein contacts on the inside of the capsid. These interactions are particularly extensive about the fivefold axes (Fig. 4.13C).

One of several important lessons learned from high-resolution structures of picornaviruses is that their design does not conform strictly to the principle of **quasiequivalence**. For example, despite the topological identity and geometric similarity of the central domains of the poliovirus proteins that form the capsid shell, the subunits do not engage in quasiequivalent bonding: interactions among VP1 molecules around the fivefold axes are not chemically or structurally equivalent to those in which VP2 or VP3 engage (Fig. 4.13A and B). These differences account for the characteristic features of the surface of the capsid. The interaction of five VP1 molecules, unique to the fivefold axes, results in a prominent protrusion extending to about 25 Å from the capsid shell (Fig. 4.13A and B). The resulting structure appears as a steep-walled plateau encircled by a valley or cleft. In the capsids of many picornaviruses, these depressions, which contain the receptor-binding sites, are so deep that they have been termed **canyons**.

An alternative icosahedral design: structure of simian virus 40. The capsids of the small DNA polyomaviruses simian virus 40 and polyomavirus, about 500 Å in diameter, are organized according to a rather different design. The structural unit is a pentamer of the major structural protein, VP1 (Fig. 4.14A). The capsid is built from 72 such structural units engaged in one of two kinds of interaction, which are described by the number of neighbors that surround any particular pentamer. Twelve structural units occupy the 12 positions of fivefold rotational symmetry, in which each is surrounded by five neighbors. Each of the remaining 60 pentamers is surrounded by 6 neighbors at positions of sixfold rotational symmetry in the capsid (Fig. 4.14A). Consequently, the 72 pentamers of simian virus 40 do not engage in identical or quasiequivalent interactions. Rather, they occupy a number of different local environments in the capsid, because of differences in packing around the five- and sixfold axes (Fig. 4.14A).

Like the three poliovirus proteins that form the capsid shell, simian virus 40 VP1 contains a large central β-barrel jelly roll domain, in this case with an N-terminal arm and a long C-terminal extension (Fig. 4.14B). However, the arrangement and packing of VP1 molecules bear little resemblance to the organization of poliovirus capsid proteins. In the first place, the VP1 β-barrels in each pentamer project outward from the surface of the capsid to a distance of about 50 Å (Fig. 4.14A), in sharp contrast to those of the poliovirus capsid proteins, which tilt along the surface of the capsid shell. As a result, the surface of simian virus 40 is much more "bristly" than that of poliovirus

BOX 4.5 EXPERIMENTS

Viral chain mail: stabilization of the bacteriophage HK97 capsid by formation of covalently bonded, interlinked subunit rings

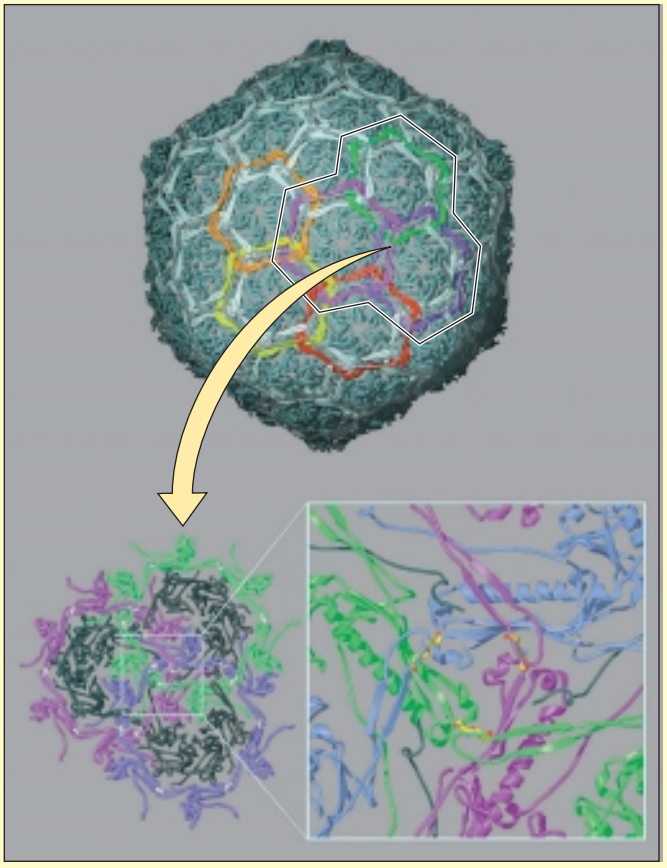
The mature capsid of the tailed, double-stranded DNA bacteriophage HK97 is a $T = 7$ structure built from hexamers and pentamers of a single viral protein, Gp5. It is formed in a multistep assembly and maturation pathway, like the virions of many other DNA bacteriophages (e.g., lambda and T4) and some animal viruses (e.g., herpesviruses). The first hints of the remarkable and unprecedented mechanism of stabilization of this structure came from biochemical experiments, which showed that

- a previously unknown covalent protein-protein linkage forms in the final reaction in the assembly of the HK97 capsid: the side chain of a lysine in every Gp5 subunit forms a covalent bond with an asparagine in an adjacent subunit. Consequently, **all** subunits are joined covalently to each other;
- this reaction is **autocatalytic**, depending only on Gp5 subunits organized in a particular conformational state: the capsid is enzyme, substrate, and product;
- HK97 mature particles are extraordinarily stable and cannot be disassembled into individual subunits by boiling in sodium dodecyl sulfate: it was therefore proposed that the cross-linking also interlinks the subunits from adjacent structural units to catenate rings of hexamers and pentamers.

The determination of the structure of the HK97 capsid to 3.6-Å resolution by X-ray crystallography has confirmed the formation of such capsid "chain mail" (see figure). The HK97 capsid is the first example of a protein catenane (an interlocked ring). This unique structure has been shown to increase the stability of the virus particle, and it may be of particular advantage as the capsid shell is very thin. The delivery of the DNA genome to host cells via the tail of the particle obviates the need for capsid disassembly and reversal of its covalent subunit-subunit bonds.

Duda, R. L. 1998. Protein chainmail: catenated protein in viral capsids. *Cell* **94:**55–60.

Wikoff, W. R., L. Liljas, R. L. Duda, H. Tsuruta, R. W. Hendrix, and J. E. Johnson. 2000. Topologically linked protein rings in the bacteriophage HK97 capsid. *Science* **289:**2129–2133.



Chain mail in the bacteriophage HK97 capsid. The exterior of the HK97 capsid is shown at the top, with hexamers and pentamers of the Gp5 protein in gray. The segments of subunits that are cross-linked into rings are colored the same, to illustrate the formation of catenated rings of subunits. The cross-linking is shown in the more detailed view below, down a quasithreefold axis with three pairs of cross-linked subunits. The K-N isopeptide bonds are shown in yellow. The cross-linked monomers (shown in blue) loop over a second pair of covalently joined subunits (green), which in turn cross over a third pair (magenta). Adapted from W. R. Wikof et al., *Science* **289:**2129–2133, 2000, with permission. Courtesy of J. Johnson, The Scripps Research Institute.

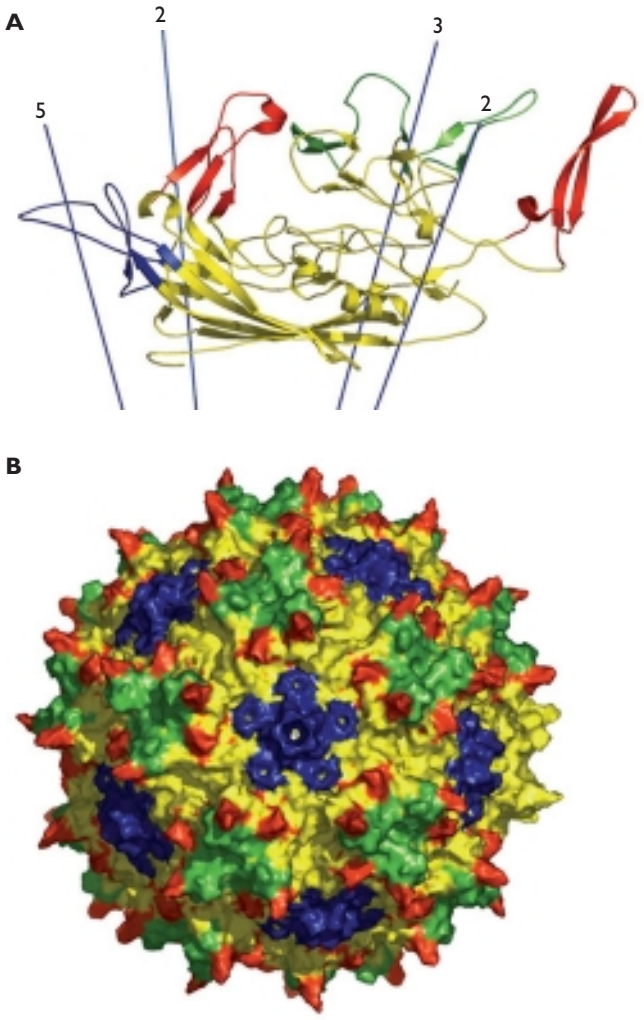


Figure 4.10 Structure of the parvovirus adeno-associated virus 2. (A) Ribbon diagram of the single subunit of the $T = 1$ particle. This comprises the C-terminal domain (about 130 amino acids) common to the VP1, VP2, and VP3 proteins, which are encoded within overlapping reading frames. As the VP1-VP2-VP3 ratio is 1:1:10 in the capsid, most subunits are contributed byVP3. The regions of the subunit that interact around the five-, three-, and twofold axes (indicated) of icosahedral symmetry are shown in blue, green, and yellow, respectively. The red segments form peaks that cluster around the threefold axes. (B) Surface view of the 3-Å-resolution structure determined by X-ray crystallography of purified virions. The regions of the single subunits from which the capsid is built are colored as in panel A. Courtesy of Michael Chapman, Florida State University. Adapted from Q. Xie et al., *Proc. Natl. Acad. Sci USA* **99:**10405–10410, 2002, with permission.

(compare Fig. 4.14A and 4.13B). Furthermore, the VP1 molecules present in adjacent pentamers in the simian virus 40 capsid do not make extensive contacts via the surfaces of their β-barrel domains. Rather, stable interactions among pentamers are mediated by their N- and C-terminal arms (Fig. 4.14). The packing of VP1 pentamers in both pentameric and hexameric arrays in the simian virus 40 capsid requires different contacts among these structural units, depending on their local environment. In fact, there are just three kinds of interpentamer contact, which are the result of alternative conformations and noncovalent interactions of the long C-terminal arms of VP1 molecules (Fig. 4.14A). The same capsid design is also exhibited by human papillomaviruses, with further strengthening of the interactions among adjacent pentamers by the formation of intermolecular disulfide bonds.

Each VP1 pentamer contains a tapering cavity that is narrowest toward the outer surface of the virion. This aperture contains a common sequence of VP2 or VP3, which associates tightly with the VP1 pentamer. Both these proteins are internal and make no contribution to the outer surface of the virion. VP2 plays an important role during entry, but why VP3 is present in virions is not known. This protein is identical in sequence to the C-terminal segment of the larger VP2 protein (Appendix, Fig. 17). It is possible that the virus particle is simply too small to accommodate 72 molecules of VP2 (as well as the DNA genome) in its interior. VP3 could serve the same structural function as the C-terminal portion of VP2, presumably stabilizing VP1 pentamers but occupying less internal space.

Simian virus 40 and poliovirus capsids differ in their surface appearance, in the number of structural units, and in the ways in which the structural units interact. Despite such differences, these virions share important features, including modular organization of the proteins that form the capsid shell and a common β-barrel domain as the capsid building block. Neither poliovirus nor simian virus 40 capsids conform to strict quasiequivalent construction: all contacts made by all protein subunits are not similar, and in the case of simian virus 40, the majority of VP1 **pentamers** are packed in **hexameric** arrays. Nevertheless, close packing with icosahedral symmetry is achieved by limited variations of the contacts, either among topologically similar, but chemically distinct, surfaces (poliovirus) or made by a flexible arm (simian virus 40).

Structurally simple icosahedral capsids or nucleocapsids in more complex virions. Several viruses that are structurally more sophisticated than those described in the previous sections nevertheless possess simple protein coats built from one or a few structural proteins. The complexity comes from the additional protein and lipid layers in which the capsid is enclosed. For example, togaviruses such as Semliki Forest virus and Ross River virus (genus *Alphavirus*), contain a $T = 4$ icosahedral capsid built from a single protein (Table 4.3) within an envelope.

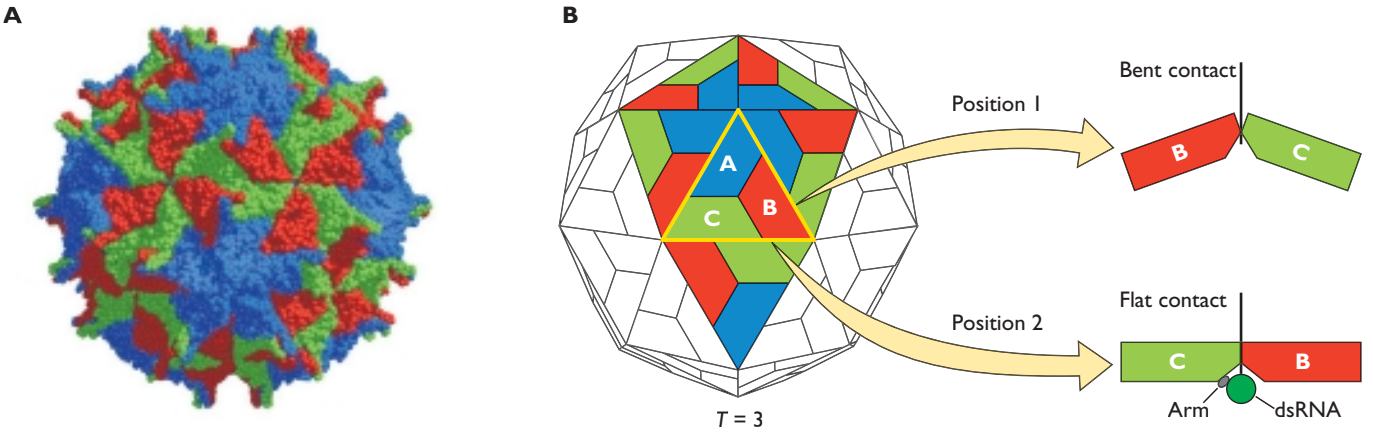


Figure 4.11 Structure of Nodamura virus. The structure of the Nodamura virus particle determined by X-ray crystallography is shown in panel **A** and summarized schematically in panel **B**. The coat protein subunits that occupy A, B, and C structural environments are colored yellow, red, and green, respectively. In panel B, the structural (asymmetric) unit is outlined in blue. The interactions between subunits at the sides of the structural units (position 1) are extensive and similar, and the asymmetric units make contact at an angle of 144°. In contrast, flat contacts are made between B and C subunits at position 2. This difference is the result of interaction of a 10-bp segment of double-stranded RNA with the internal surfaces of the subunits, and the ordering of a specific N-terminal segment of the protein only in the C subunits. (A) Courtesy of P. Natarajan and J. Johnson, The Scripps Research Institute and Virus Particle Explorer (VIPER). See V. S. Reddy et al., *J. Virol*. **75:**11943–11947, 2001.

Structurally Complex Capsids

Some naked viruses are considerably larger and more elaborate than the small RNA and DNA viruses described in the previous section. The characteristic feature of such virus particles is the presence of proteins devoted to specialized structural or functional roles. Despite such complexity, reasonably detailed pictures of the organization of this type of virion can be constructed by using combinations of biochemical, structural, and genetic methods. The well-studied human adenovirus and members of the *Reoviridae* family exemplify this approach.

Adenovirus. The most striking morphological features of the adenovirus particle (maximum diameter, 1,500 Å) are the well-defined icosahedral appearance of the capsid and the presence of long fibers at the 12 vertices (Appendix A, Fig. 1A). Each fiber, which terminates in a distal knob that binds to the adenoviral receptor, is attached to 1 of the 12 penton bases located at positions of fivefold symmetry in the capsid. The remainder of the shell is built from 240 additional subunits, the hexons, each of which is a trimer of viral protein II (Table 4.4). Formation of this capsid therefore depends on nonequivalent interactions among subunits: the hexons that surround pentons occupy a different bonding environment than those surrounded entirely by other hexons.

The intact adenovirus particle has not been studied by X-ray crystallography, but high-resolution structures have been determined for the penton base, the fiber, and the hexon. Each hexon subunit (Table 4.4) contains two β-barrel domains, each with the topology of theβ-barrels of the simpler RNA and DNA viruses described in the previous section (Fig. 4.15A). As the two β-barrels are very similar in structure, the hexon trimer exhibits pseudohexagonal symmetry, a property that facilitates its close packing in the capsid. In the trimer, the β-barrel domains of the three monomers are packed together in a hollow base from which rise three towers formed by intertwining loops from each monomer (Fig. 4.15B). The interactions among monomers are very extensive, particularly in the tower. Consequently, once the trimer has formed, the monomers cannot be dissociated easily, and the hexon is extremely stable.

The adenovirus particle contains seven additional structural proteins (Table 4.4), The presence of so many proteins and the large size of the virion have made elucidation of adenovirus structure a challenging problem. One approach that has proved generally useful in the study of complex viruses is the isolation and characterization of discrete subviral particles. For example, adenovirus particles can be dissociated into a core structure that contains the DNA genome, groups of nine hexons, and pentons. Analysis of the composition of such subassemblies, together with identification of virion proteins that contact one another (by cross-linking methods), identified two classes of virion proteins in addition to the major capsid proteins described

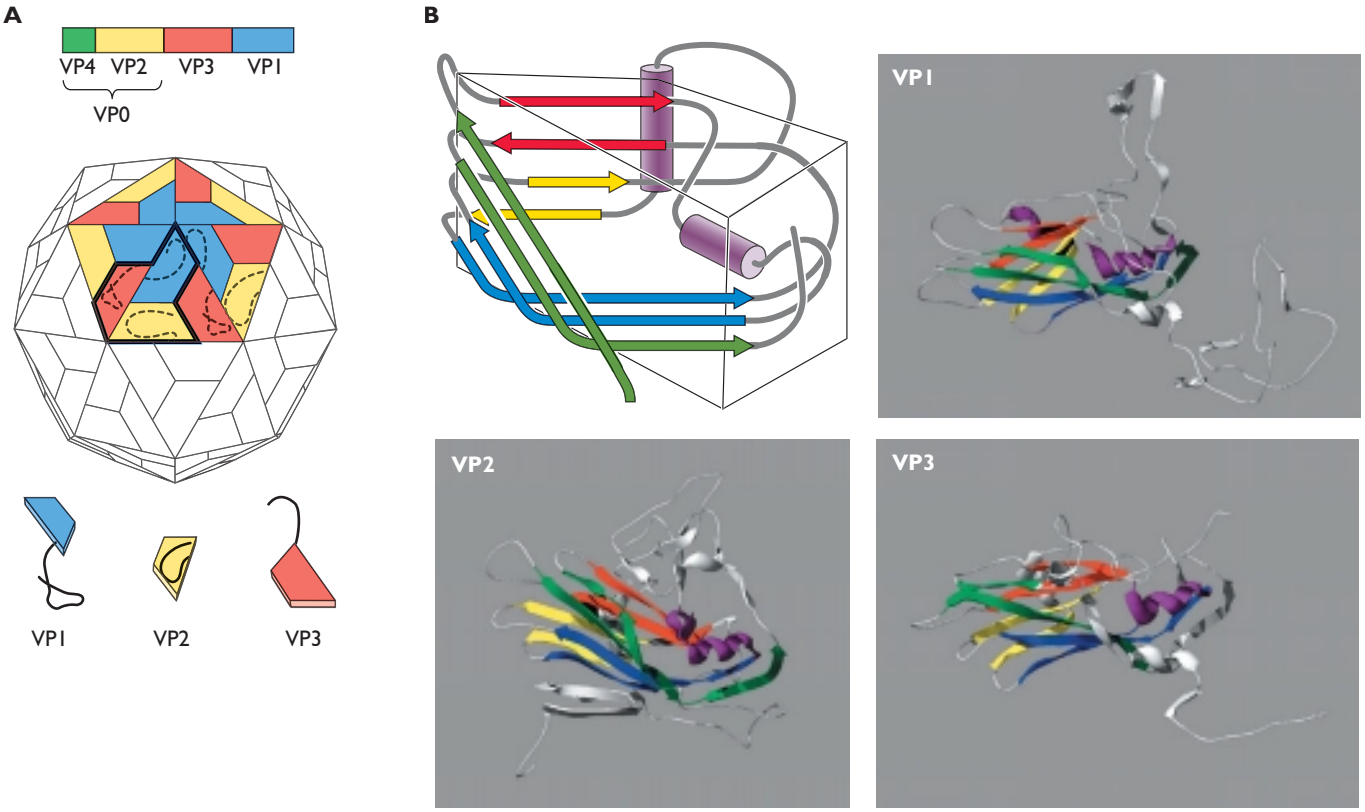


Figure 4.12 Packing and structures of poliovirus proteins. (A) The packing of the 60 VP1-VP2-VP3 structural units, represented by wedge-shaped blocks corresponding to their β-barrel domains. Note that the structural unit (outlined in black) contributes to two adjacent faces of an icosahedron rather than corresponding to a facet. When virions are assembled, VP4 is covalently joined to the N terminus of VP2. It is located on the inner surface of the capsid shell (see Fig. 4.13A). **(B)** The topology of the polypeptide chain in a β-barrel jelly roll is shown at the top left. The β-strands, indicated by arrows, form two antiparallel sheets juxtaposed in a wedgelike structure. The two α-helices (purple cylinders) that surround the open end of the wedge are also conserved in location and orientation in these proteins. As shown, the VP1, VP2, and VP3 proteins each contain a central β-barrel jelly roll domain. However, the loops that connect the β-strands in this domain of the three proteins vary considerably in length and conformation, particularly at the top of the β-barrel, which, as represented here, corresponds to the outer surface of the capsid. The N- and C-terminal segments of the protein also vary in length and structure. The very long N-terminal extension of VP3 has been truncated in this representation. Adapted from J. M. Hogle et al., *Science* **229:**1358–1365, 1985, with permission.

above. One comprises the proteins present in the core, such as protein VII, the major DNA-binding protein (Table 4.4). The remaining proteins are associated with either individual hexons or the groups of hexons that form an icosahedral face of the capsid (Table 4.4), suggesting that they stabilize the structure. Protein IX has been clearly identified as capsid "cement": a mutant virus that lacks the protein IX coding sequence produces the typical yield of virions, but these particles are much less heat stable than wild-type virions.

The locations of protein IX and other minor proteins with hexons and/or pentons have been visualized more recently by difference imaging (Fig. 4.5) and constructions of a quasiatomic model of the virion (Fig. 4.14C). In the latter approach, the crystal structures of the hexon and penton base were built into a 10-Å resolution map of the intact particle obtained by cryo-electron microscopy to reveal density associated with minor capsid proteins on both the exterior and internal surfaces of the capsid. The interactions of minor capsid proteins with the major structural units are extensive (see, e.g., Fig. 4.5), and clearly contribute to stabilizing the virus particle. During assembly, interactions among hexons and other major structural proteins must be relatively weak, so that incorrect associations can

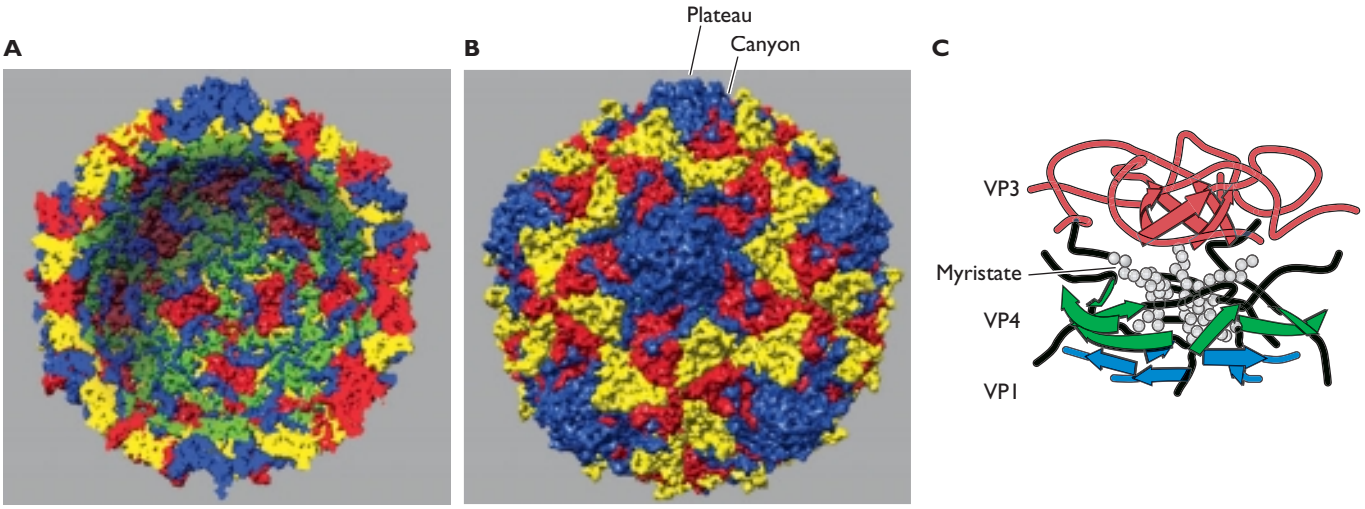


Figure 4.13 Interactions among the proteins of the poliovirus capsid. (A) Space-filling representation of the particle, with four pentamers removed from the capsid shell and VP1 in blue, VP2 in yellow, VP3 in red, and VP4 in green. Note the large central cavity in which the RNA genome resides, the dense protein shell formed by packing of the VP1, VP2, and VP3 β-barrel domains, and the interior location of VP4, which decorates the inner surface of the capsid shell. **(B)** Space-filling representation of the exterior surface showing the packing of the β-barrel domains of VP1, VP2, and VP3. Interactions among the loops connecting the upper surface of the β-barrel domains of these proteins create the surface features of the virion, such as the plateaus at the fivefold axes, which are encircled by a deep cleft or canyon. The virion is also stabilized by numerous interactions among the proteins on the inner side of the capsid. **(C)** These internal contacts are most extensive around the fivefold axes, where the N termini of five VP3 molecules are arranged in a tube-like, parallel β-sheet. The N termini of VP4 molecules carry chains of the fatty acid myristate (gray), which are added to the protein posttranslationally. The lipids mediate interaction of the β-sheet formed by VP3 N termini with a second β-sheet structure, containing strands contributed by both VP4 (green) and VP1 (blue) molecules. This internal structure is not completed until the final stages of, or after, assembly of virus particles, when proteolytic processing liberates VP2 and VP4 from their precursor, VP0. This reaction therefore stabilizes the capsid. Panels A and B were created from a PDB file.

be reversed and corrected. However, the assembled virion must be stable enough to survive passage from one host to another. It has been proposed that the incorporation of stabilizing proteins like protein IX allows these paradoxical requirements to be met.

Reoviruses. Reoviruses are naked $T = 13$ icosahedral particles, 700 to 900 Å in diameter, containing the 10 to 12 segments of the double-stranded genome and the enzymatic machinery to synthesize viral mRNA. Both adenoviruses and reoviruses are built from multiple proteins. However, reovirus particles exhibit an unusual architecture: they contain multiple protein shells. The particles of human reovirus (genus *Orthoreovirus*) contain eight proteins organized in two concentric shells, with spikes projecting from the inner layer through and beyond the outer layer at each of the 12 vertices (Fig. 4.16A). Members of the genus *Rotavirus*, which includes the leading causes of severe infantile gastroenteritis in humans (Volume II, Appendix A, Fig. 22), contain three nested protein layers, with 60 projecting spikes (Fig. 4.16B). Although differing in architectural detail, reovirus particles have common structural features, including an unusual design of the innermost protein shell.

Removal of the outermost protein layer, a process thought to occur during entry into a host cell (see Chapter 5), yields an inner core structure, comprising one shell (orthoreoviruses) or two (rotaviruses and members of the genus *Orbivirus*, such as bluetongue virus). These structures also contain the genome and virion enzymes and synthesize viral mRNAs under appropriate conditions *in vitro*. High-resolution structures have been obtained for bluetongue virus (Fig. 4.16C) and human reovirus cores, the largest viral assemblies yet to be examined by X-ray crystallography. The thin inner layer contains 120 copies of a single protein, termed VP3 in bluetongue virus and λ1 in human reovirus. These proteins are not related in their primary sequences, but they nevertheless have similar topological features and the same plate-like shape. Moreover, in both cases, the dimeric proteins occupy

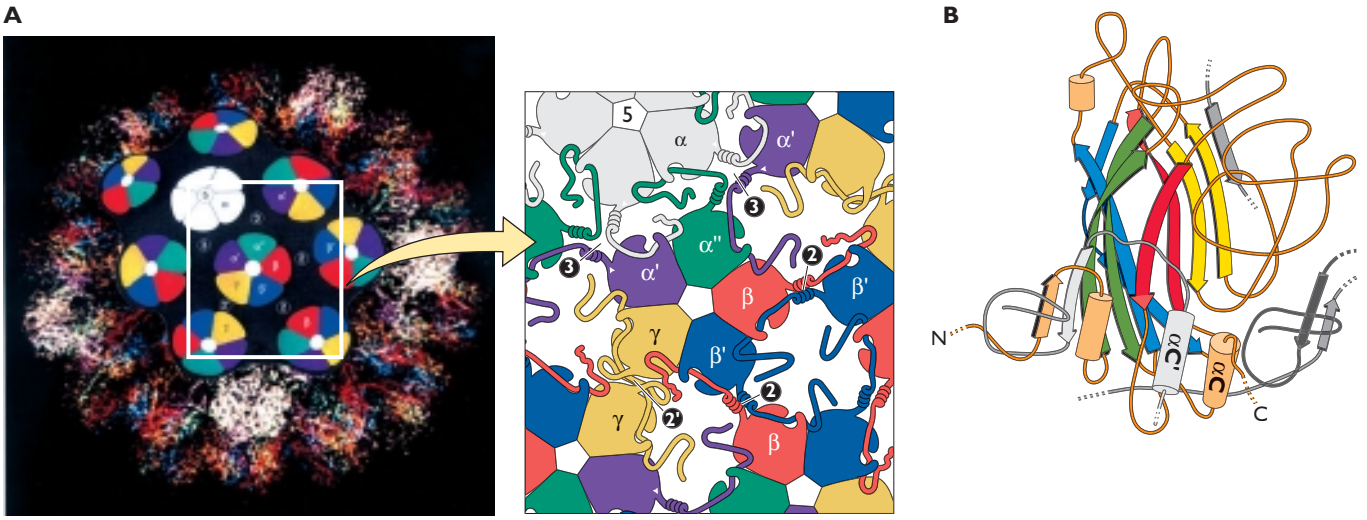


Figure 4.14 Structural features of the simian virus 40 virion. (A) View of the simian virus 40 virion showing the organization of VP1 pentamers. The 12 5-coordinated pentamers are shown in white, and the 60 pentamers present in hexameric arrays are colored. The three types of interpentameric clustering that allow 5-coordinated and 6-coordinated association of pentamers are shown in the schematic overlay and at the right. The VP1 subunits shown in white, purple, and green form a threefold cluster, designated 3. Those shown in red and blue engage in one kind of twofold interaction, designated 2, and the yellow subunits form a second kind of twofold cluster, labeled 2′. C-terminal extensions are shown as lines with coils representing the αC-helices shown in panel B. The three types of interpentamer clustering are accommodated by variation in interpentamer contacts made by the C-terminal extensions. In the threefold cluster, the C-terminal α-helices from the three interacting VP1 molecules form a three-stranded α-helical bundle. A two-chain α-helical bundle is formed at one kind of twofold cluster (2, between red and blue subunits), whereas at the second kind of twofold cluster (2′, yellow subunits) the subunits are packed so closely that there is no space to accommodate an α-helical structure. **(B)** The topology of the VP1 protein shown in a ribbon diagram, with the strands of the β-barrel jelly roll colored as in Fig. 4.12B. This β-barrel domain is radial to the capsid surface. The C-terminal arm and α-helix (orange) of the VP1 subunit invades a neighboring pentamer (not shown). The C-terminal arm and C α-helix shown in gray (αC) is the invading arm from a different neighboring pentamer (not shown), which is clamped in place by extensive interactions of its β-strand with the N-terminal segment of the subunit shown. The subunit shown also interacts with the N-terminal arm from its anticlockwise neighbor in the same pentamer (dark gray) and with the C-terminal arm of the pentamer that invades that neighbor (black). (A [left] and B) From R. C. Liddington et al., *Nature* **354:**278–284, 1991, with permission. Courtesy of S. C. Harrison, Harvard University.

one of two different structural environments, and to do so they adopt one of two distinct conformational states (Fig. 4.16C, right). Because of this arrangement, the A and B dimers are **not** quasiequivalent: virtually all contacts in which A and B monomers engage are very different. However, these differences allow the formation of VP3 assemblies with either five- or threefold rotational symmetry and hence of an icosahedral shell (Fig. 4.16C, right). The VP3 shell of bluetongue virus abuts directly on the inner surface of the middle layer, which comprises trimers of a single protein (VP7) organized into a classical $T = 13$ lattice (Fig. 4.16C, left). A large number of different (nonequivalent) contacts between VP3 and VP7 structural units weld the two layers together. These properties of reoviruses illustrate that a quasiequivalent structure is not the **only** solution to the problem of building large viral particles: viral proteins that interact with each other and with other proteins in multiple ways can provide an effective alternative.

Packaging the Nucleic Acid Genome

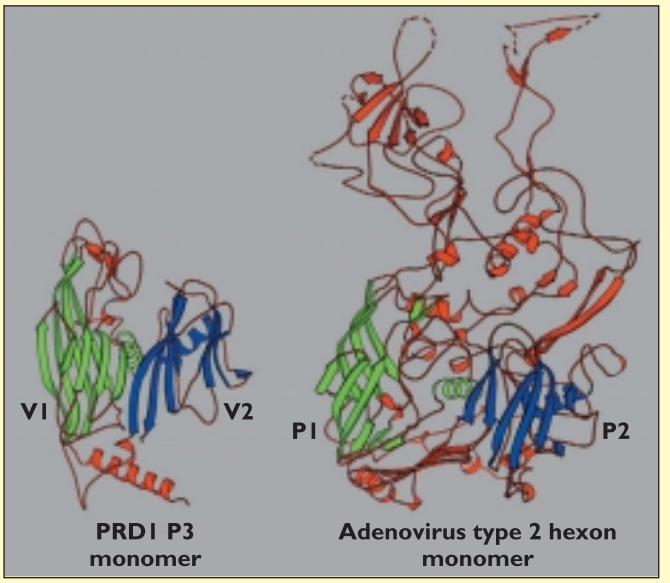
A definitive property of a virion is the presence of a nucleic acid genome. Incorporation of the genome requires its discrimination from a large population of cellular nucleic acid and its packaging. These processes are described in Chapter 13. The volumes of closed capsids or nucleocapsids are finite. Consequently, accommodation of viral genomes necessitates a high degree of condensation and compaction. A simple analogy illustrates vividly the scale of this problem; packing of the ~150-kbp DNA genome of herpes simplex virus type 1 into the viral nucleocapsid is equivalent to stuffing some 10 ft of wire into a tennis

BOX 4.6 EXPERIMENTS

Ancient evolutionary relationships deduced from structural comparisons

The specific and unusual properties of the major capsid proteins of human adenovirus and the bacteriophage PRD1 make a compelling case for common ancestry of these viruses. The adenovirus hexons form the faces of the icosahedral capsid (Fig. 4.15). This structural unit is not formed by six monomers arranged with hexagonal symmetry, but instead is a trimer. However, each hexon monomer contains two β-barrel jelly roll domains, labeled P1 and P2 in the figure below, such that the hexon exhibits pseudohexagonal symmetry. As the figure illustrates, the same arrangement is seen in the major capsid protein, P3, of bacteriophage PRD1. Moreover, the hexon and P3 monomers have similar connections within and between the β-barrel domains. These human and bacterial viruses also have in common $T = 25$ capsids, an arrangement not seen in any other virus family, and a structural unit built from distinct proteins at the positions of fivefold symmetry, from which attachment proteins project. They also share features of their genome organization and mechanism of viral DNA synthesis.

Several other large viruses, including the algal virus *Paramecium bursaria* chlorella virus and *Sulfobus* turreted icosahedral virus with an archeal host, are also built from major coat proteins with the same double-barrel fold. It is difficult to escape the conclusion that these modern viruses evolved from an ancient common ancestor.



β-Barrel jelly rolls. The human adenovirus type 2 hexon (right) and bacteriophage protein P3 (left) monomers are shown, with the two β-barrel jelly rolls present in each colored green and blue. Adapted from R. W. Hendrix, *Curr. Biol.* **9:**R914–R917, 1999, with permission. Courtesy of R. Burnett, The Wistar Institute.

Benson, S. D., J. K. H. Bamford, D. H. Bamford, and R. M. Burnett. 1999. Viral evolution revealed by bacteriophage PRD1 and human adenovirus coat protein structures. *Cell* **98:**825–833.

Benson, S. D., J. K. H. Bamford, D. H. Bamford, and R. M. Burnett. 2004. Does common architecture reveal a viral lineage spanning all three domains of life? *Mol. Cell* **16:**673–685.

Hendrix, R. W. 1999. Evolution: the long evolutionary reach of viruses. *Curr. Biol.* **9:**R914–R917.

ball! In addition, packaging of nucleic acids is an intrinsically unfavorable process because of loss of entropy as the nucleic acid becomes highly constrained in conformation. In some cases, the energy required to achieve packaging is provided, at least in part, by specialized viral proteins that harness the energy released by hydrolysis of ATP to drive the insertion of DNA. In many others, the binding of viral nucleic acids to capsid or nucleocapsid proteins appears to provide sufficient energy. Such interactions also help to neutralize the negative charge of the sugar-phosphate backbone, a prerequisite for close juxtaposition of genome sequences. Three mechanisms for condensing, and presumably organizing, nucleic acid molecules within capsids or nucleocapsids can be distinguished (Table 4.5) and are described in the following sections.

Direct Contact of the Genome with a Protein Shell

In the simplest arrangement, the viral nucleic acid makes direct contact with the protein(s) that forms a protective shell of the particle (Table 4.5). Proteins on the inner surfaces of the icosahedral capsids of many small RNA viruses interact with the viral genome. As we have seen, the interior surface of the poliovirus capsid can be described in detail. Nevertheless, we possess no structural information about the arrangement of the RNA genome, for the nucleic acid is not visible in the X-ray structure. This property indicates that the RNA genome lacks the symmetry of the virion and does not adopt the identical conformation in every virus particle. In contrast, in other small viruses with icosahedral symmetry, segments of the RNA or DNA genomes are highly ordered. For example, in the $T = 3$

Table 4.4 Specialization of adenovirus type 2 structural proteins[a]

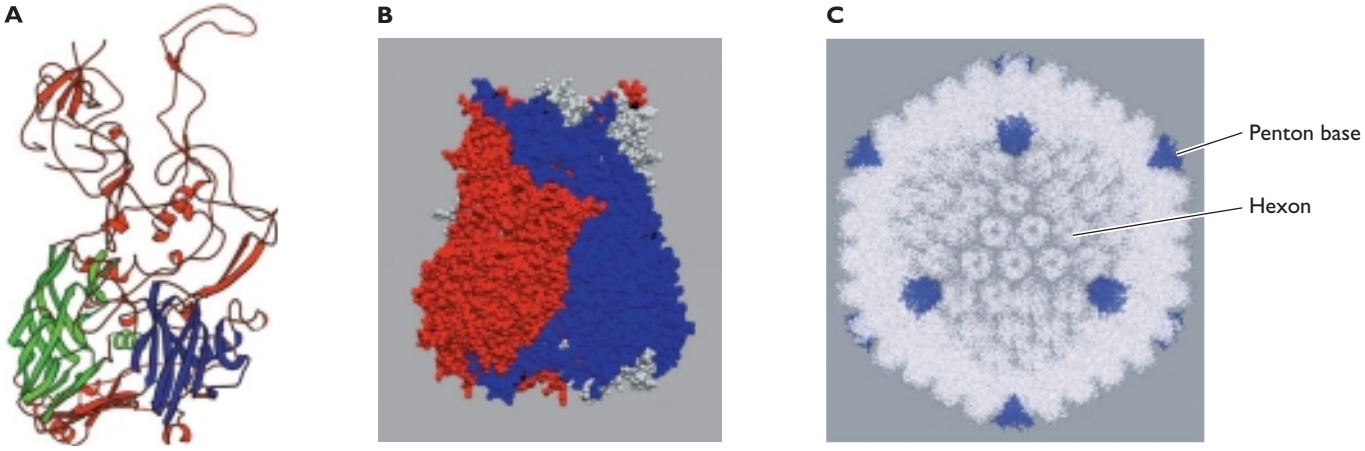
Protein	Molecular mass (kDa)	No. of copies	Location	Function
II	109,677	720	Hexon (trimer)	Formation of capsid shell
III	63,296	60	Penton base (pentamer)	Formation of capsid shell; entry
IV	61,960	36	Fiber (trimer)	Attachment to host cell
IIIa	63,287	74 ± 1	Inner capsid surface below the penton base	Stabilization of capsid
VI	23,449	342 ± 4	Hexon-associated, inner capsid surface	Stabilization of capsid; entry
VIII	14,539	211 ± 2	Hexon-associated, inner capsid surface	Stabilization of capsid
IX	14,339	247 ± 2	Outer surface of groups-of-nine hexons; edges of icosahedral faces	Stabilization of capsid
V	41,631	157 ± 1	Core, outer surface	Packaging of DNA genome
VII	19,412	835 ± 20	Core, bound to DNA	Packaging of DNA genome
μ	2,441	120 ± 1	Core	Packaging of DNA genome

[a]In addition to the structural proteins listed, the adenovirus virion contains two copies of pTP, one covalently linked to each 5′ end of the viral genome, the viral L3 protease, and several small proteins generated upon cleavage of proteins that enter the virion as precursors. Only one of these, the C-terminal extension from pVI, which activates the L3 protease, has been ascribed a function.

nodavirus Nodamura virus (Fig. 4.11), double-stranded segments of the RNA genome bind to subunits of adjacent structural units at the icosahedral twofold axes. And in virions of the small bacteriophage MS2, the entire (+) strand RNA genome appears to be icosahedrally ordered in two connected and concentric internal shells (Fig. 4.17A).

Use of the same protein or proteins both to package the genome and to build a capsid allows efficient utilization of limited genetic capacity. It is therefore an advantageous arrangement for viruses with small genomes. However, this mode of genome packing is also characteristic of some more complex viruses, notably rotaviruses and herpesviruses. The genome of rotaviruses comprises 11 segments of double-stranded RNA located within the innermost of the three protein shells of the virion. About 25% of the RNA (more than 4,000 base pairs [bp]) is highly ordered, forming a dodecahedral structure in which RNA helices are in close contact with the interior surface of the inner nucleocapsid (Fig. 4.17B).

Figure 4.15 Structural features of adenovirus particles. (A and B) Structure of the hexon. The monomer **(A)** is shown as a ribbon diagram, with gaps indicating regions that were not defined in the X-ray crystal structure at 2.9-Å resolution, and the trimer **(B)** is shown as a space-filling model with each monomer in a different color. The monomer contains two β-barrel jelly roll domains colored green and blue in panel A. The trimers are stabilized by extensive interactions within both the base and the towers. From M. M. Roberts et al., *Science* **232:**1148–1151, 1986, and F. K. Athappilly et al., *J. Mol. Biol*. **242:**430–455, 1994, with permission. Courtesy of J. Rux, S. Benson, and R. M. Burnett, The Wistar Institute. **(C)** Quasiatomic model of the capsid of human adenovirus type 5, viewed down a threefold axis. The model was made by fitting the crystal structures of the hexon (light blue) and the penton base (dark blue) into a 10-Å-resolution structure obtained by cryo-electron microscopy. Adapted from C. M. S. Fabry et al., *EMBO J.* **24:**1045–1645, 2005, with permission. Courtesy of G. Schoen, CNRS-Université Joseph Fourier, Grenoble, France.



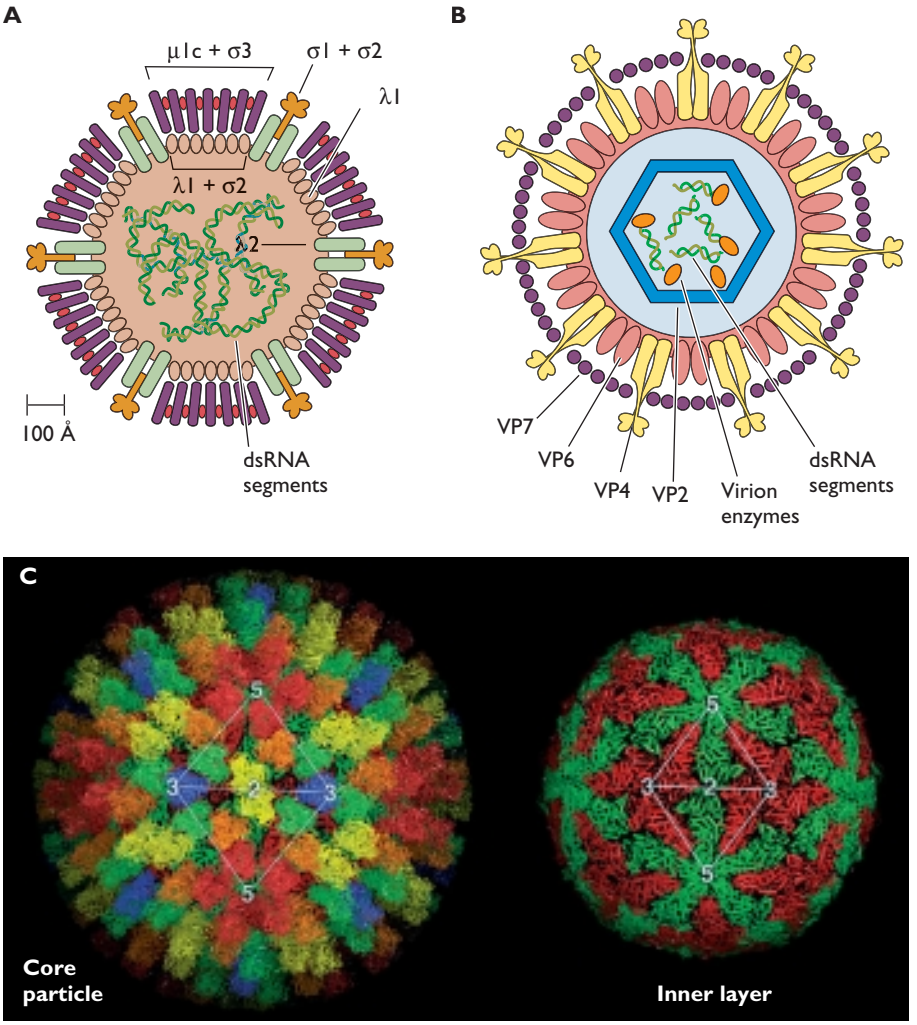


Figure 4.16 Structures of members of the *Reoviridae*. The organization of mammalian reovirus **(A)** and rotavirus **(B)** particles is shown schematically to indicate the locations of proteins, deduced from the protein composition of virions and of subviral particles that can be readily isolated from them. **(C)** X-ray crystal structure of the core of bluetongue virus, a member of the *Orbivirus* genus of the *Reoviridae*, showing the core particle (left) and the inner scaffold (right). Trimers of VP7 project radially from the outer layer of the core particle. Each icosahedral asymmetric unit, two of which are indicated by the white lines, contains 13 copies of VP7 arranged as five trimers colored red, orange, green, yellow, and blue, respectively. The outer layer is organized with classical $T = 13$ icosahedral symmetry. As shown on the right, the inner layer is built from VP3 dimers that occupy one of two completely different structural environments, designated A and B and colored green and red, respectively. A-type monomers span the icosahedral twofold axes and interact in rings of five around the icosahedral fivefold axes. In contrast, B-type monomers are organized as triangular "plugs" around the threefold axes. Differences in the interactions among monomers at different positions allow close packing to form the closed shell, the equivalent of a $T = 2$ lattice. As might be anticipated, VP7 trimers in pentameric or hexameric arrays in the outer layer make different contacts with the two classes of VP3 monomer in the inner layer. Nevertheless, each type of interaction is extensive and, in total, these contacts compensate for the symmetry mismatch between the two layers of the core. The details of these contacts suggest that the inner shell both defines the size of the virus particle and provides a template for assembly of the outer $T = 13$ structure. From J. M. Grimes et al., *Nature* **395:**470–478, 1998, with permission. Courtesy of D. I. Stuart, University of Oxford.

Table 4.5 Mechanisms of packaging the viral genome

Mechanism	Example(s)	Protein(s) contacting nucleic acid
By direct contact with nucleocapsid or capsid proteins	Alphavirus	C
	Herpesvirus	?
	Parvovirus	Ca
	Picornavirus	VP4
	Reovirus	λ1
By specialized viral nucleic acid-binding proteins	Adenovirus	VII, V, μ
	Orthomyxovirus	NP
	Poxvirus	L4R, A10L
		A3L, A4L
		F17R, I1
	Retrovirus	NC
	Rhabdovirus	N
By cellular DNA-binding proteins	Papillomavirus, polyomavirus	Histones H2A, H2B, H3, and H4

One of the most surprising properties of the large herpesviral nucleocapsid (described in "Complex Viruses" below) is the absence of internal proteins associated with viral DNA: despite intense efforts, no such core proteins have been identified. The viral genome has not yet been visualized in the herpesviral nucleocapsid. In contrast, cryo-electron microscopy has allowed visualization of the large, double-stranded DNA genome of bacteriophage T4, which is organized in closely opposed concentric layers (Fig. 4.18). This arrangement illustrates graphically the remarkably dense packing needed to accommodate such large viral DNA genomes in closed structures of fixed dimensions. This type of organization must require neutralization of the negative charges of the sugar-phosphate backbone. In herpesviral nucleocapsids, such neutralization might be accomplished by proteins that form the inner surface of the nucleocapsid, or by incorporation of small, positively charged, cellular molecules like spermine and spermidine.

Packaging by Specialized Virion Proteins

In many other virus particles, the genome is associated with specialized nucleic acid-binding proteins, such as the nucleocapsid proteins of (–) strand RNA viruses and retroviruses, or the core proteins of adenoviruses described above. An important function of such proteins is to condense and protect viral genomes. Consequently, they do not recognize specific nucleic acid sequences but rather bind nonspecifically to RNA or DNA genomes. This mode of binding is exemplified by the organization of the vesicular stomatitis virus N protein-RNA complexes, in which 9 nucleotides of RNA are tightly but nonspecifically bound in a cavity formed between the two domains of each N protein molecule (Fig. 4.7). These protein-RNA interactions both sequester the RNA within the protein ring and organize it into a helical structure. Electron micrographs of nucleocapsids of other RNA viruses, including members of the *Paramyxoviridae* and *Filoviridae*, suggest that organization of RNA genomes into helical ribonucleoproteins by two-domain RNA-binding proteins may be a common genome packaging mechanism.

Electron microscopy of cores released from adenovirus particles suggested that the internal nucleoprotein is also organized in some regular fashion. However, this structure has proved difficult to study in detail because the core is not stable once released from virions, nor have structures of core proteins been determined. The fundamental DNA packaging unit is a multimer of protein VII, which appears as beads on a string of adenoviral DNA when other core proteins are removed. Protein VII and the other core proteins are basic, as would be expected for proteins that bind to a negatively charged DNA molecule without sequence specificity.

Retrovirus virions contain approximately 2,000 molecules of the nucleocapsid (NC) protein that bind to the two copies of the encapsidated (+) strand RNA genome (Appendix, Fig. 21). The NC protein is also responsible for recognition of a specific packaging signal in the RNA during assembly and therefore binds both specifically and nonspecifically to the genome. Like other viral genome-binding proteins, NC is positively charged and, in most retroviruses, contains at least one copy of a well-characterized nucleic acid-binding motif. The structures of human immunodeficiency virus type 1 NC and of NC bound to the RNA packaging signal determined by NMR methods indicate that a long, N-terminal helix rich in basic residues

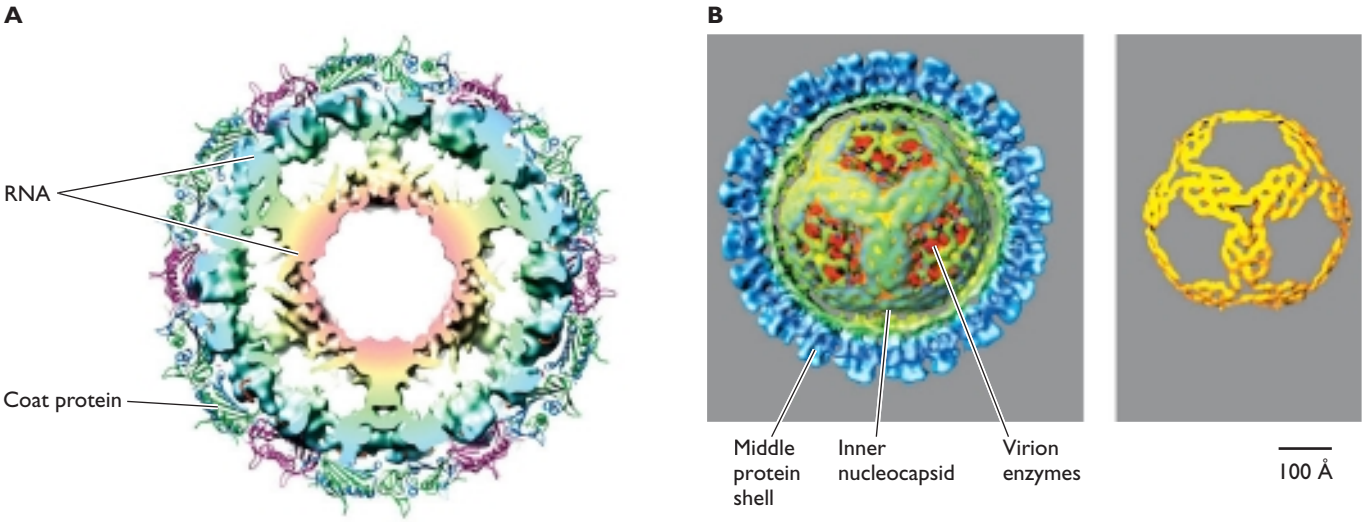


Figure 4.17 Structural organization of RNA genomes. (A) Single-stranded bacteriophage MS2 RNA. This structure (9-Å resolution) was determined by cryo-electron microscopy and difference imaging. Shown is a 40-Å section through the virion, viewed down the threefold axis. The crystal structure of the coat protein dimers is shown in cartoon form, with different conformers of the coat protein dimmer colored blue, green, or red. The regions of the cryo-electron microscopy map corresponding to the RNA genome are shown as radially colored density ranging from pale blue ($r \sim 108$ Å) to pink ($r \sim 42$ Å). One RNA shell lies immediately beneath, and makes close contacts with, the inner surface of the capsid protein shell. It is connected around the fivefold axes to a second, inner shell of RNA. It has been estimated that this ordered RNS represents ~90% of the genome. From K. Tropova et al., *J. Mol. Biol.* **375:**824–836, 2008, with permission. Courtesy of N. Ranson, University of Leeds, Leeds, United Kingdom. **(B)** Double-stranded rotaviral RNA. This structure was determined by cryo-electron microscopy, image reconstruction, and difference imaging of infectious double-layered particles and various virus-like particles. The 19-Å-resolution structure of the double-layered particle is shown on the left. The largely enclosed double-stranded RNA, which is packed around the structures formed by the virion enzymes (red), is shown in yellow. The dodecahedral shell of ordered RNA, in which each strand is about 20 Å in diameter as expected for RNA in double-stranded helices is shown on the right. From B. V. V. Prasad et al., *Nature* **382:**471–473, 1996, with permission. Courtesy of B. V. V. Prasad, Baylor College of Medicine.

interacts nonspecifically with the RNA. Whether this interaction is responsible for coating the entire RNA genome in the virion and how NC molecules condense the genome are not yet known. Retroviral ribonucleoproteins are encased within a protein shell built from the capsid (CA) protein to form an internal core. Although they contain the same components, these cores vary considerably in morphology (Fig. 4.19). Studies of the structures of the human immunodeficiency virus type 1 CA protein and the assemblies it forms *in vitro* (Box 4.7) indicate that this protein determines the conical shape of the core of this retrovirus.

Packaging by Cellular Proteins

The final mechanism of packaging the viral genome, by cellular proteins, is unique to polyomaviruses, such as simian virus 40, and papillomaviruses. The circular, double-stranded DNA genomes of these viruses are organized into nucleosomes that contain the four cellular core histones, H2A, H2B, H3, and H4. These genomes are organized within the virion (and in infected cells) like cellular DNA in chromatin to form a minichromosome. This packaging mechanism is elegant, with two major advantages: none of the limited viral genetic information needs to be devoted to DNA-binding proteins, and the viral genome, which is transcribed by cellular RNA polymerase II, enters the infected cell nucleus as a nucleoprotein closely resembling the cellular templates for this enzyme.

In each simian virus 40 particle, the 20 or so nucleosomes that package the viral genome condense the DNA by a factor of approximately 7. Within the virion, the minichromosome must be further compacted, presumably as a result of its interactions with the internal proteins of the capsid, VP2 and VP3, and perhaps the N-terminal arms of VP1 that lie on the interior surface. Both VP2 and VP3 can bind nonspecifically to DNA as well as to cellular histones, and all three proteins associate with the minichromosome during virion assembly.

Although three different ways of condensing and organizing genomic nucleic acids within virions can be distinguished readily (Table 4.5), few of these packaging

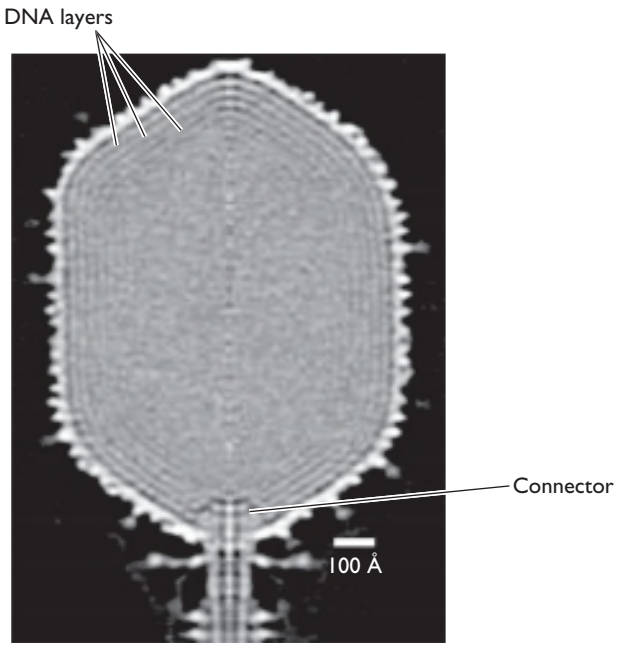


Figure 4.18 Dense packing of the double-stranded DNA genome in the head of bacteriophage T4 DNA. The central section of a 22-Å cryo-electron microscopy reconstruction of the head of bacteriophage T4 viewed perpendicular to the fivefold axis is shown. The concentric layers seen underneath the capsid shell have been attributed to the viral DNA genome. The connector, which is derived from the portal structure by which the DNA genome enters the head during assembly, connects the head to the tail. Adapted from A. Fokine et al., *Proc. Natl. Acad. Sci. USA* **101:**6003–6008, 2004, with permission. Courtesy of M. Rossmann, Purdue University.

arrangements are understood in detail. High-resolution structural descriptions of virion interiors, and of the nucleoproteins that reside within them, would undoubtedly provide important insights into mechanisms of condensation of the nucleic acid. Such structural information might also improve our understanding of the advantages conferred by the various packaging mechanisms described above.

Viruses with Envelopes

Many viruses contain structural elements in addition to the capsids or nucleocapsids described previously. All such virus particles possess an envelope formed by a viral protein-containing membrane derived from the host cell, but they vary considerably in size, morphology, and complexity. Furthermore, viral membranes differ in lipid composition, the number of proteins they contain, and their location. The envelopes form the outermost layer of enveloped animal viruses, but in bacteriophages of the PRD1 family the membrane lies **beneath** an icosahedral capsid. Typical features of viral envelopes and their proteins are described in the next section, to set the stage for consideration of the structures of envelope proteins and the various ways in which they interact with internal components of the virion (Fig. 4.20).

Viral Envelope Components

The foundation of all viral envelopes is a lipid membrane acquired from the host cell during assembly. The precise lipid composition is variable, for viral envelopes can be derived from different kinds of cellular membrane. Embedded in the membrane are viral proteins, the great majority of which are **glycoproteins** that carry covalently linked sugar chains, or **oligosaccharides** (Fig. 4.21). Sugars are added to the proteins posttranslationally, during transport to the cellular membrane at which progeny virions assemble. Intra- or interchain disulfide bonds, another common chemical feature of these proteins, are also acquired during transport to assembly sites. These covalent bonds stabilize the tertiary or quaternary structures of viral glycoproteins (Table 4.6).

Envelope Glycoproteins

Viral glycoproteins are **integral membrane proteins** firmly embedded in the lipid bilayer by a short **membrane-spanning domain** (Fig. 4.21). The membrane-spanning domains of viral proteins are hydrophobic α-helices of sufficient length to span the lipid bilayer. They generally separate large external domains that are decorated with oligosaccharides from smaller internal domains (Fig. 4.21). The former contain binding sites for cell surface virus receptors, major antigenic determinants, and sequences that mediate fusion of viral with cellular membranes during entry. Internal domains, which make contact with other components of the virion, are often essential for virus assembly.

With few if any exceptions, the structures formed by viral membrane glycoproteins are oligomeric. These oligomers vary considerably in composition. Some comprise multiple copies of a single protein, but in many cases each subunit contains two or more protein chains (Table 4.6). The subunits are held together by noncovalent interactions and disulfide bonds. On the exterior of the virion, these oligomers form surface projections, often called spikes. Because of their critical roles in initiating infection, the structures of many viral glycoproteins have been determined.

The hemagglutinin (HA) protein of human influenza A virus is a trimer of disulfide-linked HA1 and HA2 molecules. This protein contains a globular head with a top surface that is projected about 135 Å from the viral membrane by a long stem (Fig. 4.22A). The latter is formed and stabilized by the coiling of α-helices present in each monomer. The membrane-distal globular domain contains the binding site for the virus receptor. This important

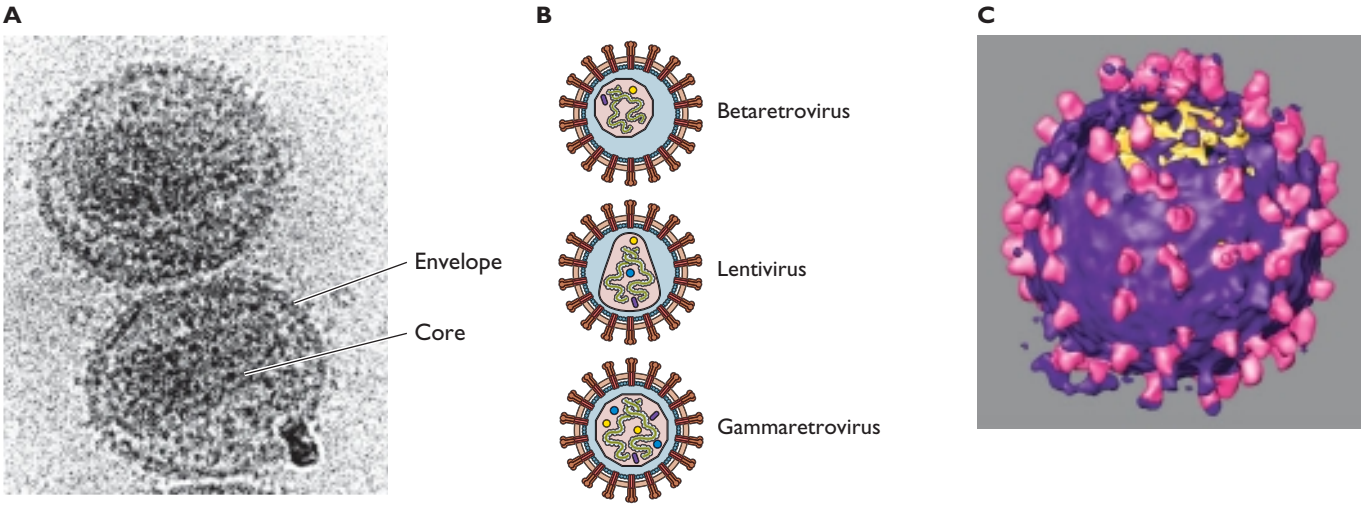


Figure 4.19 Morphology of retroviruses. (A) Cryo-electron micrograph of mature human immunodeficiency virus type 1 showing the elongated internal cores of these particles. Courtesy of T. Wilk, European Molecular Biology Laboratory. **(B)** Variations in the morphology of retroviruses shown schematically. Although retrovirus particles are assembled from the same components, some contain roughly spherical cores, whereas the cores of lentiviruses like human immunodeficiency virus type 1 are elongated and conical. In beta retroviruses the position of the capsid is variable and in some case is not central. The tertiary structures of retroviral CA proteins are highly conserved, and all examined form hexameric arrays in the absence of other viral proteins. It has therefore been proposed that the differences in morphology among retroviral capsids result from alternative positioning of the 12 pentamers required to form a closed lattice (see Box 4.6). **(C)** Cryo-electron tomogram of a Moloney murine leukemia virus particle, showing the dense but irregular packing of Env glycoprotein spikes (magenta) on the lipid bilayer (purple) of the viral envelope. Adapted from F. Förster et al., *Proc. Natl. Acad. Sci. USA* **102:**4729–4734, 2005. Courtesy of F. Förster, Max-Planck-Institut für Biochemie, Martinsried, Germany.

functional region is located more than 100 Å away from the lipid membrane of influenza virus particles. Other viral glycoproteins that mediate cell attachment and entry, such as the E protein of the flavivirus tick-borne encephalitis virus, are quite different in structure; the external domain of E protein is a flat, elongated dimer that would lie on the surface of the viral membrane rather than projecting from it (Fig. 4.22B). Despite their lack of common structural features, both the HA protein and the E protein are primed for dramatic conformational change to allow entry of internal virion components into a host cell.

The particles of the majority of other enveloped viruses contain one or two glycoproteins with typical properties. For example, the surfaces of all retroviruses are covered by a dense array of projections (Fig. 4.19C) formed by the Env proteins TM (transmembrane) and SU (surface unit). However, some enveloped viruses contain a larger collection of glycoproteins. One of the several remarkable features of the virions of herpesviruses is the large number of envelope proteins: so far, more than 12 viral glycoproteins have been identified (Table 4.7). As expected, some of them are important for attachment of the virus to host cells and for entry.

The high-resolution viral glycoprotein structures mentioned above are those of the large external domains of the proteins cleaved from the viral envelope by proteases. This treatment facilitated crystallization, but of course precluded determination of the structure of membrane-spanning or internal segments of the proteins, both of which play important structural or functional roles. Membrane-spanning domains can contribute to the stability of oligomeric glycoproteins, as in influenza virus HA, and transmit signals from the exterior to the interior of the virion. Internal domains can participate in anchoring the envelope to internal virion structures (Fig. 4.20). Recent improvements in resolution achieved by application of cryo-electron microscopy and image reconstruction (Fig. 4.5) have allowed visualization of these segments of glycoproteins of some enveloped viruses. This important advance has provided much previously inaccessible information, as discussed in "Simple Enveloped Viruses: Direct Contact of External Proteins with the Capsid or Nucleocapsid" below.

Other Envelope Proteins

The envelopes of some more complex viruses, including orthomyxoviruses, herpesviruses, and poxviruses,

BOX 4.7 EXPERIMENTS

A fullerene cone model of the human immunodeficiency virus type 1 core

(A) Purified human immunodeficiency virus type 1 CA-NC protein self-assembles into cylinders and cones when incubated with a segment of the viral RNA genome *in vitro*. Although the RNA facilitates formation of these structures, it is not essential. The cones assembled *in vitro* are capped at both ends, and many appear very similar in dimensions and morphology to cores isolated from viral particles.

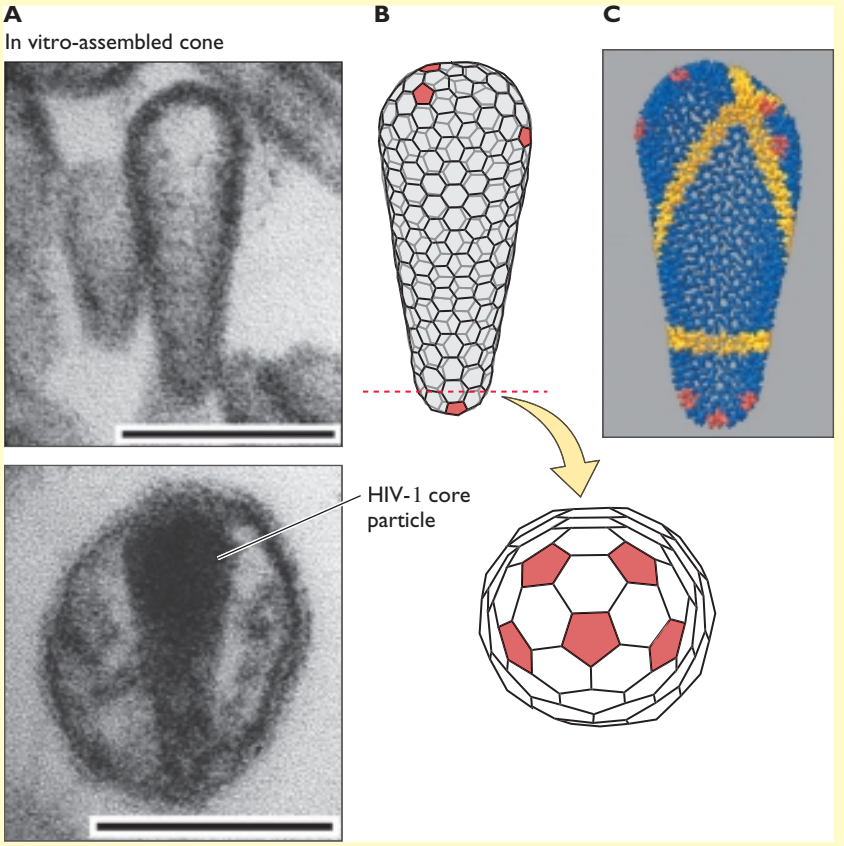
The very regular appearance of the synthetic CA-NC cones suggested that, despite their asymmetry, they are constructed from a regular, underlying lattice (analogous to the lattices that describe structures with icosahedral symmetry discussed in Box 4.4). In fact, these human immunodeficiency virus type 1 cores can be modeled using the geometric principles that describe cones formed by carbon. Such elemental carbon cones comprise helices of hexamers closed at each end by caps of buckminsterfullerene, which are structures that contain pentamers surrounded by hexamers. As in structures with icosahedral symmetry (Box 4.4), the positions of pentamers determine the geometry of cones. However, in cones, pentamers are present **only** in the terminal caps. **(B)** The human immunodeficiency virus type 1 cones formed *in vitro* and isolated from mature virions can be modeled as a fullerene cone assembling on a curved hexagonal lattice with five pentamers at the narrow end of the cone, as shown in the expanded view. The wide end would be closed by an additional 7 pentamers (because 12 pentamers are required to form a closed structure from a hexagonal lattice). In this type of structure, the cone angle at the narrow end can adopt only one of five allowed angles, determined by the number of pentamers. A narrow cap with five pentamers, as in the model shown in panel B, should exhibit a cone angle of 19.2°. Approximately 90% of all the synthetic CA-NC cores examined met this prediction, consistent with the fullerene cone model. *In vitro*, the purified CA protein can form tubes, which are built from CA hexamers. The N-terminal domain of the CA protein, which is essential for capsid assembly, forms the hexameric rings, and adjacent hexamers interact via the C-terminal dimerization domain. In tubes, the planes of hexameric rings are parallel to the helix axis. Electron microscopy and image reconstruction of authentic cores established that the structures are also constructed from hexameric rings of the CA protein. **(C)** As illustrated by the yellow CA hexamers, to form cones the planes of the hexameric rings must be tilted with respect to the cone axis. Consequently, hexamers are organized in spirals that change gradually in pitch, diameter, and eventually helical handedness. This model requires that CA hexameric rings adopt many different orientations. The flexible hinge between the N- and C-terminal domains of each CA monomer and loose packing of adjacent rings of hexamers appear to provide the necessary conformational flexibility. Pentamers are shown in red. (A and C) From B. K. Ganser et al., *Science* **283:**80–83, 1999, with permission. Courtesy of W. Sundquist.

Briggs, J. A. G., T. Wik, R. Welker, H.-G. Kraüsslich, and S. D. Fuller. 2003. Structural organization of authentic, mature HIV-1 virions and cores. *EMBO J.* **22:**1707–1715.

Ganser, B. K., S. Li, V. Y. Klishko, J. T. Finch, and W. I. Sundquist. 1999. Assembly and analysis of conical models for the HIV-1 core. *Science* **283:**80–83.

Ganser-Pormillos, B. K., and U. K. von Schwedler. 2004. Assembly properties of the human immunodeficiency virus type 1 CA protein. *J. Virol* **78:**2545–2552.

Li, S., C. P. Hill, W. I. Sundquist, and J. T. Finch. 2000. Image constructions of helical assemblies of the HIV-1 CA protein. *Nature* **407:**409–413.



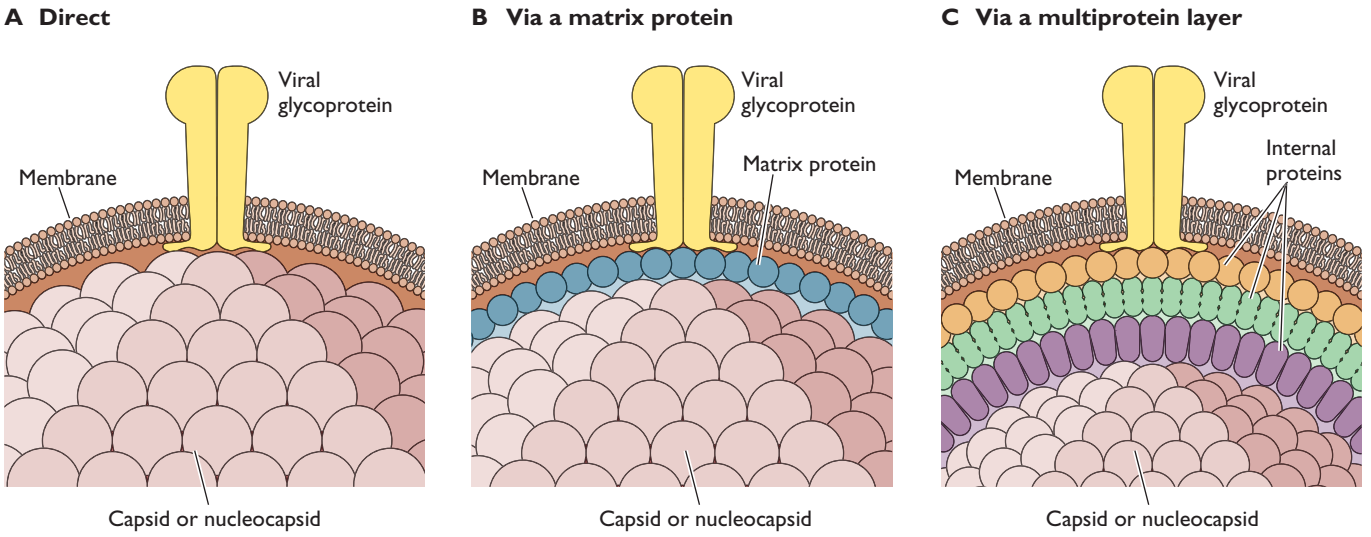


Figure 4.20 Schematic illustration of three modes of interaction of capsids or nucleocapsids with envelopes of virus particles.

contain integral membrane proteins that lack large external domains or possess multiple membrane-spanning segments (Table 4.7). Among the best characterized of these is the influenza A virus M2 protein. This small (97-amino-acid) protein is a minor component of virions, estimated to range from 14 to 68 copies per particle. In the viral membrane, two disulfide-linked M2 dimers associate to form a noncovalent tetramer that functions as an ion channel. The M2 ion channel is the target of the influenza virus inhibitor amantadine (Volume II, Fig. 9.11). The effects of this drug, as well as of mutations in the M2 coding sequence, indicate that M2 can play important roles in entry and assembly by controlling the pH of the local environments in which virus particles and HA molecules are present.

Figure 4.21 Structural and chemical features of a typical viral envelope glycoprotein shown schematically. The protein is inserted into the lipid bilayer via a single membrane-spanning domain. This segment separates a larger external domain, decorated with N-linked oligosaccharides (purple) and containing disulfide bonds (green), from a smaller internal domain.

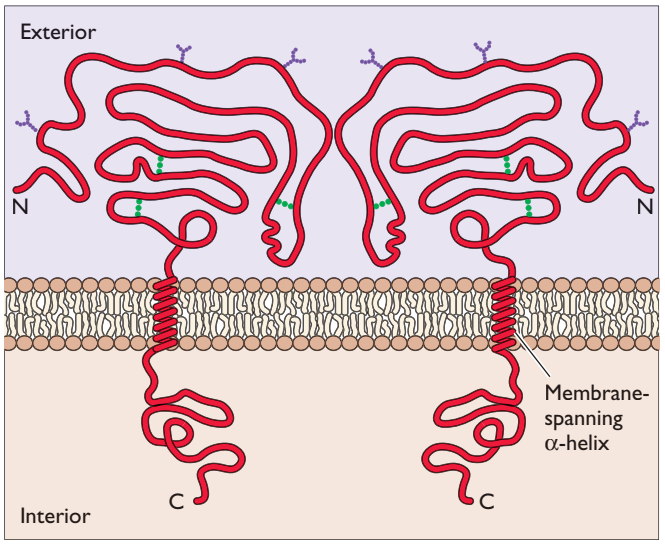


Table 4.6 Oligomeric structures of some viral membrane proteins[a]

Virus	Protein	Quaternary structure
Alphavirus		
Semliki Forest virus	E1, E2, E3	$(E1E2E3)_3$
Ross River virus	E1, E2	$(E1E2)_3$
Herpesvirus		
Herpes simplex virus type 1	gH, gL	$(gHgL)_?$
Orthomyxovirus		
Influenza virus	HA	$(HA1\text{-}HA2)_3$
	NA	$(NA\text{-}NA)_2$
	M2	$(M2\text{-}M2)_2$
Retrovirus		
Avian sarcoma virus	Env	$(SU\text{-}TM)_3$
Rhabdovirus		
Vesicular stomatitis virus	G	$(G)_3$

[a]The best-predicted oligomeric structures of the viral membrane proteins listed are shown in column 3, with hyphens indicating disulfide-bonded protein chains. The proteins that comprise many of the heteromeric subunits listed (e.g., influenza virus HA, retroviral Env) are produced by proteolytic processing of precursors during transport to the cell surface.

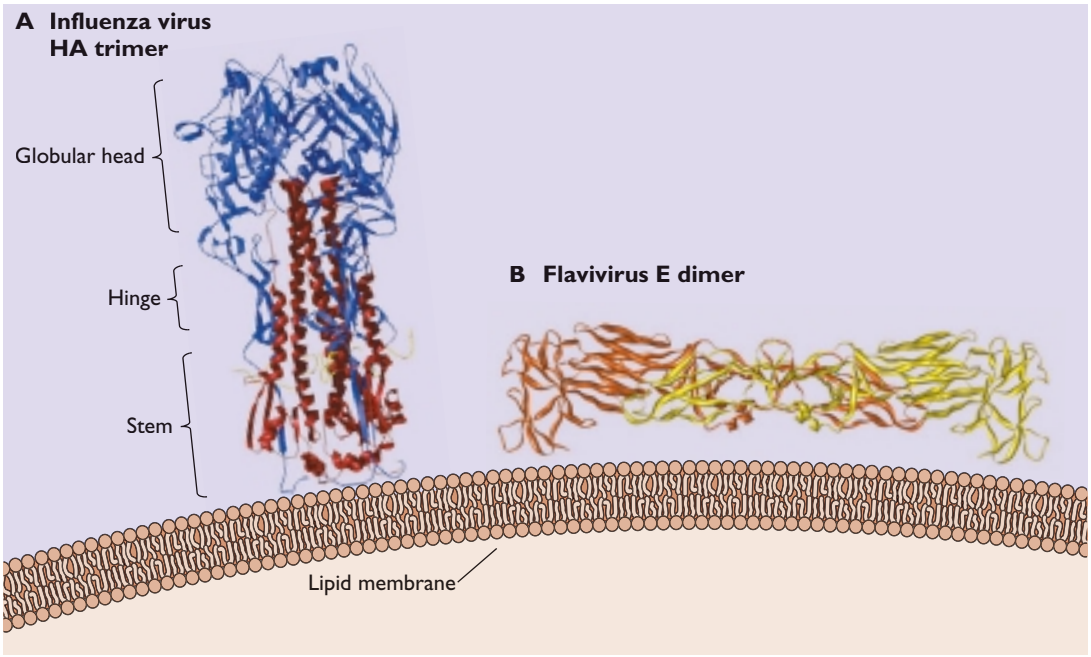


Figure 4.22 Structures of extracellular domains of viral glycoproteins. (A) X-ray crystal structure of the influenza virus HA glycoprotein trimer. Each monomer comprises HA1 (blue) and HA2 (red) subunits covalently linked by a disulfide bond. Adapted from J. Chen et al., Cell **95:**409–417, 1998, with permission. **(B)** X-ray structure of the tick-borne encephalitis virus (a flavivirus) E protein dimer, with the subunits shown in orange and yellow. Adapted from F. A. Rey et al., *Nature* **375:**291–298, 1995, with permission.

Simple Enveloped Viruses: Direct Contact of External Proteins with the Capsid or Nucleocapsid

In the simplest enveloped viruses, exemplified by (+) strand RNA alphaviruses such as Semliki Forest and Ross River viruses, the envelope directly abuts an inner nucleocapsid containing the (+) strand RNA genome. This inner protein layer is a $T = 4$ icosahedral shell built from 240 copies of a single capsid (C) protein arranged as hexamers and pentamers. The outer glycoprotein layer also contains 240 copies of the envelope proteins (Table 4.6). They cover the surface of the particle, such that the lipid membrane is not exposed on the exterior. Strikingly, the glycoproteins are also organized into a $T = 4$ icosahedral shell (Fig. 4.23A), as a result of binding of their internal domains to the C-protein subunits of the underlying nucleocapsid.

The structure of Sindbis virus has been determined by cryo-electron microscopy and image reconstruction to 9-Å resolution (Fig. 4.23A), the highest yet achieved for an enveloped virus. The structures of the E1 and C proteins of the related Semliki Forest virus have been solved at high resolution. The organization of the alphavirus envelope, including the transmembrane anchoring of the outer glycoprotein layer to structural units of the nucleocapsid, can therefore be described with unprecedented precision (Fig. 4.23). The transmembrane segments of the E1 and E2 glycoproteins form a pair of tightly associated α-helices, with the cytoplasmic domain of E2 in close opposition to a cleft in the capsid protein (Fig. 4.23C and D). On the outer surface of the membrane, the external portions of these glycoproteins, together with the E3 protein, form an unexpectedly elaborate structure: a thin $T = 4$ icosahedral protein layer (called the skirt) covers most of the membrane and supports the spikes, which are hollow, three-lobed projections (Fig. 4.23B and C).

The structures formed by external domains of membrane (E) proteins of the important human pathogens West Nile virus and dengue virus *(Flaviviridae)* are quite different: they lie flat on the particle surface, rather than forming protruding spikes. Nevertheless, the external domains of these E proteins are also icosahedrally ordered, and the envelopes of viruses of these families are described as **structured**. In contrast, the arrangement of membrane proteins generally exhibits little relationship to the structure of the capsid or nucleocapsid) when virions contain additional protein layers.

Table 4.7 Some herpes simplex virus type 1 virion proteins

Location	Protein	Gene	Function or properties
Nucleocapsid	VP5	UL19	Major capsid protein; forms both hexamers and pentamers
	VP19C/VP23	UL38/UL18	The heterotrimer ("triplex") connecting VP5 subunits on surface
	VP24	UL26	Protease; synthesized as a precursor
	VP26	UL35	Caps VP5 hexons but not pentons
	Portal	UL6	Present at one vertex; required for entry of DNA
Tegument	VP1-3	UL36	Very large (~273 kDa)
	VP16	UL48	Abundant structural protein; activator of IE gene transcription
	VP18.8	UL13	Protein kinase
	VP22	UL49	Hyperacetylation and stabilization of microtubules
	Vhs	UL41	Host shutoff factor
	US11p	US11	Myristylated protein; envelopment and transport of nascent virions
Envelope	gB	UL27	Fusion; binds heparan sulfate; binds tegument proteins, e.g., VP16
	gC	UL44	Attachment protein; binds heparan sulfate
	gD	US6	Binds cell surface receptors; entry
	gE/gI	US8/US7	Heterodimer; binds Fc domain of immunoglobulin G; cell-cell spread
	gH/gL	UL22/UL1	Heterodimer; fusion
	gG	US4	Not known
	gJ	US5	Not known
	gK	UL53	Virus-induced cell fusion; egress
	gM/gN	UL10/UL49.5	Heterodimer; function not known
	US9p	US9	Function not known for HSV-1; no large external domain
		UL20	Egress; probably contains multiple membrane-spanning domains

Enveloped Viruses with an Additional Protein Layer

Virions of several enveloped viruses contain an additional protein layer that mediates interactions of the genome-containing structure with the viral envelope. In the simplest case, a single viral structural protein, termed the matrix protein, welds an internal ribonucleoprotein to the envelope (Fig 4.20B). This arrangement is found in members of several groups of (−) strand RNA viruses (Appendix, Fig. 8 and 23). Retrovirus particles also contain an analogous, membrane-associated matrix protein, which makes contact with an internal capsid in which the viral ribonucleoprotein is encased.

Because the internal capsids or nucleocapsids of these more complex enveloped viruses are not in direct contact with the envelope, the organization and symmetry of the internal structure are not necessarily evident from the external appearance of the surface glycoprotein layer. Nor does the organization of these proteins reflect the symmetry of the capsid or nucleocapsid. For example, the outer surface of all retroviruses appears as a dense, roughly spherical array of projecting knobs or spikes, regardless of whether the internal core is spherical or cone shaped (Fig. 4.19). Likewise, influenza virus particles, which contain helical nucleocapsids, are generally roughly spherical particles 800 to 1,200 Å in diameter (Appendix, Fig. 8) although long, filamentous forms are common in clinical isolates. In general, the interior architecture of these enveloped viruses cannot be described in detail. However, high-resolution structures have been obtained for several matrix proteins. In conjunction with the results of *in vitro* assays for lipid binding and mutational analyses, such structural information allows molecular modeling of matrix protein-envelope interactions.

Internal proteins that mediate contact with the viral envelope are not embedded within the lipid bilayer, but rather bind to its inner face. Such viral proteins are targeted to, and interact with, membranes by means of specific signals, which are described in more detail in Chapter 12. For example, a posttranslationally added fatty acid chain is important for membrane binding of the MA proteins of most retroviruses. The human immunodeficiency virus type 1 MA protein was the first viral peripheral membrane protein for which a high-resolution structure was determined, initially by NMR methods (Fig. 4.3). Subsequent analysis by X-ray crystallography established that MA is a trimer (Fig. 4.24). Each MA molecule comprises a compact, globular domain of α-helices capped by a

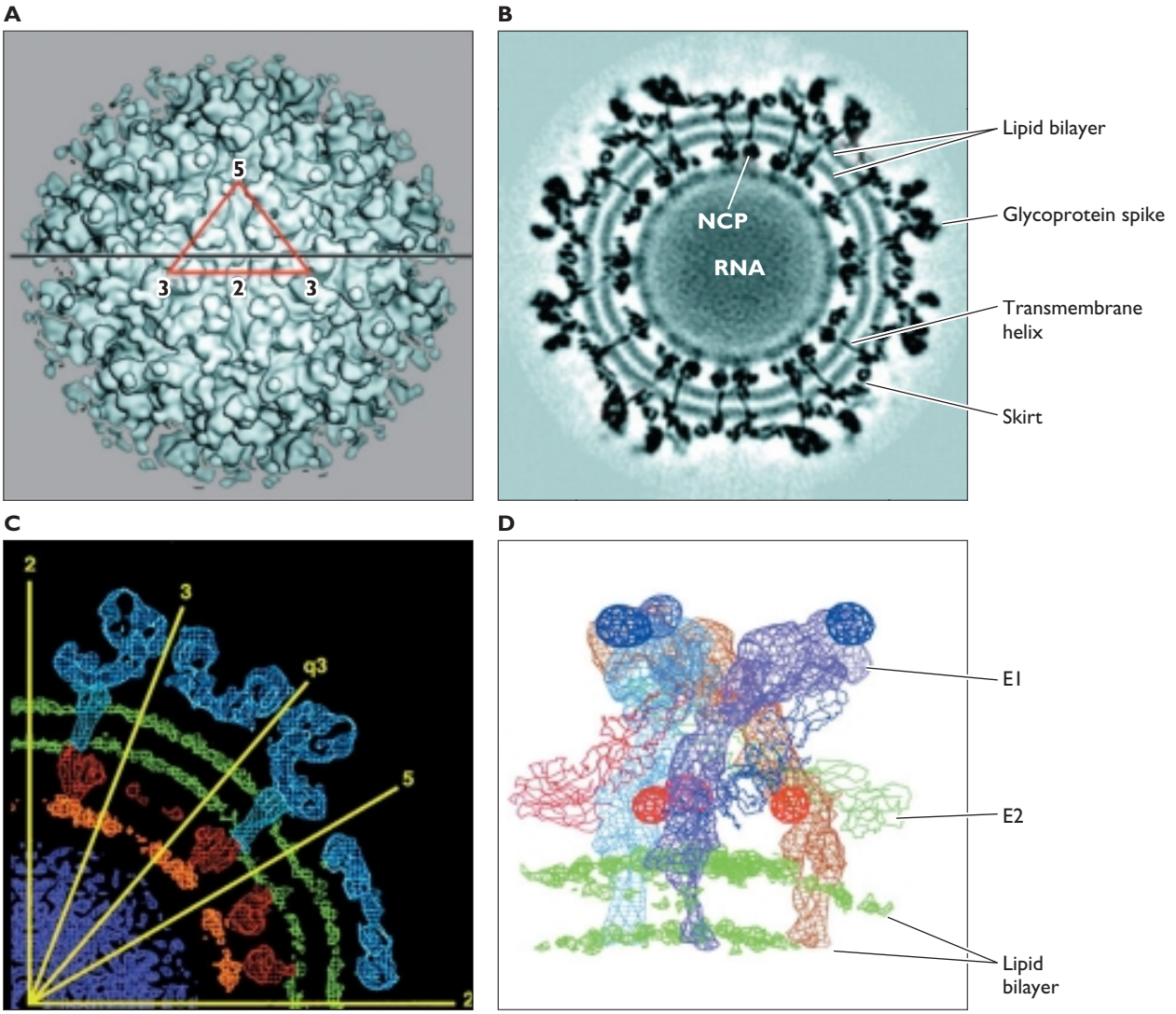


Figure 4.23 Structure of a simple enveloped virus, Sindbis virus. (A) The surface structure of Sindbis virus, a member of the alphavirus genus of the *Togaviridae*, at 20-Å resolution determined by cryo-electron microscopy. The boundaries of the structural (asymmetric) unit are demarcated by the red triangle, on which the icosahedral five-, three-, and twofold axes of rotational symmetry are indicated. This outer surface is organized as a $T = 4$ icosahedral shell studded with 80 spikes, each built from three copies of each of the transmembrane glycoproteins E1 and E2. These spikes are connected by a thin, external protein layer, termed the skirt. **(B)** Cross section through the density map at 11-Å resolution, along the black line shown in panel A. The lipid bilayer of the viral envelope is clearly defined at this resolution, as are the transmembrane domains of the glycoproteins. **(C)** Different layers of the particle, based on the fitting of a high-resolution structure of the E1 glycoprotein into a 9-Å reconstruction of the virus particle. The nucleocapsid (red) surrounds the genomic (+) strand RNA. The RNA is the least well-ordered feature in the reconstruction, although segments (orange) lying just below the capsid (C) protein appear to be ordered by interaction with this protein. The C protein penetrates the inner leaflet of the lipid membrane, where it interacts with the cytoplasmic domain of the E2 glycoprotein (blue). The membrane is spanned by rod-like structures that are connected to the skirt by short stems. **(D)** The structure of the E1 and E2 glycoproteins, obtained by fitting the crystal structure of the closely related Semliki Forest virus E1 glycoprotein into the 11-Å density map and assigning density unaccounted for to the E2 glycoprotein. The view shown is around a quasi-threefold symmetry axis (q3 in panel C), with the three E2 glycoprotein molecules in a trimeric spike colored light blue, brown, and purple and the E1 molecules shown as backbone traces colored red, green and dark blue. The E1 glycoprotein is largely tangential to the surface of the particle. The portions of the proteins that cross the lipid bilayer are helical, twisting around one another in a left-handed coiled coil. Courtesy of Michael Rossmann, Purdue University. Adapted from W. Zhang et al., *J. Virol.* **76:**11645–11658, 2002, with permission.

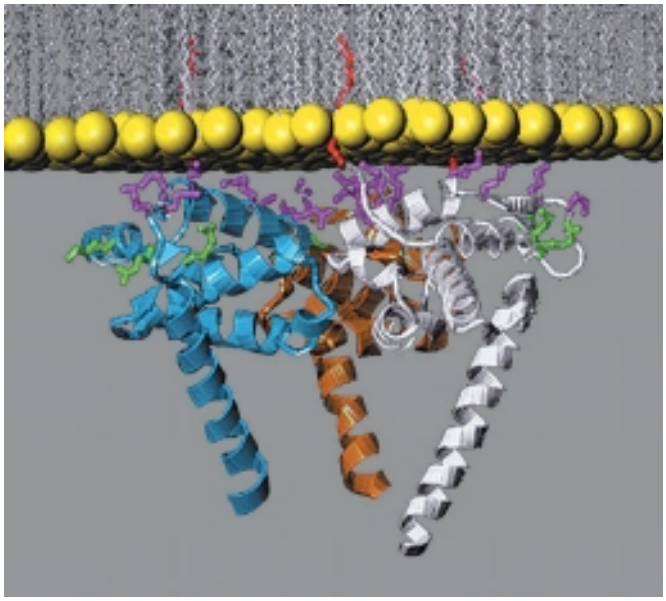


Figure 4.24 Model of the interaction of human immunodeficiency virus type 1 MA protein with the membrane. The membrane is shown with the polar head groups of membrane lipids in yellow. This model is based on the X-ray crystal structure of recombinant MA protein synthesized in *E. coli* and consequently lacking the N-terminal (myristate) 14-carbon fatty acid normally added in human cells. The three monomers in the MA trimer are shown in different colors. Basic residues in the β-sheet that caps the globular α-helical domain are magenta or green. Substitution of those shown in magenta impairs replication of the virus in cells in culture. The position of the myrsitate (red) was modeled schematically. From C. P. Hill et al., *Proc. Natl. Acad. Sci. USA* **93:**3099–3104, 1996, with permission. Courtesy of C. P. Hill and W. I. Sundquist, University of Utah.

β-sheet that contains positively charged amino acids that are also necessary for membrane binding. As illustrated in the model of MA oriented on a membrane shown in Fig. 4.24, the basic residues form a positively charged surface, positioned for interaction with phospholipid head groups on the inner surface of the envelope. The matrix proteins of (–) strand RNA viruses such as vesicular stomatitis virus and influenza virus also contain positively charged domains required for membrane binding, despite having three-dimensional folds that are quite different from those of retroviral MA proteins (and from one another).

Complex Viruses

Virus particles that house large DNA genomes are structurally far more complex than any considered in previous sections. Such virions comprise obviously distinct components with different symmetries and/or multiple layers. We illustrate these properties using as examples bacteriophage T4, herpes simplex virus type 1, and vaccinia virus.

Bacteriophage T4

Bacteriophage T4, which has been studied for over 50 years, is the classic example of a structurally complex virus that lacks an envelope. The T4 virion, which is built from about 50 of the proteins encoded in the ~170-kbp double-stranded DNA genome, is a structurally elegant machine tailored for active delivery of the genome to host cells. The most striking feature is the presence of morphologically distinct and functionally specialized structures, notably the head containing the genome and a long tail that terminates in a baseplate from which six long tail fibers protrude (Fig. 4.25A). A set of short fibers are also attached to the baseplate, while yet another set, termed whiskers, decorate the junction between head and tail.

The head of the mature T4 particle, an elongated prolate icosahedron, is built from hexamers of a single viral protein (gp23*). In contrast to the other capsids or nucleocapsids considered so far, two T numbers are needed to describe the organization of gp23* in the two end structures ($T = 13$) and in the elongated midsection ($T = 20$). As in adenoviral capsids, the pentamers that occupy the vertices contain a different viral protein, and additional proteins reside on the outer or inner surfaces of the icosahedral shell (Fig. 4.25B). One of the 12 vertices is occupied by a unique structure termed the connector, which joins the head to the tail. Such structures are derived from the nanomachine that pulls DNA into immature heads termed the **portal**. Portals are a characteristic feature of the nucleocapisds of other families of DNA-containing bacteriophages, as well as of herpesviruses.

In contrast to the head, the ~100-nm-long tail, which comprises two protein layers, exhibits helical symmetry (Fig 4.25A). The outer layer is a contractile sheath that functions in injection of the viral genome into host cells. The tail is connected to the head via a hexameric ring and at its head-distal end to a complex, dome-shaped structure termed the baseplate that contains at least 16 different proteins (Fig 4.25C). Both long and short tail fibers project from the baseplate. The former, which are long and bent, are the primary receptor-binding structures of bacteriophage T4. As discussed in Chapter 5, remarkable conformational changes induced upon receptor binding by the tips of the long fibers are transmitted via the baseplate to initiate injection of the DNA genome.

Herpesviruses

Virions of the *Herpesviridae* contain many more proteins than any animal virus described in previous sections, and exhibit a number of unusual architectural features. Over half of the more than 80 genes of herpes simplex virus type 1 encode proteins found in virus particles (Table 4.7), which are correspondingly large, about 2,000 Å in

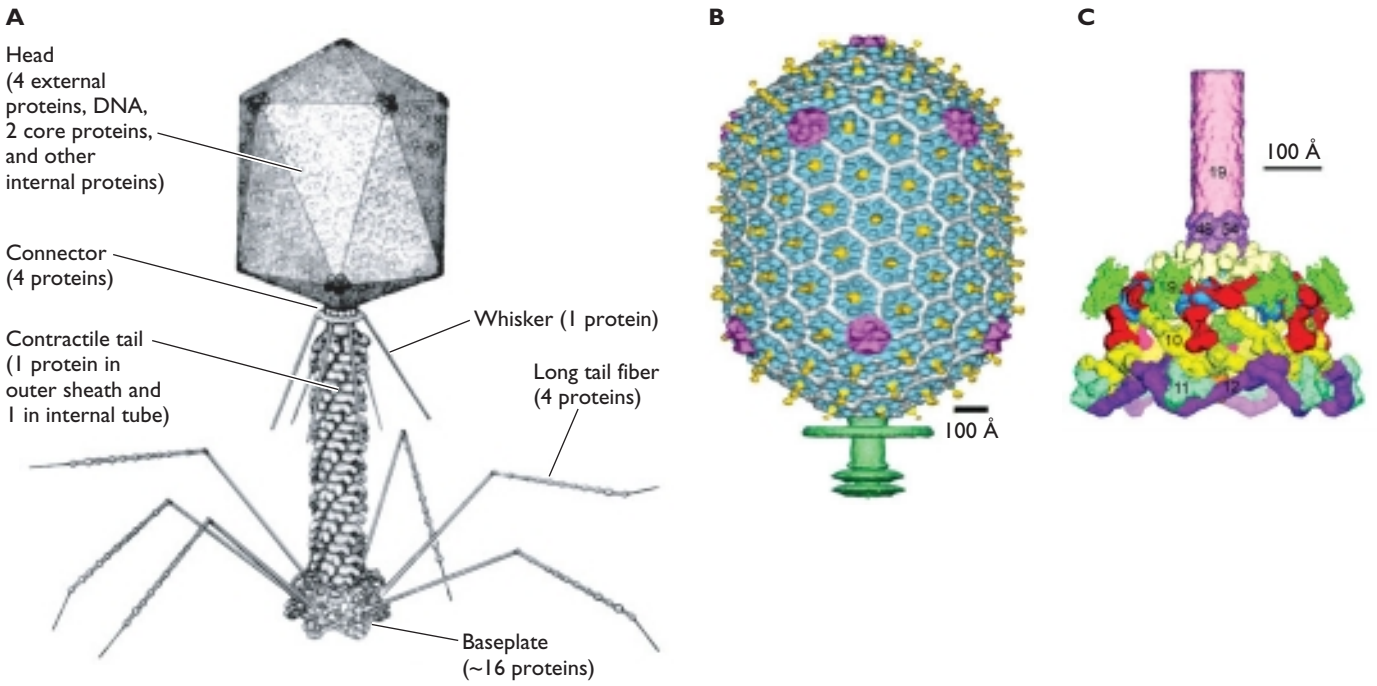


Figure 4.25 Morphological complexity of bacteriophage T4. (A) A Model of the virion. Adapted from P.G. Leiman et al., *Cell Mol. Life Sci.* **60:**2356–2370, 2003, with permission. **(B)** structure of the head (22-Å resolution) determined by cryo-electron microscopy, with the major capsid proteins shown in blue (gp23*) and magenta (gp24*), the protruding noc protein in yellow, the protein that binds between gp23* subunits in white, and the beginning of the tail in green. Adapted from A. Fokine et al., *Proc. Natl. Acad. Sci. USA* **101:**6003–6008, 2004, with permission. **(C)** Side view of the baseplate connected to the inner tube of the tail (protein 19), obtained by fitting the X-ray structures of individual baseplate proteins (numbered and shown in different colors) in a 12-Å-resolution reconstruction of the baseplate-tail tube complex. The baseplate is a complex dome-shaped structure constructed on a hexameric base. Adapted from V. A. Kostyachenko et al., *Nat. Struct. Biol.* **10:**688–693, 2003, with permission. (B and C) Courtesy of M. Rossmann, Purdue University.

diameter. These proteins are components of the envelope (Table 4.7) from which glycoprotein spikes project, or of two distinct internal structures. The latter are the nucleocapsid surrounding the DNA genome and the protein layer encasing this structure, called the **tegument** (Fig. 4.26A).

A single protein (VP5) forms both the hexons and the pentons of the $T = 16$ icosahedral nucleocapsid of herpes simplex virus type 1 (Fig. 4.26B). Like the structural units of the smaller simian virus 40 capsid, these VP5-containing assemblies make direct contact with one another. However, the large (~1,500-Å-diameter) herpesviral nucleocapsid is stabilized by additional proteins, VP19C and VP23. These two proteins form triplexes that link the major structural units (Fig. 4.26B). Although apparently a typical and quite simple icosahedral shell, this viral nucleocapsid is in fact an asymmetric structure: in infectious virions, 1 of the 12 vertices is occupied not by a VP5 penton but by a unique portal (Fig. 4.26C). This structure comprises 12 copies of the UL6 protein (Table 4.7). The portal assembled from the UL6 protein made in insect cells is a squat hollow cylinder that is wider at one end and surrounded by a two-tiered ring at the wider end (Fig. 4.26C). The asymmetry of the herpesviral nucleocapsid and the incorporation of the portal have important implications for the mechanism of assembly (see Chapter 13).

The tegument contains at least 13 viral proteins, viral RNAs, and cellular components. Descriptions of this structure have changed considerably with the application of increasingly sophisticated methods of electron microscopy (Box 4.8). It is generally agreed that specific tegument proteins are icosahedrally ordered, as a result of direct contacts with the structural units of the nucleocapsid (Fig. 4.26D). However, some tegument proteins are **not** uniformly distributed around the nucleocapsid. Rather, they are concentrated on one side of the capsid, where they form a well-defined cap-like structure (Fig 4.26E). As this totally unanticipated asymmetry of herpesviral particles has been

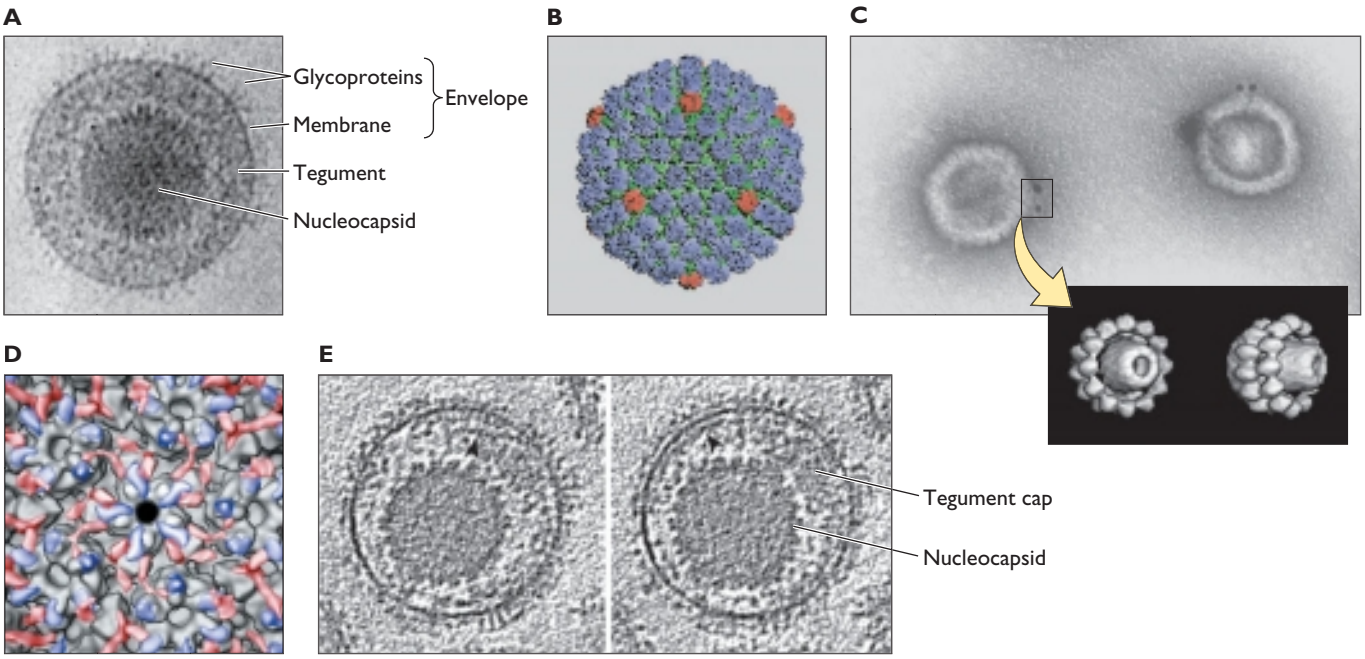


Figure 4.26 Structural features of herpesvirus particles. (A) Electron micrograph of a frozen, hydrated herpes simplex virus type 1 virion. From F. J. Rixon, *Semin. Virol.* **4:**135–144, 1993, with permission. Courtesy of F. Rixon, Institute of Virology, Glasgow, United Kingdom, and W. Chiu, Baylor College of Medicine. **(B)** Reconstruction of the herpes simplex virus type 1 nucleocapsid (8.5-Å resolution), with VP5 hexamers and pentamers colored blue and red, respectively, and the triplexes that reinforce the connections among these structural units in green. VP5 hexamers, but not pentamers, are capped by a hexameric ring of VP26 protein molecules (not shown). Adapted from Z. H. Zhou et al., *Science* **288:**877–880, 2000, with permission. Courtesy of W. Chiu, Baylor College of Medicine. **(C)** The single portal of herpes simplex virus type 1 nucleocapsids visualized by staining with an antibody specific for the viral UL6 protein conjugated to gold beads. The gold beads are electron dense and appear as dark spots in the electron micrograph. They are present at a single vertex in each nucleocapsid, which therefore contains one portal. The inset shows a 16-Å reconstruction of the UL6 protein portal based on cryo-electron microscopy. Adapted from W. W. Newcomb et al., *J. Virol.* **75:**10923–10932, 2001, and B. L. Trus et al., *J. Virol.* **78:**12668–12671, 2004, with permission. Courtesy of A. C. Steven, National Institutes of Health. **(D)** Interactions of two tegument proteins with the simian cytomegalovirus nucleocapsid. Tegument proteins that bind to hexons plus pentons and to triplexes are shown in blue and red, respectively. These proteins were visualized by cryo-electron microscopy, image reconstruction (to 22-Å resolution), and difference mapping of nucleocapsids purified from the nucleus and cytoplasm of virus-infected cells. The latter carry the tegument, but the former do not. Adapted from B. L. Trus et al., *J. Virol.* **73:**2181–2192, 1999, with permission. Courtesy of A. C. Steven, National Institutes of Health. **(E)** Two slices through a cryo-electron tomogram of a single herpes simplex virus type 1 particle, showing the eccentric tegument cap. Adapted from K. Grunewald et al., *Science* **302:**1396–1398, 2003, with permission. Courtesy of A. C. Steven, National Institutes of Health.

viewed only at low resolution, the molecular organization of the cap is not yet understood. Herpesvirus particles completely lacking certain tegument proteins appear morphologically normal and are fully infectious, suggesting that this layer may be structurally plastic.

Poxviruses

Like bacteriophage T4 and herpesvirus particles, those of poxviruses such as vaccinia virus comprise multiple, distinct structural elements. Two forms of infectious particles, termed mature virions and enveloped extracellular virions, are produced in vaccinia virus-infected cells (see Chapter 13). Mature virions are large, enveloped structures (~330 × 360 × 125 nm) comprising at least 75 proteins that appear in the electron microscope as brick- or barrel-shaped (Fig. 4.27A). Whether one or two envelopes are present has been the subject of long-standing debate. However, there is now a growing consensus for the presence of just a single membrane. At least 25 membrane proteins, most of which contain one or two membrane-spanning domains, have been identified. An unusual feature of these viral membranes is that they are not glycosylated.

A number of internal structures have been observed by examination of thin sections through purified particles

BOX 4.8 METHODS

Evolution of descriptions of the organization of the herpesviral tegument with technical advances

~1975 to 1993: An amorphous tegument, an irregular structure with no obvious organization or symmetry. This view was based on negative staining of purified virions, a technique that we now know resulted in distortion of the tegument (and envelope).

1993 to 2003: A regular, uniformly organized tegument revealed by cryo-electron microscopy (see, e.g., Fig. 4.26A), which avoids artifacts associated with negative staining. However, image reconstruction assumed icosahedral symmetry. Consequently, structures with different, or no, symmetry cannot be visualized.

2003 to present: A regular structure that is asymmetrically organized within virions to form a cap on one side of the nucleocapsid (Fig. 4.26E). This view is based on cryo-electron tomography, in which no assumptions about symmetry are made during image reconstruction.

(Fig 4.27B). These features include the core wall, which surrounds the central core that contains the ~200-kbp DNA genome. Remarkably, the core contains at least 30 enzymes with many different activities. The outer surface of the core wall abuts the inner surface of the virion membrane, except where the two central masses termed lateral bodies are located (Fig 4.27B). Although viral proteins that contribute to these various structures have been identified, our understanding of vaccinia virus architecture remains at low resolution.

Other Components of Virions

Some virus particles comprise only the nucleic acid genome and structural proteins necessary for protection and delivery into a host cell. However, many contain additional viral proteins or other components, which are generally present at much lower concentrations but play essential or important roles in establishing an efficient infectious cycle (Table 4.8).

Virion Enzymes

Many types of virus particle contain enzymes necessary for synthesis of viral nucleic acids. Such enzymes generally catalyze reactions unique to virus-infected cells, such as synthesis of viral mRNA from an RNA template or of viral DNA from an RNA template. However, virions of vaccinia virus contain a DNA-dependent RNA polymerase, analogous to cellular RNA polymerases, as well as several enzymes that modify viral RNA transcripts (Table 4.8). This complement of enzymes is necessary because transcription

Figure 4.27 Structural features of the poxvirus vaccinia virus. (A) Electron micrograph of a negatively stained purified virion, showing the protrusions termed surface tubular elements. From S. Willon et al., *Virology* **214:**503–511, 1995. Courtesy of S. Dales, University of Western Ontario, London, Canada. **(B)** Electron micrograph of frozen section of a purified virion. Adapted from M. Hollinshead et al., *J. Virol.* **73:**1503–1517, 1999, with permission. Courtesy of D. J. Vaux, University of Oxford, Oxford, England.

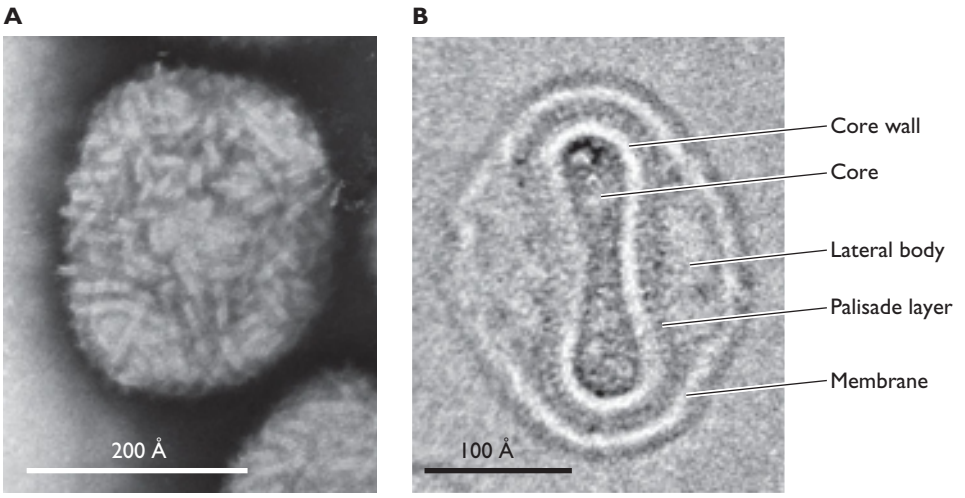


Table 4.8 Some virion enzymes

Virus	Protein	Function(s)
Adenovirus		
Human adenovirus type 2	L3 23k	Protease, production of infectious virions
Herpesvirus		
Herpes simplex virus type 1	VP24	Protease, capsid maturation for genome encapsidation
	UL13	Protein kinase
	Vhs	RNase
Orthomyxovirus		
Influenza A virus	P proteins	RNA-dependent RNA polymerase; synthesis of viral mRNA and vRNA; cap-dependent endonuclease
Poxvirus		
Vaccinia virus[a]	DNA-dependent RNA polymerase (8 subunits)	Synthesis of viral mRNA
	Poly(A) polymerase (2 subunits)	Synthesis of poly(A) on viral mRNA
	Capping enzyme (2 subunits)	Addition of 5′ caps to viral pre-mRNA
	DNA topoisomerase	Sequence-specific nicking of viral DNA
	Proteases 1 and 2	Virion morphogenesis
	Glutaredoxin	Thiol transferase; nonessential
Reovirus		
Reovirus type 1	λ2	Guanyl transferase
	λ3	Double-stranded RNA-dependent RNA polymerase
Retrovirus		
Avian sarcoma virus	Pol	Reverse transcriptase, proviral DNA synthesis
Human immunodeficiency virus type 1	IN	Integrase, integration of proviral DNA into the cellular genome
	PR	Protease, production of infectious virions
Rhabdovirus		
Vesicular stomatitis virus	L	RNA-dependent RNA polymerase, synthesis of viral mRNA and vRNA

[a]Vaccinia virions contain at least 17 enzymes, only a few of are listed.

of the viral double-stranded DNA genome takes place in the cytoplasm of infected cells, whereas cellular DNA-dependent RNA polymerases and the RNA-processing machinery are restricted to the nucleus. Other types of enzyme found in virions include integrase, cap-dependent endonuclease, and proteases (Table 4.8). The proteases, which eliminate covalent connections among specific proteins from which virions assemble, are necessary for the production of infectious particles.

Other Viral Proteins

More complex virions may also contain additional viral proteins that are not enzymes, but nonetheless are important for an efficient infectious cycle (Table 4.9). Among the best characterized examples of this class are several tegument proteins of herpesviruses. The VP16 protein activates transcription of viral immediate-early genes to initiate the viral program of gene expression. In contrast to the majority of viral proteins discussed in this section, VP16 is present at high concentration, because it is also necessary for virion assembly. Other herpesvirus tegument proteins induce the degradation of cellular mRNA, or block the cellular mechanism by which viral proteins are presented to the host's immune system. Complex retroviruses like human immunodeficiency virus type 1 also contain additional proteins required for efficient viral replication in certain cell types, such as Nef and Vpr. These proteins are discussed in Volume II, Chapter 6.

Nongenomic Viral Nucleic Acid

The **viral** nucleic acids present in virions have, by definition, been considered to be genomes. It was therefore quite a surprise to find other, functional viral nucleic acids within virus particles. This unexpected property was first

Table 4.9 Some additional viral components of virions

Virus	Component	Function
Proteins		
Herpesvirus		
Herpes simplex virus type 1	VP16	Structural protein, activation of IE gene transcription
Human cytomegalovirus	pp65	Inhibition of presentation of viral IE proteins as antigens
Poxvirus		
Vaccinia virus	VETF	Binds to early promoters; essential for their transcription
	RAP94	Associated with virion RNA polymerase, specificity factor
	I6L protein	DNA packaging; binds ends of genome
Retrovirus		
Human immunodeficiency virus type 1	Vpr	Required for efficient infection in some cell types
	Nef	
Other viral nucleic acids		
Herpesvirus		
Human cytomegalovirus	5 late mRNAs	?

reported for human cytomegalovirus, a betaherpesvirus with one of the largest DNA genomes known, and an important human pathogen (Volume II, Appendix A, Fig. 9). As discussed in more detail below, it can be difficult to distinguish macromolecules that are assembled specifically into enveloped virus particles from those that are incorporated nonspecifically. However, of the 200 or so viral mRNAs synthesized in human cytomegalovirus-infected cells, only 4 can be readily detected in purified virions. Moreover, these same viral mRNAs were found within cells very soon after infection in the presence of a drug that prevents synthesis of **all** mRNAs. These viral mRNAs must therefore be packaged specifically. The virion-delivered mRNAs, which probably reside within the tegument, are translated in the cytoplasm of newly infected cells. Neither the functions of the viral proteins made from these viral mRNAs nor the mechanism by which the mRNAs enter assembling virus particles is yet understood.

Cellular Macromolecules

Virus particles can also contain cellular macromolecules that play important roles during the infectious cycle (Table 4.9), such as the cellular histones that package polyomaviral and papillomaviral DNAs. Because they are formed by budding, enveloped viruses can readily incorporate cellular proteins and other macromolecules. Cellular glycoproteins may not be excluded from the membrane from which the viral envelope is derived. Moreover, as a bud enlarges and pinches off during virus assembly, internal cellular components may be trapped within it. In addition, enveloped viruses are generally more difficult to purify than naked viruses. As a result, preparations of these viruses may be contaminated with vesicles formed from cellular membranes during virus purification. Consequently, it can be difficult to distinguish cellular components specifically incorporated into enveloped virus particles from those trapped randomly or copurifying with the virus. Nevertheless, in some cases it is clear that cellular molecules are important components of virus particles: these molecules are reproducibly observed in virions at a specific stoichiometry and can be shown to play essential or important roles in the infectious cycle. The cellular components captured in retrovirus particles have been particularly well characterized, but cellular proteins are also present in other virions. For example, the herpesviral tegument contains substantial quantities of actin, as well as other cytoskeletal proteins (Table 4.9).

The primer for initiation of synthesis of the (−) strand DNA during reverse transcription is invariably a cellular transfer RNA (tRNA). This RNA is incorporated into virions by virtue of its binding to a specific sequence in the RNA genome and to reverse transcriptase. A variety of cellular proteins are also present in some retroviral particles. One of the most unusual properties of human immunodeficiency virus type 1 is the presence of cellular cyclophilin A, a **chaperone** that assists or catalyzes protein folding. This protein is a member of a ubiquitous family of peptidyl-prolyl isomerases, which catalyze the intrinsically slow *cis-trans* isomerization of peptide bonds preceding proline residues in newly synthesized proteins. Cyclophilin A is the major cytoplasmic member of this family. It is incorporated within human immunodeficiency virus type 1 particles via specific interactions with the central portion of (CA) the capsid protein, and it catalyzes isomerization of

a single Gly-Pro bond in CA. Although incorporation of cyclophilin A is not a prerequisite for assembly, particles that lack this cellular chaperone have reduced infectivity. It is thought that in human cells, cyclophilin provides protection against an intrinsic antiviral defense mechanism, but this mechanism has not been identified.

Cellular membrane proteins, such as Icam-1 and Lfa1 (see Chapter 5), can also be incorporated in the viral envelope and can contribute to attachment and entry of human immunodeficiency virus type 1 particles. They may also influence pathogenesis (see Volume II, Chapter 6). Other cellular proteins assembled into viral particles, such as actin found in herpesviral virions, may facilitate assembly or budding reactions (see Chapter 13).

The majority of cellular components present in virus particles serve to facilitate virus replication, a property exemplified by the cellular tRNA primers for retroviral reverse transcription. However, it has become clear recently that incorporation of cellular components can also provide antiviral defense. As discussed in Volume II, Chapters 3 and 6, packaging of a cellular enzyme that converts cytosine to uracil (Apobec3) into retrovirus particles at the end of one infectious cycle leads to degradation and hypermutation of viral DNA synthesized early in the next cycle of infection.

It is clear from these examples that virus particles contain a surprisingly broad repertoire of molecular functions that are delivered to their host cells (Tables 4.8 and 4.9). This repertoire is undoubtedly larger than we presently appreciate, for the precise molecular functions and/or roles in the infectious cycles of many virion components have yet to be established.

Perspectives

Virus particles are among the most elegant and visually pleasing structures found in nature, properties emphasized by the images presented in this chapter. Now that many structures of particles or their components have been examined, we can appreciate the surprisingly diverse architectures exhibited by virions. Nevertheless, the simple principles of their construction proposed over 50 years ago remain pertinent: with few exceptions, the capsid or nucleocapsid shells that encase and protect nucleic acid genomes are built from a small number of protein subunits arranged with helical or icosahedral symmetry.

The detailed views of nonenveloped virions provided by X-ray crystallography emphasize just how well these protein shells provide protection of the genome during passage from one host cell or organism to another. They have also identified several mechanisms by which identical or nonidentical subunits can interact to form icosahedrally symmetric structures. More complex virus particles, which may contain additional protein layers, a lipid envelope carrying viral proteins, and enzymes or other proteins necessary to initiate the infectious cycle, cannot be described in the exquisite detail provided by a high-resolution structure. For many years we possessed only schematic views of these structures, deduced from negative-contrast electron microscopy and biochemical or genetic methods of analysis. Within the past decade, the development and refinement of cryo-electron microscopy and techniques of image reconstruction have revolutionized structural studies of larger and more complex viruses. To cite but a few examples, these methods have yielded remarkable views of genome organization and interactions among envelope and internal proteins. The recent improvement in the resolution that can be achieved by these techniques to <10 Å, the power of difference imaging methods, and the now routine ability to produce large quantities of viral proteins promise many new insights into the structure, molecular organization, and function of virus particles. Such information will improve our understanding, presently quite limited, of mechanisms of packaging of nucleic acid genomes and of tethering envelopes (when present) to internal structures. Some surprises are undoubtedly in store. But we can predict with some confidence that future studies will reinforce and elaborate the general principles of virus structure described here.

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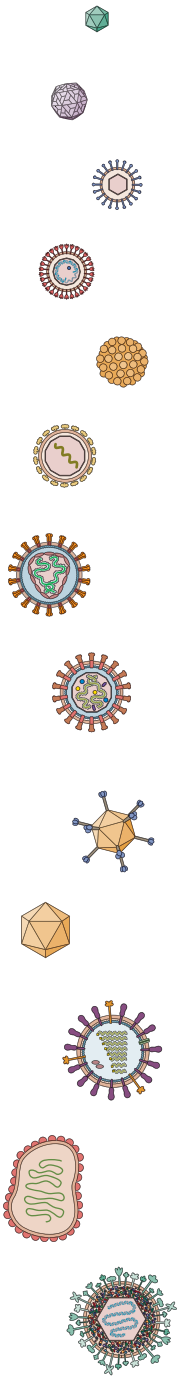


5

Attachment and Entry

Who hath deceived thee so often as thyself?
BENJAMIN FRANKLIN

Introduction

Because viruses are obligate intracellular parasites, the viral genome must enter a cell for the viral replication cycle to occur. The physical properties of the virion are obstacles to this seemingly simple goal. Virions are too large to diffuse passively across the plasma membrane. Furthermore, the viral genome is encapsidated in a stable coat that shields the nucleic acid as it travels through the harsh extracellular environment. These impediments must all be overcome during the process of viral entry into cells. When viruses encounter the surface of a susceptible host cell, a series of events lead to entry of the viral genome into the cytoplasm or nucleus. The first step in entry is adherence of virus particles to the plasma membrane, an interaction mediated by binding to a specific **receptor** molecule on the cell surface.

The cellular receptor plays an important role in **uncoating**, the process by which the viral genome is exposed, so that gene expression and genome replication can begin. Interaction of the virus particle with its cell receptor may initiate conformational changes that prime the capsid for uncoating. Alternatively, the cell receptor may direct the virion into endocytic pathways, where uncoating may be triggered by low pH or by the action of proteases. These steps bring the genome into the cytoplasm, where the genomes of most RNA-containing viruses replicate. The genomes of viruses that replicate in the nucleus are brought to that location by cellular transport pathways. Viruses that replicate in the nucleus include all DNA-containing viruses except poxviruses, RNA-containing retroviruses, influenza viruses, and Borna disease virus.

Early studies of virus entry into host cells, from the 1950s until the late 1970s, led to the view that viruses enter by an entirely passive process: virus particles attach to the cell surface, are taken up into the cell, and release their genomes, which are then replicated. No active role for the receptor in uncoating was envisioned. Beginning in the 1980s, the techniques of cellular, molecular, and structural biology were applied to elucidate the earliest events in viral infection. It is now understood that virus entry into cells is

not a passive process but, rather, relies on viral usurpation of normal cellular processes, including endocytosis, membrane fusion, vesicular trafficking, and transport into the nucleus. Because of the limited functions encoded by viral genomes, virus entry into cells absolutely depends on cellular processes.

Attachment of Viruses to Cells

General Principles

Infection of cells by many, but not all, viruses requires binding to a receptor on the cell surface. Exceptions include viruses of yeasts and fungi, which have no extracellular phases, and plant viruses, which are thought to enter cells through openings produced by mechanical damage, such as those caused by farm machinery or insects. In some cases, the receptor is the only cell surface molecule required for entry into cells. In others, binding to a cellular receptor is not sufficient for infection: an additional cell surface molecule, or **coreceptor**, is required for entry (Box 5.1).

The cell receptor may determine the **host range** of a virus, i.e., its ability to infect a particular animal or cell culture. For example, poliovirus infects primates and primate cell cultures but not mice or mouse cell cultures. Mouse cells synthesize a protein that is homologous to the poliovirus receptor but sufficiently different that poliovirus cannot attach to it. In this example, the poliovirus receptor is **the** determinant of poliovirus host range. However, production of the receptor in a particular cell type does **not** ensure that virus replication will occur. Some primate cell cultures produce the poliovirus receptor but cannot be infected. The restricted host range of the virus in such cells is most probably due to a block in viral replication beyond the attachment step. Cell receptors can also be determinants of tissue **tropism**, the predilection of a virus to invade and replicate in a particular cell type. However, there are many other determinants of tissue tropism. For example, the sialic acid residues on membrane glycoproteins or glycolipids, which are receptors for influenza virus, are found on many tissues, yet viral replication in the host is restricted. The basis for such restriction is discussed in Volume II, Chapter 1.

Our understanding of the earliest interactions of viruses with cells comes almost exclusively from analysis of synchronously infected cells in culture. The initial association of virions with cells is probably via electrostatic forces, as they are sensitive to low pH or high concentrations of salt. Subsequent high-affinity binding relies mainly on hydrophobic and other short-range forces whose strength and specificity are governed primarily by the conformations of the interacting viral and cellular interfaces. Although the affinity of a receptor for a single virus particle is low, the presence of multiple receptor-binding sites on the virion and the fluid nature of the plasma membrane allows engagement of multiple cell receptors. Consequently the avidity of virus binding to cells is usually very high. Virion binding can usually occur at 4°C (even though entry does not) as well as at body temperature (e.g., 37°C). Infection of cultured cells can therefore be synchronized by allowing binding to take place at a low temperature and then shifting the cells to a physiological temperature to allow the initiation of subsequent steps.

The first steps in virus attachment are governed largely by the probability that a virion and a cell will collide, and therefore by the concentrations of free virions and host cells. The rate of attachment can be described by the equation

$$dA/dt = k[V][H]$$

where $[V]$ and $[H]$ are the concentrations of virions and host cells, respectively, and k is a rate constant. Values of k for animal viruses vary greatly from a maximal value that represents the limits of diffusion to one that is as much as 5 orders of magnitude lower. It can be seen from this

BOX 5.1 TERMINOLOGY

Receptors and coreceptors

By convention, the first cell surface molecule that is found to be essential for virus binding is called its **receptor**. Sometimes, such binding is not sufficient for entry into the cell. When binding to another cell surface molecule is needed, that protein is called a **coreceptor**. For example, human immunodeficiency virus binds to cells via a receptor, CD4, and then requires interaction with a second cell surface protein such as CXCR4, the coreceptor.

In practice, the use of receptor and coreceptor can be confusing and inaccurate. A particular cell surface molecule that is a coreceptor for one virus may be a receptor for another. Furthermore, as is the case for the human immunodeficiency viruses, binding only to the coreceptor may be sufficient for entry of some members. Distinguishing receptors and coreceptors by the order in which they are bound is difficult to determine experimentally and is likely to be influenced by cell type and multiplicity of infection. Furthermore, some viruses can infect cells that synthesize only the coreceptor. Usage of the terms "receptor" and "coreceptor" is convenient when describing virus entry, but the appellations may not be entirely accurate.

equation that if a mixture of viruses and cells is diluted after virions have been allowed to attach, subsequent binding is greatly reduced. For example, a 100-fold dilution of the mixture reduces the attachment rate 10,000-fold (i.e., $1/100 \times 1/100$). Dilution can be used to prevent subsequent virus adsorption and hence to synchronize an infection.

Identification of Cell Receptors for Virus Particles

Early investigations of viral receptors exploited a variety of enzymes to characterize the cell surface components that are required for virus attachment. The first cell receptor discovered, sialic acid, which binds influenza virus, was identified because the enzyme neuraminidase removes this carbohydrate from cells and blocks virus attachment. In a similar way, experiments with proteases showed that many receptors are proteins. These types of analyses provided the first clues concerning the chemical nature of cell surface components to which virions become attached. It was also possible to determine whether different viruses share receptors, by determining whether saturating cells with one kind of virion prevented binding of a second.

Despite these approaches, identification of cell receptors for viruses languished because biochemical purification of these molecules proved difficult. As late as 1985, only one cell receptor, the sialic acid receptor of influenza viruses, had been identified unequivocally. The development of three crucial technologies rapidly changed this situation. The first, production of monoclonal antibodies, provided a powerful means of isolating and characterizing individual cell surface proteins. Hybridoma cell lines which secrete monoclonal antibodies that block virus attachment are obtained after immunizing mice with intact cells. Such antibodies can be used to purify the receptor protein by immunoprecipitation (Box 5.2) or affinity chromatography.

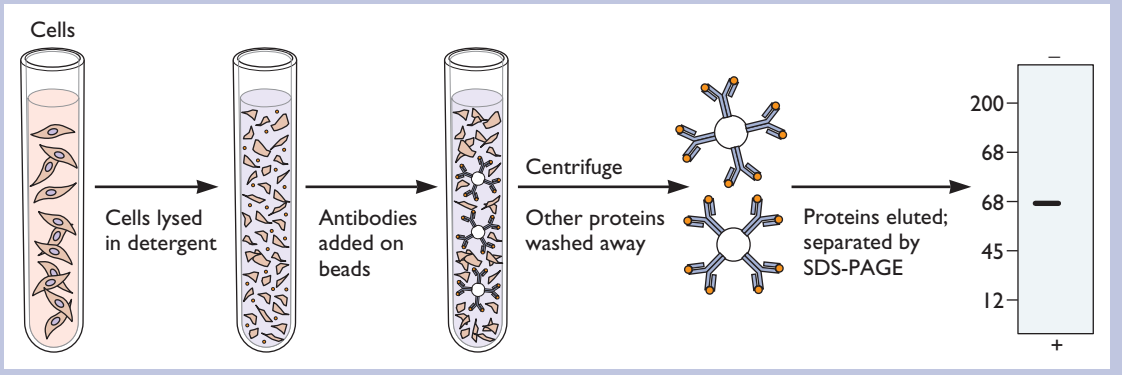
A second technology that advanced the cell receptor field was the development of DNA-mediated transformation. This method was crucial for isolating genes encoding receptors following introduction of DNA from susceptible cells into nonsusceptible cells (see Fig. 5.1). Cells that acquire DNA encoding the receptor and carry the corresponding protein on their surface are able to bind virus specifically. Clones of such cells are recognized and selected, for example, by the binding of receptor-specific

BOX 5.2 METHODS

Immunoprecipitation

Immunoprecipitation depends on the interaction of specific antibodies with proteins in solubilized extracts of cells or tissues (see figure). The antibody-protein complexes are isolated, and the proteins are dissociated from the complex and fractionated by electrophoresis in polyacrylamide gels. If the antibody is sufficiently specific, it may be possible to identify the protein that is bound by the antibody. For example, if the antibody used blocks virus attachment and is directed against a cell membrane protein, immunoprecipitation can provide information on the size of the protein. To identify the protein, it can be extracted from the gel and a partial amino acid sequence can be determined.

Isolation of proteins by immunoprecipitation. Cells are lysed with a detergent to solubilize proteins. Antibodies directed against the desired protein are coupled to beads and then added to the cell lysate. The beads are removed by centrifugation and washed free of protein not bound by the antibody. The bound proteins can then be fractionated by gel electrophoresis and visualized by staining. The heavy and light chains of the antibody molecules are not shown. The numbers next to the gel on the right are molecular masses in kilodaltons. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.



monoclonal antibodies. The receptor genes can then be isolated from these selected cells by using a third technology, molecular cloning. Although these technologies have led to the identification of many cell receptors for viruses, each method has associated uncertainties (Box 5.3).

The availability of cloned receptor genes has made it possible to investigate the details of receptor interaction with viruses by site-directed mutagenesis. Receptor proteins can be synthesized in heterologous systems and purified, and their properties can be studied *in vitro*, while animal cells producing altered receptor proteins can be used to test the effects of alterations on virus attachment. Because of their hydrophobic membrane-spanning domains, many of these cell surface proteins are relatively insoluble and difficult to work with. Soluble forms obtained by the expression of genes encoding truncated proteins lacking the membrane-spanning domain have been essential for structural studies of receptor-virus interactions. Cloned receptor genes have also been used to produce transgenic mice that synthesize receptor proteins. Such transgenic animals can serve as useful models in the study of human viral diseases.

Examples of Cell Receptors

Many different cell surface molecules can serve as receptors for the attachment of viruses. Some viruses attach to more than one, and viruses of different families share some receptors. Diverse molecules that serve as receptors for viruses are discussed below.

The Cell Receptor for Poliovirus, CD155

Members of the enterovirus genus of the *Picornaviridae* include human polioviruses, coxsackieviruses, echoviruses, and enteroviruses. These viruses are stable at acidic pH and multiply in the gastrointestinal tract. However, they also can replicate in other tissues, such as nerve, heart, and muscle. The cell receptor for poliovirus, CD155, was identified by using a DNA transformation and cloning strategy (Fig. 5.1). It was well known that mouse cells cannot be infected with poliovirus because they do not produce the cell receptor. Introduction of infectious poliovirus RNA into mouse tissue cell cultures leads to poliovirus replication, indicating that there is no intracellular block to virus multiplication. Cloning of the human gene from receptor-positive mouse cells established that the CD155 glycoprotein is a member of the immunoglobulin (Ig) superfamily (Fig. 5.2). The first of the three Ig-like domains is essential and sufficient for receptor function, but efficient (wild-type) binding requires all three. A structure of CD155 bound to poliovirus has been determined (Fig. 5.3A). This structure confirmed the results of mutational studies, which indicate that only domain 1 of CD155 contacts the viral capsid.

As mouse cells are permissive for poliovirus replication and as susceptibility appeared to be limited **only** by the absence of CD155, a small-animal model for the disease was developed by producing transgenic mice that synthesize this receptor. Inoculation of CD155 transgenic mice with poliovirus by various routes produces paralysis, tremors, and death, as is observed in human poliomyelitis. These CD155-synthesizing mice were the first new animal model created by transgenic technology for the study of viral disease. Similar approaches have subsequently led to animal models for viral diseases caused by measles virus and echoviruses.

The observation that even some human cells that make CD155 are resistant to poliovirus infection prompted the search for a second cellular protein that could regulate

BOX 5.3 BACKGROUND

Criteria for identifying cell receptors for viruses

The combination of monoclonal antibodies, molecular cloning, and DNA-mediated transformation provides a powerful approach for identifying cellular proteins that are receptors for viruses. Each method has associated uncertainties. For example, a monoclonal antibody that blocks virus attachment might recognize not the receptor but a closely associated membrane protein (see "The Cell Receptor for Poliovirus, CD155"). To prove that the protein recognized by the monoclonal antibody **is** a receptor, the protein must be isolated and its DNA must be cloned and introduced into cells to demonstrate that it can confer virus-binding activity. Any of the approaches outlined in Fig. 5.1 can result in identification of a cellular gene that encodes a putative receptor. However, the encoded protein might not be a receptor but may modify another cellular protein so that it can serve as a receptor. Proof that the DNA codes for a receptor would come from the identification of a monoclonal antibody that blocks virus attachment and is directed against the encoded protein.

For some viruses, synthesis of the receptor on cells leads to binding but not infection. In such cases a coreceptor is required, either for internalization or for membrane fusion. The techniques of molecular cloning also can be used to identify coreceptors. For example, production of CD4 on mouse cells leads to binding of human immunodeficiency virus type 1 but not infection, because fusion of viral and cell membranes does not occur. To identify the coreceptor, a DNA clone was isolated from human cells that allowed membrane fusion catalyzed by viral SU protein in mouse cells synthesizing CD4.

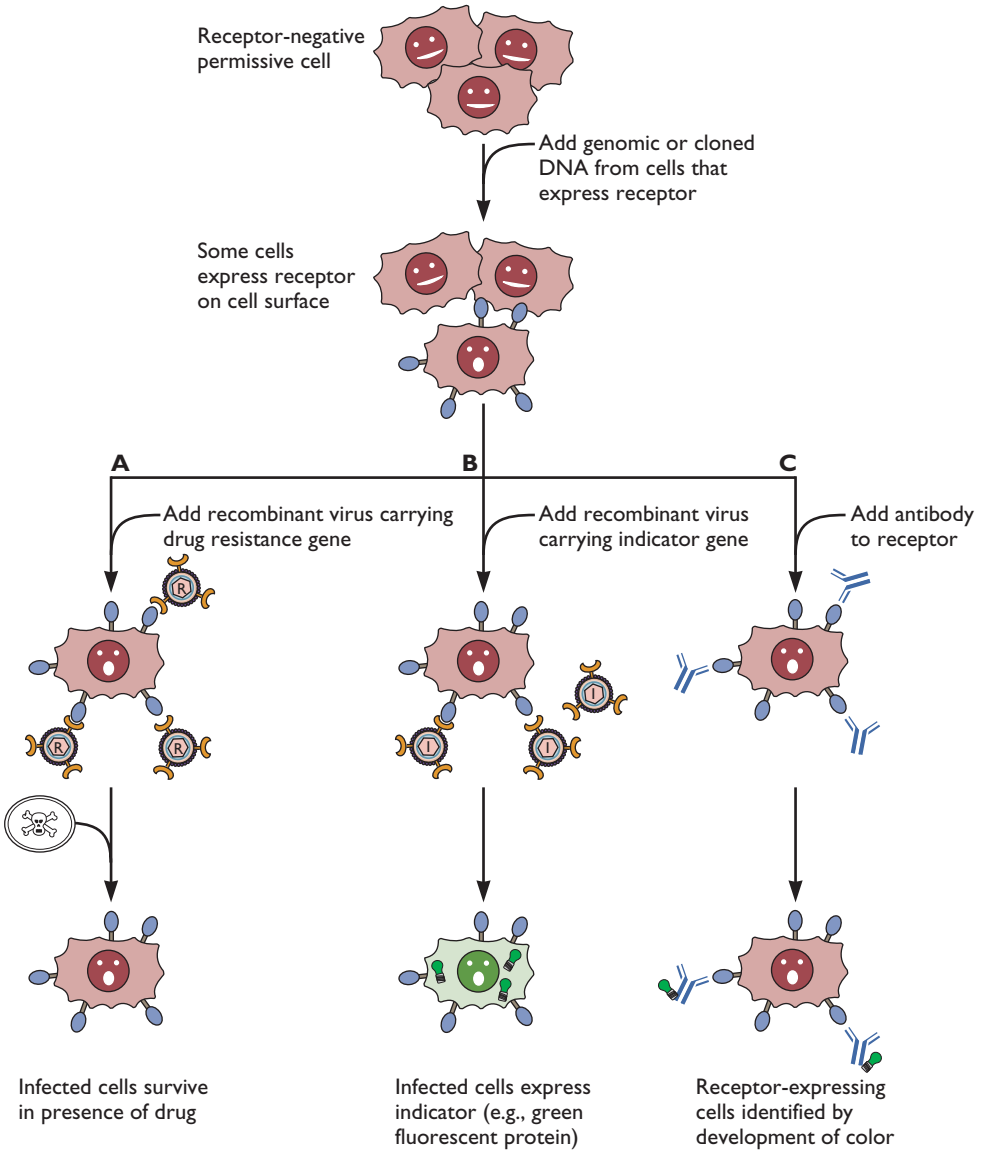


Figure 5.1 Experimental strategies for identification and isolation of genes encoding cell receptors for viruses. Genomic DNA or pools of DNA clones from cells known to synthesize the receptor are introduced into receptor-negative permissive cells. A small number of recipient cells express the receptor. Three different strategies for identifying such rare receptor-expressing cells are outlined. **(A)** The cells are infected with a virus that has been engineered so that it carries a gene encoding drug resistance. Cells that express the receptor will become resistant to the drug. This strategy works only for viruses that persist in cells without killing them. **(B)** For lytic viruses, an alternative is to engineer the virus to express an indicator, such as green fluorescent protein or β-galactosidase. Cells that make the correct receptor and become infected with such viruses can be distinguished by a color change, such as green in the case of green fluorescent protein. **(C)** The third approach depends on the availability of an antibody directed against the receptor, which binds to cells that express the receptor gene. Bound antibodies can be detected by an indicator molecule. When complementary DNA (cDNA) cloned in a plasmid is used as the donor DNA, pools of individual clones (usually 10,000 clones per pool) are prepared and introduced individually into cells. The specific DNA pool that yields receptor-expressing cells is then subdivided, and the screening process is repeated until a single receptor-encoding DNA is identified.

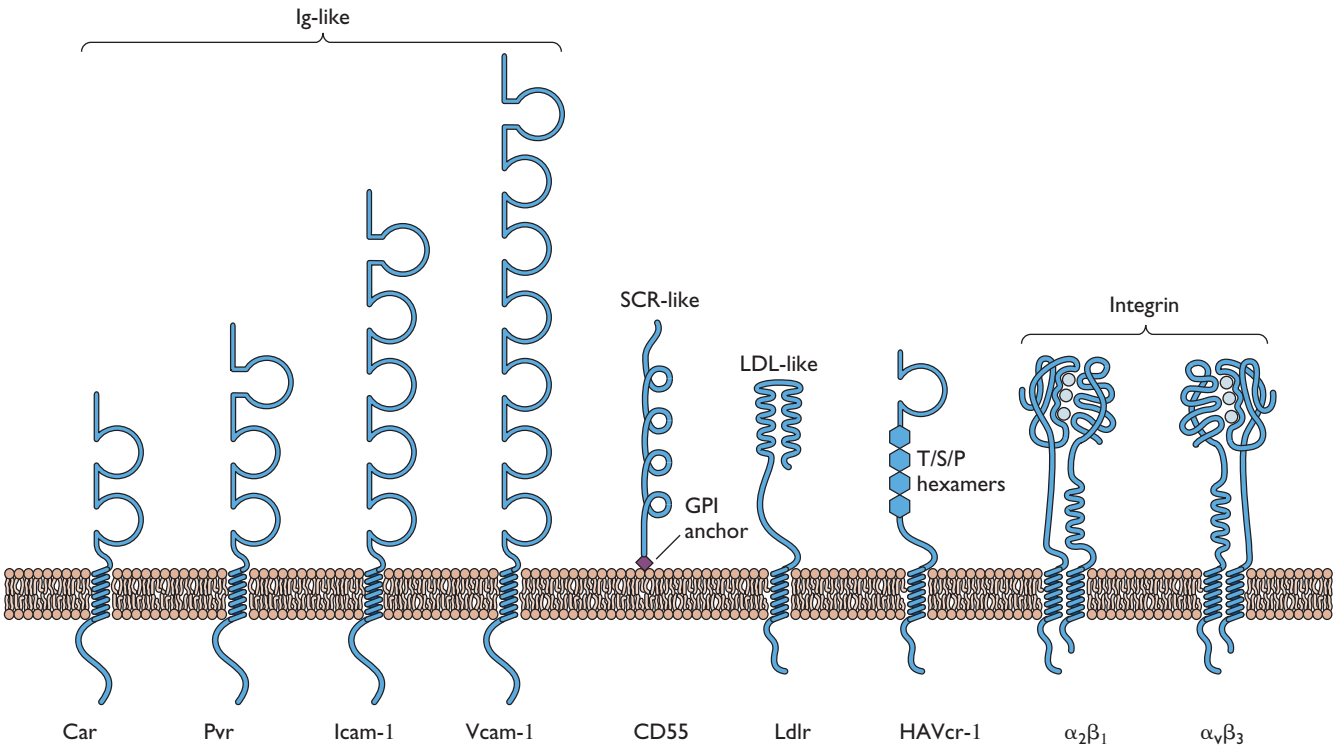


Figure 5.2 Cell receptors for picornaviruses. A schematic diagram of the cell proteins that function as receptors for different picornaviruses is shown. The different domains (Ig-like, short consensus repeat-like [SCR-like], low-density lipoprotein-like [LDL-like], and threonine/serine/proline [T/S/P]) are labeled. Car, coxsackievirus and adenovirus receptor; Vcam-1, vascular cell adhesion molecule 1; Ldlr, low-density lipoprotein receptor. Adapted from D. J. Evans and J. W. Almond, *Trends Microbiol*. **6:**198–202, 1998, with permission.

virus entry. One candidate was the lymphocyte homing receptor, CD44, which was thought to be a coreceptor for poliovirus, because a monoclonal antibody against it blocks poliovirus binding to cells. This multifunctional 100-kDa membrane glycoprotein normally helps direct the migration of lymphocytes to the lymph nodes and regulates lymphocyte adhesion and other functions. However, CD44 is not a receptor for poliovirus, nor is it required for poliovirus infection of cells that produce CD155. It is thought that CD155 and CD44 are associated in the plasma membrane and that anti-CD44 antibodies may block poliovirus attachment by sterically hindering the poliovirus-binding site on CD155. These results emphasize that monoclonal antibodies that block virus binding do not necessarily identify the receptor on cells. The resistance to poliovirus infection of certain cells that carry CD155 is determined by the type I interferon response (see Volume II, Chapter 3).

The Cell Receptor for Rhinovirus, Intercellular Adhesion Molecule 1

Members of the *Rhinovirus* genus of the *Picornaviridae* are unstable below pH 5 to 6 and multiply primarily in the upper respiratory tract. Up to 50% of all human common colds are caused by members of the major group of rhinoviruses. The cell surface receptor for these rhinoviruses (~90 serotypes) was identified by screening monoclonal antibodies for their ability to block rhinovirus infection. When such a monoclonal antibody was identified, it was used to isolate a 95-kDa cell surface glycoprotein by affinity chromatography. Amino acid sequence analysis of the purified protein, which bound to rhinovirus *in vitro*, identified it as the integral membrane protein intercellular adhesion molecule 1 (Icam-1). The cell receptor for the remaining rhinovirus serotypes is the low-density lipoprotein receptor.

Icam-1 is a member of the Ig superfamily. It has five domains that are homologous to one another and to the constant domains of antibody molecules. Each domain is stabilized by intrachain disulfide bonds. Icam-1 is found on the surface of many cell types, including those of the nasal epithelium, the normal entry site for rhinoviruses. The natural ligand of Icam-1 is another integral membrane glycoprotein, the integrin known as lymphocyte function-associated antigen 1 (Lfa-1). The normal function of Icam-1

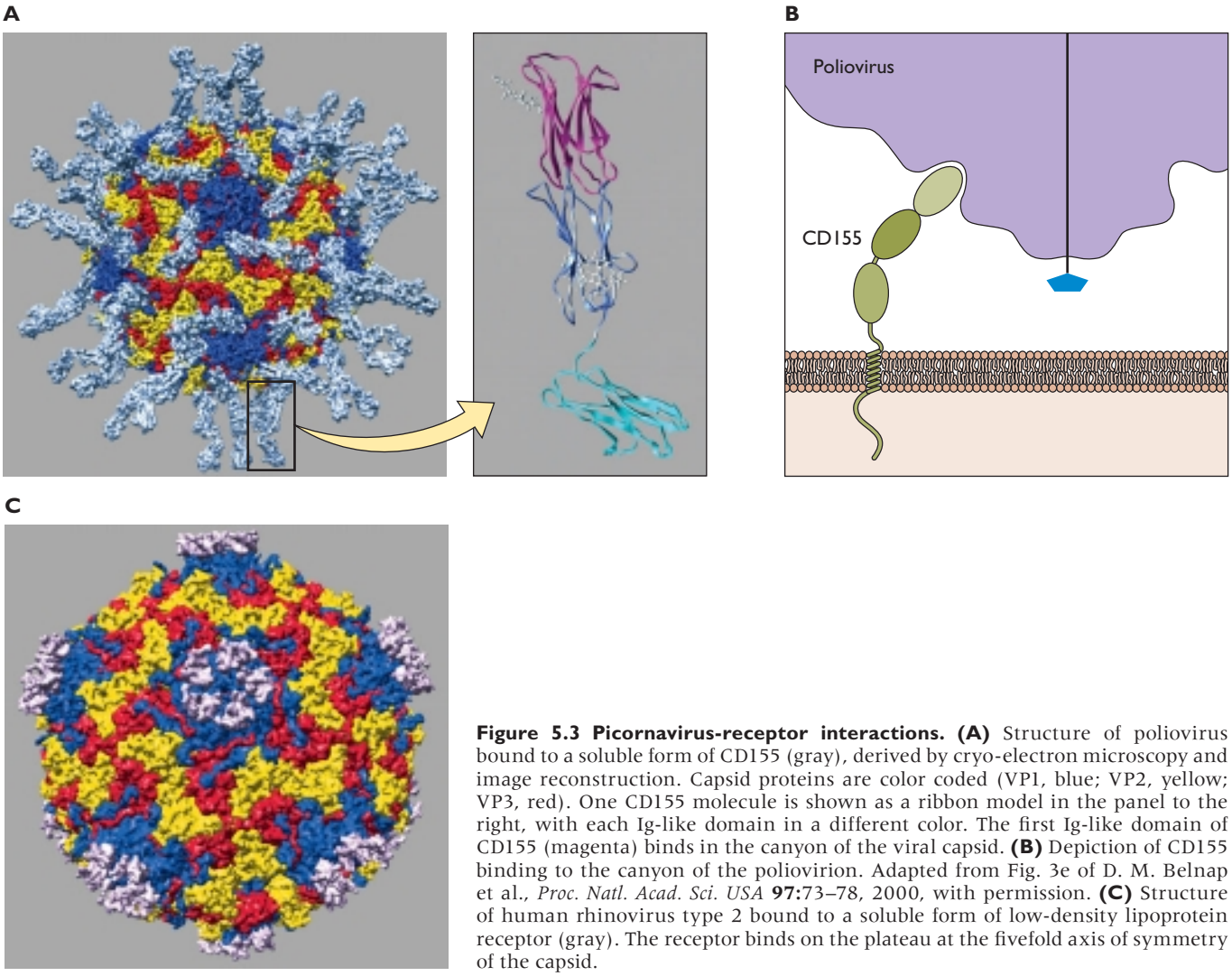


Figure 5.3 Picornavirus-receptor interactions. (A) Structure of poliovirus bound to a soluble form of CD155 (gray), derived by cryo-electron microscopy and image reconstruction. Capsid proteins are color coded (VP1, blue; VP2, yellow; VP3, red). One CD155 molecule is shown as a ribbon model in the panel to the right, with each Ig-like domain in a different color. The first Ig-like domain of CD155 (magenta) binds in the canyon of the viral capsid. **(B)** Depiction of CD155 binding to the canyon of the poliovirion. Adapted from Fig. 3e of D. M. Belnap et al., *Proc. Natl. Acad. Sci. USA* **97:**73–78, 2000, with permission. **(C)** Structure of human rhinovirus type 2 bound to a soluble form of low-density lipoprotein receptor (gray). The receptor binds on the plateau at the fivefold axis of symmetry of the capsid.

is to bind Lfa-1 on the surface of lymphocytes and promote a wide variety of immunological and inflammatory responses. Mediators of inflammation induce increased synthesis of Icam-1. Therefore, the initial reaction of host defenses to rhinoviruses, which leads to the production of such mediators, might actually induce the appearance of Icam-1 in nearby cells and thereby enhance subsequent spread of the virus.

Mutational analyses of cloned Icam-1 DNA established that the binding sites for rhinovirus and Lfa-1 are located in domains 1 and 2, with critical contact points in both cases mapping to domain 1, the most membrane distal and accessible. Although the binding sites for rhinovirus and Lfa-1 partially overlap, they are distinct. Amino acids in first N-terminal domains of CD155 and Icam-1 that are crucial for virus binding are different, demonstrating the diverse interactions that may occur even among structurally related viruses and receptors.

For other picornaviruses, one type of cell receptor is not enough for infection. Decay-accelerating protein (CD55), a member of the complement cascade, is the cell receptor for many enteroviruses (Fig. 5.2), but infection also requires the presence of a coreceptor. For example, coxsackievirus A21 can bind to cell surface decay-accelerating protein, but this interaction does not lead to infection unless Icam-1 is also present. It is thought that virions first bind to decay-accelerating protein and then interact with Icam-1 to allow cell entry. A similar process, with different receptors and coreceptors, is likely to occur during infection with other enteroviruses.

The Cell Receptor for Influenza Virus, Sialic Acid

The family *Orthomyxoviridae* comprises the three genera of influenza viruses, A, B, and C. Influenza A viruses are the best-studied members of this family. This virus binds to negatively charged, terminal sialic acid moieties present in oligosaccharide chains that are covalently attached to cell surface glycoproteins or glycolipids. The presence of sialic acid on most cell surfaces accounts for the ability of influenza virions to attach to many types of cell. The interaction of influenza virus with individual sialic acid moieties is of low affinity. However, the opportunity for multiple interactions among the numerous hemagglutinin (HA) molecules on the surface of the virion and multiple sialic acid residues on cellular glycoproteins and glycolipids results in a high overall avidity of the virus particle for the cell surface. The surfaces of influenza viruses were shown in the early 1940s to contain an enzyme that, paradoxically, removes the receptors for attachment from the surface of cells. Later this enzyme was identified as the virus-encoded envelope protein neuraminidase, which cleaves the glycoside linkages of sialic acids (Fig. 5.4). This enzyme is required for release of virions bound to the surfaces of infected cells, facilitating virus spread through the respiratory tract (Volume II, Chapter 9).

Glycolipids, Unusual Cell Receptors for Polyomaviruses

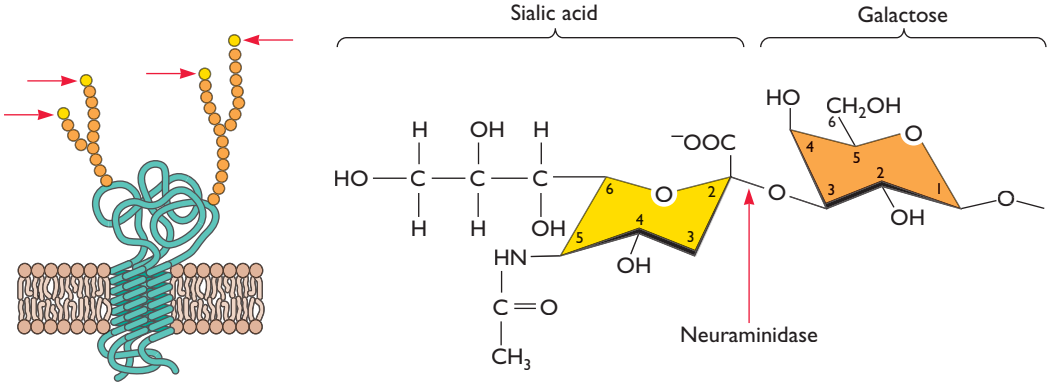
The family *Polyomaviridae* includes simian virus 40, mouse polyomavirus, and human BK virus. These viruses are unusual because they bind to ganglioside cell receptors. Gangliosides are glycosphingolipids with one or more sialic acids linked to a sugar chain. There are over 40 known gangliosides, which differ in the position and number of sialic acid residues. Simian virus 40, polyomavirus, and BK virus bind to three different types of ganglioside. Structural studies have revealed that sialic acid linked to galactose by an α(2,3) linkage binds to a pocket on the surface of the polyomavirus capsid. Gangliosides are highly concentrated in lipid rafts (Chapter 2, Box 2.1) and participate in signal transduction, two properties that play roles during polyomavirus entry into cells.

CD4, the Cell Receptor for Human Immunodeficiency Virus Type 1

Animal retroviruses have long been of interest because of their ability to cause a variety of serious diseases, especially cancers (caused by oncogenic retroviruses) and neurological disorders (caused by lentiviruses). The worldwide acquired immunodeficiency syndrome (AIDS) epidemic has focused enormous attention on the lentivirus human immunodeficiency virus type 1 and its close relatives. The cell surface receptors of this virus have been among the most intensively studied and currently are the best understood.

The cell receptor for human immunodeficiency virus type 1 is CD4 protein, a 55-kDa rodlike molecule that is a member of the Ig superfamily and has four Ig-like domains. A variety of techniques have been used to identify the site of interaction with human immunodeficiency virus type 1, including site-directed mutagenesis and X-ray crystallographic studies of a complex of CD4 and the viral attachment protein SU (Fig. 5.5). The interaction site

Figure 5.4 Sialic acid receptors for influenza viruses. An integral membrane glycoprotein is shown at left; the arrows point to terminal sialic acid units that are attachment sites for influenza virus. The structure of a terminal sialic acid moiety that is recognized by the viral envelope protein hemagglutinin is shown at right. Sialic acid is attached to galactose by an α(2,3) linkage in the example shown; certain HA subtypes preferentially bind to molecules with an α(2,6) linkage. The site of cleavage by the influenza virus envelope protein neuraminidase is indicated. The sialic acid shown is *N*-acetylneuraminic acid, which is the preferred receptor for influenza A and B viruses. These viruses do not bind to 9-*O*-acetyl-*N*-neuraminic acid, the receptor for influenza C viruses.



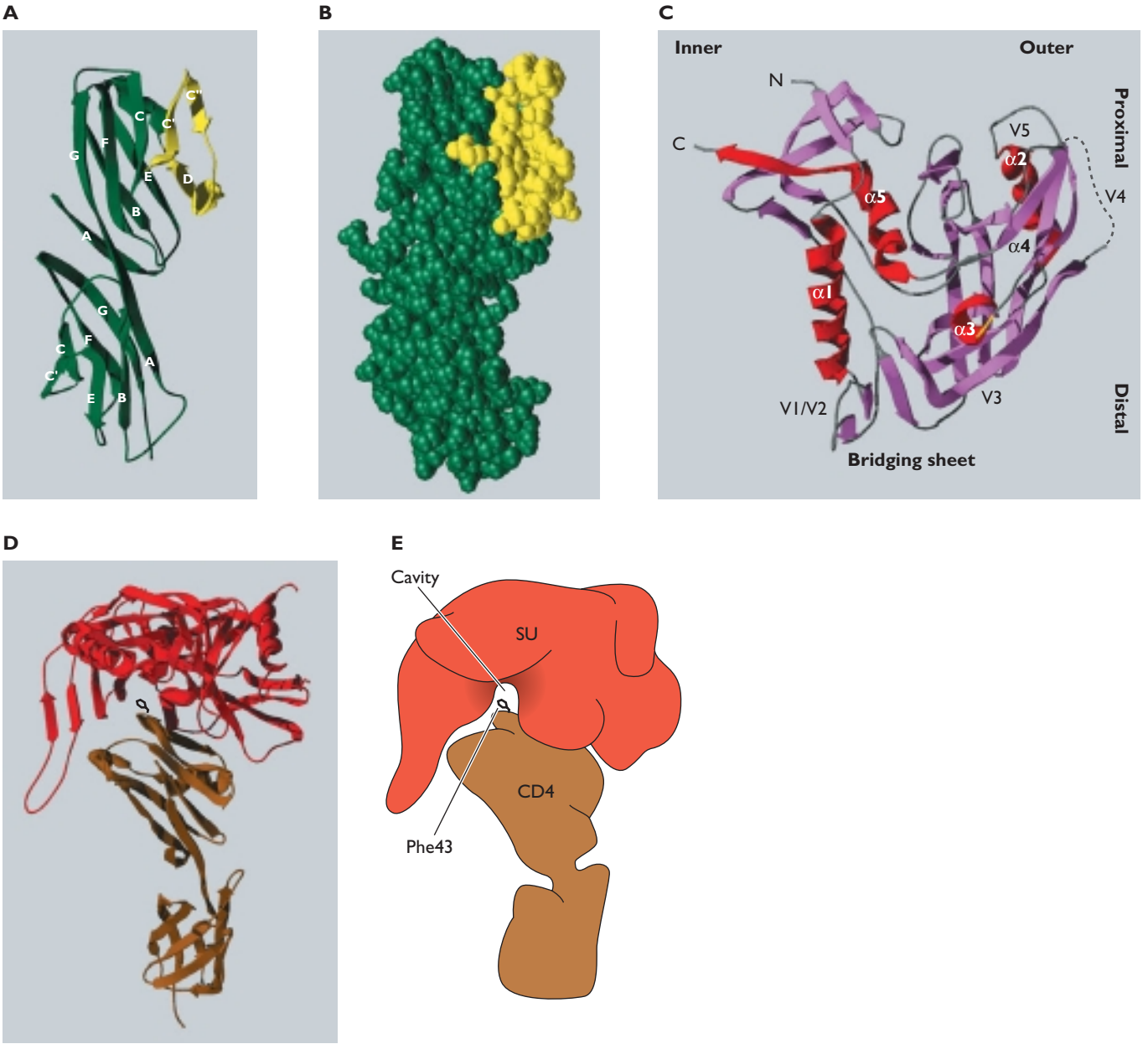


Figure 5.5 Interaction of human immunodeficiency virus type 1 SU with its cell receptor, CD4. **(A)** Ribbon model of the backbone carbons of CD4 domains 1 and 2 (residues 1 to 182), derived from X-ray crystallography. **(B)** Space-filling model of CD4. Shown in yellow in panels A and B are residues that interact with human immunodeficiency virus type 1 during attachment, as revealed by mutagenesis. **(C)** Ribbon diagram of a core of SU, derived from X-ray crystallographic data. This modified SU binds CD4 with an affinity comparable to that of the native protein. α-helices are red, β-strands are magenta, and β-strand 15, which forms an antiparallel β-sheet with strand C'' of CD4, is yellow. **(D)** Ribbon diagram of SU (red) bound to CD4 (brown), derived from X-ray crystallographic data. The side chain of CD4 Phe43 is shown. **(E)** Cartoon of the CD4-SU complex. Mutagenesis has identified CD4 Phe43 as a residue critical for binding to SU. Phe43 is shown penetrating the hydrophobic cavity of SU. This amino acid, which makes 23% of the interatomic contacts between CD4 and SU, is at the center of the interface and appears to stabilize the entire complex. Adapted from J. Wang et al., *Nature* **348:**411–418, 1990 (A and B), and P. D. Kwong et al., *Nature* **393:**648–659, 1998 (D), with permission.

for SU in domain 1 of CD4 is in a region analogous to the site in CD155 that binds to poliovirus. Because two viruses with entirely different virion architectures bind to analogous surfaces of these Ig-like domains, some feature of this region seems likely to be especially advantageous for virus attachment.

One of the first strategies to be considered for the treatment of AIDS was the use of soluble CD4 protein, which lacks the membrane-spanning domain, to inhibit viral infection. The rationale for such treatment was that soluble CD4 should bind virus particles and block their attachment to CD4 on host cell surfaces. Although inhibition of viral infectivity could be demonstrated in cell culture experiments, clinical trials gave disappointing results. This failure can be attributed in part to the fact that each viral envelope includes many copies (~30) of the structure that binds CD4. Consequently, relatively high concentrations in serum would be required to block all of them. This problem was further compounded by the short half-life of soluble CD4 in the blood. Furthermore, human immunodeficiency virus can also be spread from cell to cell by fusion, a process that is not readily blocked by circulating, soluble CD4.

Cell Receptors for Adenoviruses

The results of competition experiments indicated that members of two different virus families, group B coxsackieviruses and adenoviruses, share a cell receptor. This receptor is a 46-kDa member of the Ig superfamily called Car (Coxsackievirus and adenovirus receptor). Binding to this receptor is not sufficient for infection by most adenoviruses. Interaction with a coreceptor, the α_v integrin $\alpha_v\beta_3$ or $\alpha_v\beta_5$, is required for uptake of the capsid into the cell by receptor-mediated endocytosis. An exception is adenovirus type 9, which can infect hematopoietic cells after binding directly to α_v integrins. Adenoviruses of subgroup B bind CD46, which is also a cell receptor for some strains of measles virus, an enveloped member of the *Paramyxoviridae*.

Cell Receptors for Alphaherpesviruses

The alphaherpesvirus subfamily of the *Herpesviridae* includes herpes simplex virus types 1 and 2, pseudorabies virus, and bovine herpesvirus. Initial contact of these viruses with the cell surface is made by low-affinity binding to glycosaminoglycans (preferentially heparan sulfate), abundant components of the extracellular matrix. This interaction concentrates virus particles near the cell surface and facilitates subsequent attachment to an integral membrane protein, which is required for entry into the cell. Members of at least two different protein families serve as entry receptors for alphaherpesviruses. One of these families, the nectins, comprises the poliovirus receptor CD155 and related proteins, yet another example of receptors shared by different viruses. When members of these two protein families are not present, 3-O-sulfated heparan sulfate can serve as an entry receptor for alphaherpesviruses.

Alternative Receptors

Some examples of the use of alternative cell surface molecules as receptors for the same virus have already been discussed. Two additional examples illustrate how receptor usage depends on the nature of the virus isolate or the cell line. Infection with foot-and-mouth disease virus type A12 requires the RGD-binding integrin $\alpha_v\beta_3$. However, the receptor for the O strain of foot-and-mouth disease virus, which has been extensively passaged in cell culture, is not integrin $\alpha_v\beta_3$ but cell surface heparan sulfate. On the other hand, the type A12 strain cannot infect cells that lack integrin $\alpha_v\beta_3$, even if heparan sulfate is present. In a similar way, adaptation of Sindbis virus to cultured cells has led to the selection of variants that bind heparan sulfate. When cell receptors are rare, viruses that can bind to the more abundant glycosaminoglycan are readily selected.

How Virions Attach to Receptors

Animal viruses have multiple receptor-binding sites on their surfaces. Of necessity, one or more of the capsid proteins of nonenveloped viruses specifically interacts with the cell receptor. Receptor-binding sites for enveloped viruses are surface glycoproteins that have been incorporated into their cell-derived membranes. Although the details vary among viruses, most virus-receptor interactions follow one of several mechanisms illustrated by the best-studied examples described below.

Nonenveloped Viruses Bind via the Capsid Surface or Projections

Attachment via surface features: canyons and loops. The RNA genomes of picornaviruses are protected by capsids made up of four virus-encoded proteins, VP1, VP2, VP3, and VP4, arranged with icosahedral symmetry (see Fig. 4.12). Three-dimensional structures have been determined by X-ray crystallography for at least one member of each of the five picornavirus genera. The picornavirus capsid is built from 60 subunits arranged as 12 pentamers (see Fig. 4.12A). Each subunit contains the four capsid proteins in an identical arrangement, with portions of the first three exposed on the surface. Although the arrangement is similar, the surface architecture of the three exposed proteins varies among the family members, a property that accounts for the different serotypes and the different modes of interaction that can take place with cell receptors. For example, the capsids of rhinoviruses

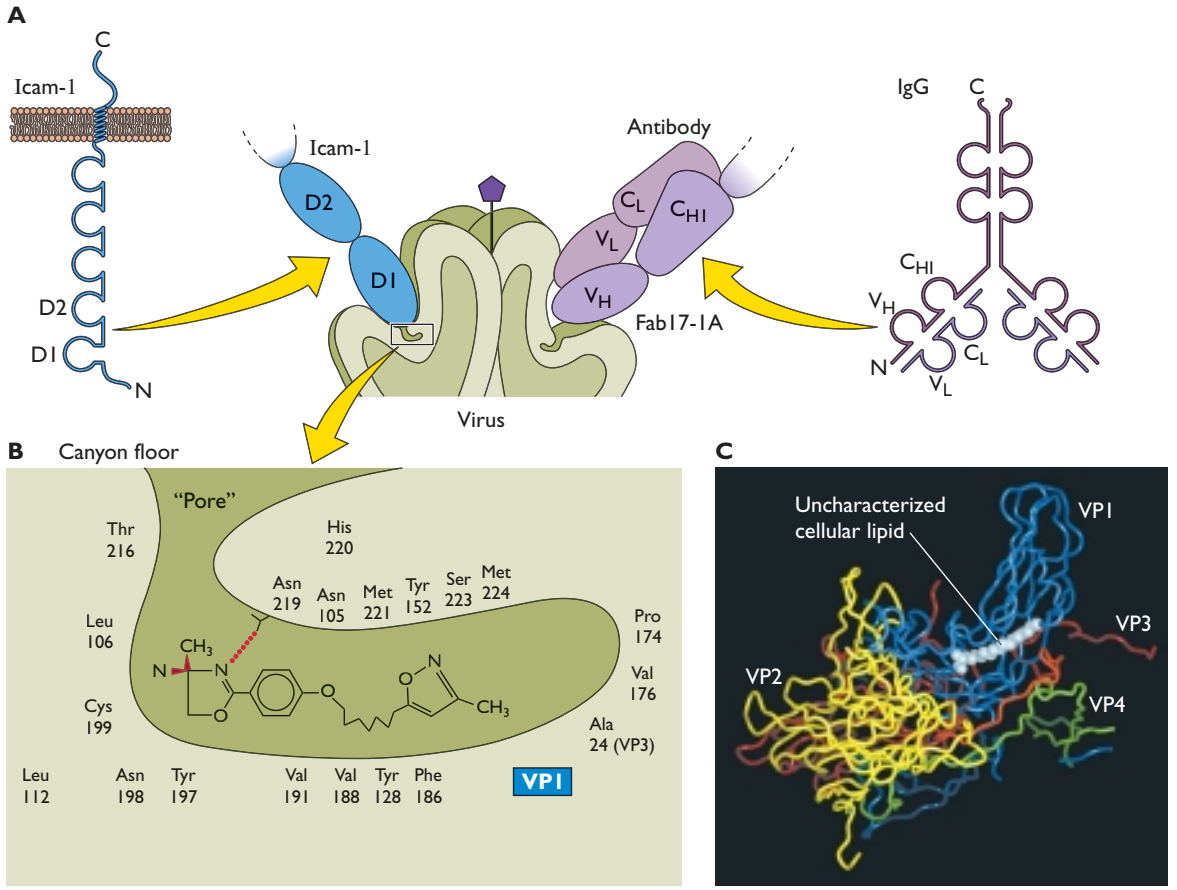


Figure 5.6 Receptor, antibody, and drug binding to the picornavirus capsid. (A) Schematic diagram of the canyon in the human rhinovirus capsid. The domain structure of the cell receptor Icam-1 is illustrated at the left, and the model in the center shows the tip of domain 1 dipping into the canyon. The Fab portion of the antibody contacts a good deal of the canyon but not residues at the deepest regions. Antibodies that bind to the virus in this manner neutralize viral infectivity by blocking entry of receptor into the canyon. **(B)** Location of a WIN compound in a hydrophobic pocket below the canyon floor. **(C)** Location of lipid, possibly sphingosine, in the capsid of poliovirus type 1. Shown is a protomer consisting of one copy of VP1 (blue), VP2 (yellow), VP3 (red), and VP4 (green). The lipid, shown as gray spheres, is bound in the hydrophobic tunnel beneath the canyon floor. Adapted from T. Smith et al., *Nature* **383:**350–354, 1996 (A), and J. Badger et al., *Proc. Natl. Acad. Sci. USA* **85:**3304–3308, 1988 (B), with permission.

and some enteroviruses such as poliovirus have deep canyons surrounding the 12 fivefold axes of symmetry (Fig. 5.3), whereas cardioviruses and aphthoviruses lack this feature.

The canyons in the capsids of some rhinoviruses and enteroviruses are the sites of interaction with cell surface receptors (Fig. 5.3). Amino acids that line the canyons are more highly conserved than any others on the viral surface, and their substitution can alter the affinity of binding to cells. Poliovirus bound to a receptor fragment comprising CD155 domains 1 and 2 has been visualized in reconstructed images from cryo-electron microscopy. The results indicate that the first domain of CD155 binds to the central portion of the canyon in an orientation oblique to the surface of the virion (Fig. 5.3B).

Although canyons are present in the capsid of rhinovirus type 2, they are not the binding sites for the cellular receptor, low-density lipoprotein receptor. Rather, the binding site on the capsid is located on the star-shaped plateau at the fivefold axis of symmetry (Fig. 5.3C). Sequence and structural comparisons have revealed why different rhinovirus serotypes bind distinct receptors. The key amino acid that interacts with a negatively charged cluster of low-density lipoprotein receptor is a lysine of VP1 conserved in all rhinoviruses that bind this receptor. This lysine is not found in VP1 of rhinoviruses that bind Icam-1.

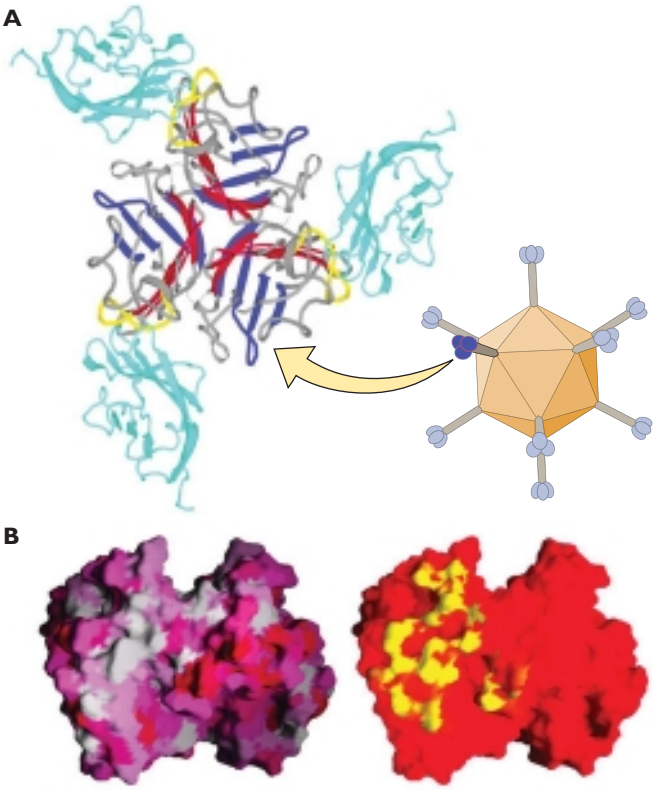


Figure 5.7 Structure of the adenovirus 12 knob complexed with the Car receptor. **(A)** Ribbon diagram of the knob-Car complex as viewed down the axis of the viral fiber. The trimeric knob is in the center. The AB loop of the knob protein, which contacts Car, is in yellow. The first Ig-like domains of three Car molecules bound to the knob are colored blue. **(B)** Surface models of the interface between the knob and Car domain 1. Both models depict two knob monomers and are viewed looking down at the interface with Car. (Left) Conservation of amino acids among all known adenovirus knob protein sequences is represented by a color scale from white (conserved amino acids) to red (nonconserved amino acids). The white strip of conserved amino acids is covered when Car is bound. (Right) Amino acids are colored according to whether they do (yellow) or do not (red) contact Car. From M. C. Bewley et al., *Science* **286:**1579–1583, 1999, with permission.

The canyons of picornaviruses were at first thought to be too deep and narrow to permit entry of antibody molecules with adjacent Ig domains. It was hypothesized that this physical barrier would allow amino acids crucial for receptor interactions to be hidden from the immune system. However, X-ray crystallographic analyses of a specific rhinovirus-antibody complex have shown that the antibody penetrates deep into the canyon, as does Icam-1: the shape of the canyon is not likely to play a role in immune escape (Fig. 5.6).

For picornaviruses with capsids that do not have prominent canyons, including coxsackievirus A and foot-and-mouth disease virus, attachment is to VP1 surface loops that include RGD motifs recognized by their integrin receptors. Alteration of the RGD sequence in VP1 of foot-and-mouth disease virus blocks virus binding.

Attachment via protruding fibers. The nonenveloped DNA-containing adenoviruses are much larger than picornaviruses, and their icosahedral capsids are more complex, comprising at least 10 different proteins. Electron microscopy shows that adenovirus particles have fibers protruding from each pentamer (Fig. 5.7; see the appendix, Fig. 1A). The fibers are composed of homotrimers of the adenovirus fiber protein and are anchored in the pentameric penton base protein; both proteins have roles to play in virus attachment and uptake.

For many adenovirus serotypes, attachment via the fibers is necessary but not sufficient for infection. A region comprising the N-terminal 40 amino acids of each subunit of the fiber protein is bound noncovalently to the penton base. The central shaft region is composed of repeating

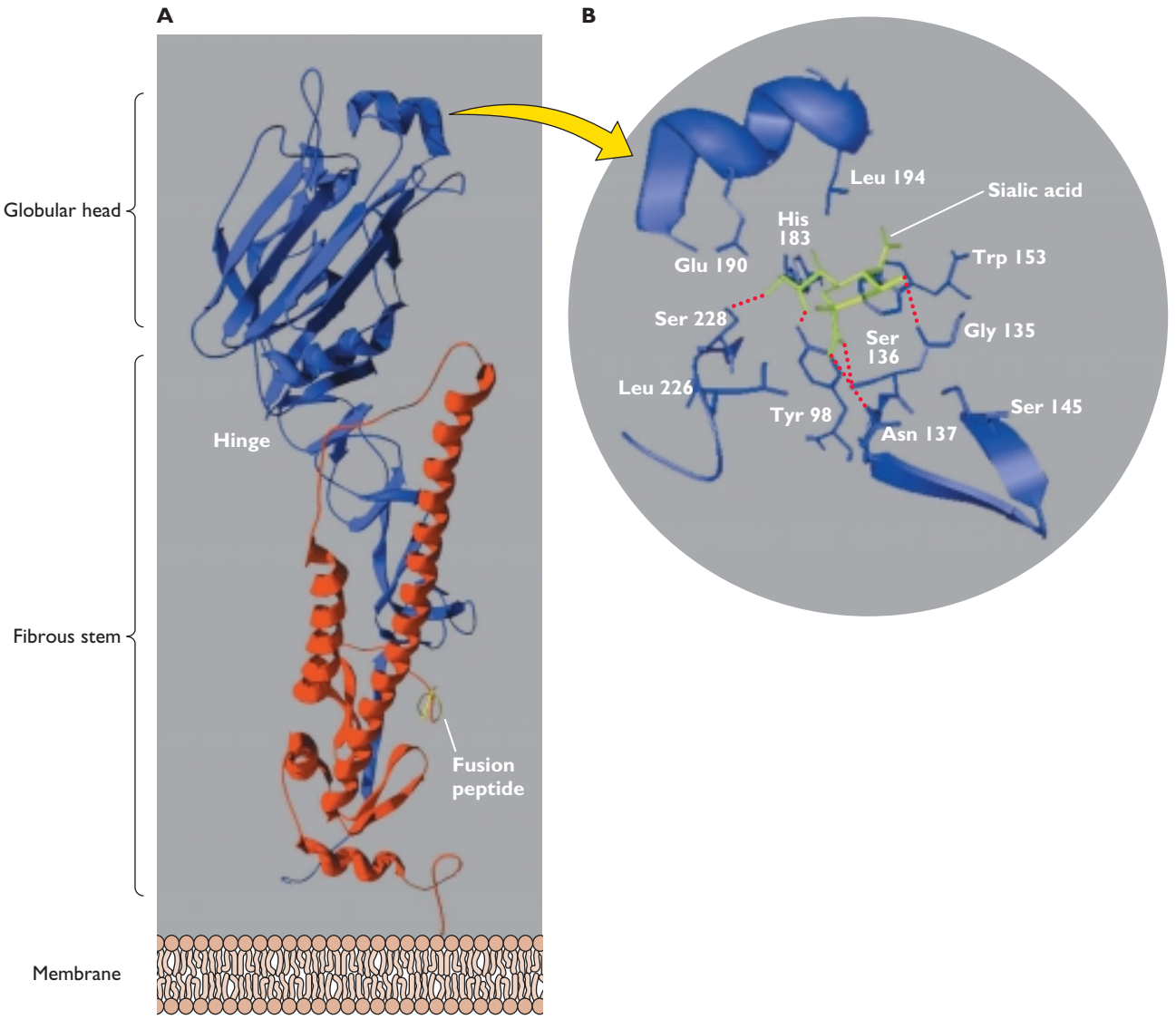


Figure 5.8 Structure of a monomer of influenza virus HA protein and details of the receptor-binding site. (A) HA monomer modeled from the X-ray crystal structure of the natural trimer. HA1 (blue) and HA2 (red) subunits are held together by a disulfide bridge as well as by many noncovalent interactions. The fusion peptide at the N terminus of HA2 is indicated (yellow). **(B)** Close-up of the receptor-binding site with a bound sialic acid molecule. Side chains of the conserved amino acids that form the site and hydrogen-bond with the receptor are included.

motifs of approximately 15 amino acids; the length of the shaft in different serotypes is determined by the number of these repeats. The three constituent shaft regions appear to form a rigid triple-helical structure in the trimeric fiber. The C-terminal 180 amino acids of each subunit interact to form a terminal knob. Genetic analyses and competition experiments indicate that determinants for the initial, specific attachment to host cell receptors reside in this knob. The structure of this receptor-binding domain bound to Car reveals that surface loops of the knob contact one face of Car (Fig. 5.7).

Enveloped Viruses Bind via Transmembrane Glycoproteins

As noted above, the lipid membranes of enveloped viruses originate from the cells they infect. The process of

virion assembly includes insertion into membranes of specific viral proteins that carry membrane-spanning domains analogous to those of cellular integral membrane proteins. Attachment sites (i.e., viral ligands) on one or more of these envelope proteins bind to specific cell receptors. The two best-studied examples of enveloped virus attachment and its consequences are provided by the interactions of influenza A virus and the retrovirus human immunodeficiency virus type 1 with their cell receptors.

Influenza virus HA. Influenza virus HA is the viral glycoprotein that binds to the cell receptor sialic acid. The HA monomer is synthesized as a precursor that is glycosylated and subsequently cleaved to form HA1 and HA2 subunits. Each HA monomer consists of a long, helical stalk anchored in the membrane by HA2 and topped by a large HA1 globule, which includes the sialic acid-binding pocket (Fig. 5.8). While attachment of all influenza A virus strains requires sialic acid, strains vary in their affinities for different sialyloligosaccharides. For example, human virus strains are preferentially bound by sialic acids attached to galactose via an α(2,6) linkage, the major sialic acid present on human respiratory epithelium (Fig. 5.4). Avian virus strains bind preferentially to sialic acids attached to galactose via an α(2,3) linkage, the major sialic acid in the duck gut epithelium. Amino acids in the sialic acid-binding pocket of HA (Fig. 5.8) determine which sialic acid is preferred and can therefore determine viral host range. An example is the origin of the 1918 influenza virus strain, which may have evolved from an avian virus. It is thought that an amino acid change in the sialic acid-binding pocket of the avian HA allowed it to recognize the α(2,6)-linked sialic acids that predominate in human cells.

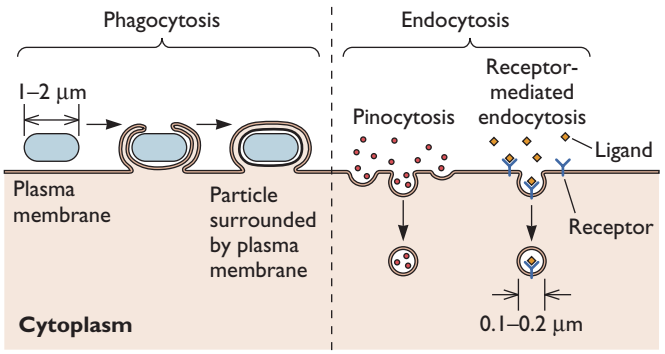
The envelope glycoprotein of human immunodeficiency virus type 1. When examined by electron microscopy, the envelopes of human immunodeficiency virus type 1 and other retroviruses appear to be studded with "spikes" (see Fig. 4.19). These structures are composed of trimers of the single viral envelope glycoprotein. The spikes bind the cell receptor CD4 (Fig. 5.5). The monomers of the spike protein are synthesized as heavily glycosylated precursors that are cleaved by a cellular protease to form SU and TM. The latter is anchored in the envelope by a single membrane-spanning domain and remains bound to SU by numerous noncovalent bonds.

The atomic structure of a complex of human immunodeficiency virus type 1 SU, a two-domain fragment of CD4, and a neutralizing antibody against SU has been determined by X-ray crystallography (Fig. 5.5). The polypeptide of SU is folded into an inner and an outer domain linked by an antiparallel four-stranded "bridging sheet." A depression at the interface of the outer and inner domains and the bridging sheet forms the binding site for CD4. The CD4-binding site in SU is a deep cavity, and the opening of this cavity is occupied by CD4 amino acid Phe43, which is critical for SU binding. Comparison with the structure of SU in the absence of CD4 indicates that receptor binding induces conformational changes in SU. These changes expose binding sites on SU for the chemokine receptors, which are required for fusion of viral and cell membranes (see "Uncoating at the Plasma Membrane" below).

Endocytosis of Virions by Cells

Many viruses enter cells by the same pathways by which cells take up macromolecules. The plasma membrane, the limiting membrane of the cell, permits nutrient molecules to enter and waste molecules to leave, thereby ensuring an appropriate internal environment. Water, gases, and small hydrophobic molecules such as ethanol can freely traverse the lipid bilayer, but most metabolites and certain ions (Ca^{2+}, H^+, K^+, and Na^+) cannot diffuse through the membrane. These essential components enter the cell by specific transport processes. Integral membrane proteins are responsible for the transport of ions, sugars, and amino acids, while proteins and large particles are taken into the cell by phagocytosis or endocytosis. The former process (Fig. 5.9) is nonspecific, which means that any particle or molecule can be taken into the cell.

Figure 5.9 Mechanisms for the uptake of macromolecules from extracellular fluid. During phagocytosis, large particles such as bacteria or cell fragments that come in contact with the cell surface are engulfed by extensions of the plasma membrane. Phagosomes ultimately fuse with lysosomes, resulting in degradation of the material within the vesicle. Macrophages use phagocytosis to ingest bacteria and destroy them. Endocytosis comprises the invagination and pinching off of small regions of the plasma membrane, resulting in the nonspecific internalization of molecules (pinocytosis or fluid-phase endocytosis) or the specific uptake of molecules bound to cell surface receptors (receptor-mediated endocytosis). Adapted from J. Darnell et al., *Molecular Cell Biology* (Scientific American Books, New York, NY, 1986), with permission.



Specific molecules are selectively taken into cells from the extracellular fluid by **receptor-mediated endocytosis** (Fig. 5.9 and 5.10); this is also the mechanism of entry of many viruses. Ligands in the extracellular medium bind to cells via specific plasma membrane receptor proteins. The receptor-ligand complex diffuses along the membrane until it reaches an invagination that is coated on its cytoplasmic surface by a cagelike lattice composed of the fibrous protein clathrin (Fig. 5.10). Such clathrin-coated pits can comprise as much as 2% of the surface area of a cell, and some receptors are clustered over these areas even in the absence of their ligands. Following the accumulation of receptor-ligand complexes, the clathrin-coated pit invaginates and then pinches off to form a clathrin-coated vesicle containing the ligand-receptor complex. Within a few seconds, the clathrin coat is lost and the vesicles fuse with small, smooth-walled vesicles located near the cell surface, called early **endosomes.** The lumen of early endosomes is mildly acidic (pH 6.5 to 6.0), a result of energy-dependent transport of protons into the interior of the vesicles by a membrane proton pump. The contents of the early endosome are then transported via endosomal carrier vesicles to late endosomes located close to the nucleus. The lumen of late endosomes is more acidic (pH 6.0 to 5.0). Some ligands dissociate from their receptors in the acidic environment of the endosome, and the receptors are recycled to the cell surface by transport vesicles that bud from the endosome and fuse with the plasma membrane. Late endosomes in turn fuse with **lysosomes,** which are vesicles containing a variety of enzymes that degrade sugars, proteins, nucleic acids, and lipids. Ligands that reach the lysosomes are degraded by enzymes for further use of their constituents. In some cases, the entire ligand-receptor complex travels to the lysosomal compartment, where it is degraded. Viruses usually enter the cytoplasm from the early or late endosomes, and a few enter from lysosomes.

Clathrin-mediated endocytosis is a continuous but regulated process. For example, the uptake of vesicular stomatitis virus into cells may be influenced by over 90 different cellular protein kinases. Influenza virus and reovirus particles are taken into cells, not into preexisting pits but mainly by clathrin-coated pits that form after virus binds to the cell surface. It is not known how virus binding to the plasma membrane induces the formation of the clathrin-coated pit.

Although uptake of most viruses occurs by the clathrin-mediated endocytic pathway, other pathways are also involved. These include caveolin- (or raft-mediated) and clathrin-independent endocytosis (Fig. 5.10). The caveolar pathway requires cholesterol (a major component of lipid rafts). Three types of caveolar endocytosis have been identified. Endocytosis by caveolin 1-containing **caveolae** is observed in cells infected with simian virus 40 and polyomavirus. Dynamin 2-dependent, noncaveolar, raft-mediated endocytosis occurs during echovirus and rotavirus infection, while dynamin-independent, noncaveolar, raft-mediated endocytosis is also observed during simian virus 40 and polyomavirus infection. Caveolae are distinguished from clathrin-coated vesicles by their flask-like shape, their size (50 to 70 nm in diameter), the absence of a clathrin coat, and the presence of a marker protein called caveolin. In the uninfected cell, caveolae participate in transcytosis, signal transduction, and uptake of membrane components and extracellular ligands. When a virus particle binds the caveolae, a signal transduction pathway involving tyrosine phosphorylation is activated. Such signaling is required for pinching off of the vesicle, which then moves within the cytoplasm. Disassembly of filamentous actin also occurs, presumably to facilitate movement of the vesicle deeper into the cytoplasm. There it fuses with the **caveosome**, a larger membranous organelle that contains caveolin (Fig. 5.10). In contrast to endosomes, the pH of the caveosome lumen is neutral. Some viruses (e.g., echovirus type 1) penetrate the cytoplasm from the caveosome. Others (simian virus 40, polyomavirus, coxsackievirus B3) are sorted to the endoplasmic reticulum by a transport vesicle that lacks caveolin. These viruses enter the cytoplasm by a process mediated by thiol oxidases present in the lumen of the endoplasmic reticulum and by a component of the protein degradation pathway present in the membrane.

The study of virus entry by endocytosis can be confusing because some viruses may enter cells by multiple routes, depending on cell type and multiplicity of infection. For example, herpes simplex virus can enter cells by three different routes and influenza A virus may enter cells by both clathrin-dependent and clathrin-independent pathways.

Membrane Fusion

The formation of vesicles during the process of endocytosis requires the fusion of cell membranes. For example, during endocytosis, fusion produces the intracellular vesicle following invagination of a small region of the plasma membrane (Fig. 5.10). Membrane fusion also takes place during many other cellular processes, such as cell division, myoblast fusion, and exocytosis.

Membrane fusion must be regulated in order to maintain the integrity of the cell and its intracellular compartments. Consequently, membrane fusion does not occur spontaneously but proceeds by specialized mechanisms mediated by proteins. The two membranes must first come into close proximity. This reaction is mediated by interactions of integral membrane proteins that protrude from the lipid bilayers, a targeting protein on one membrane and a docking protein on the other. The next step, fusion,

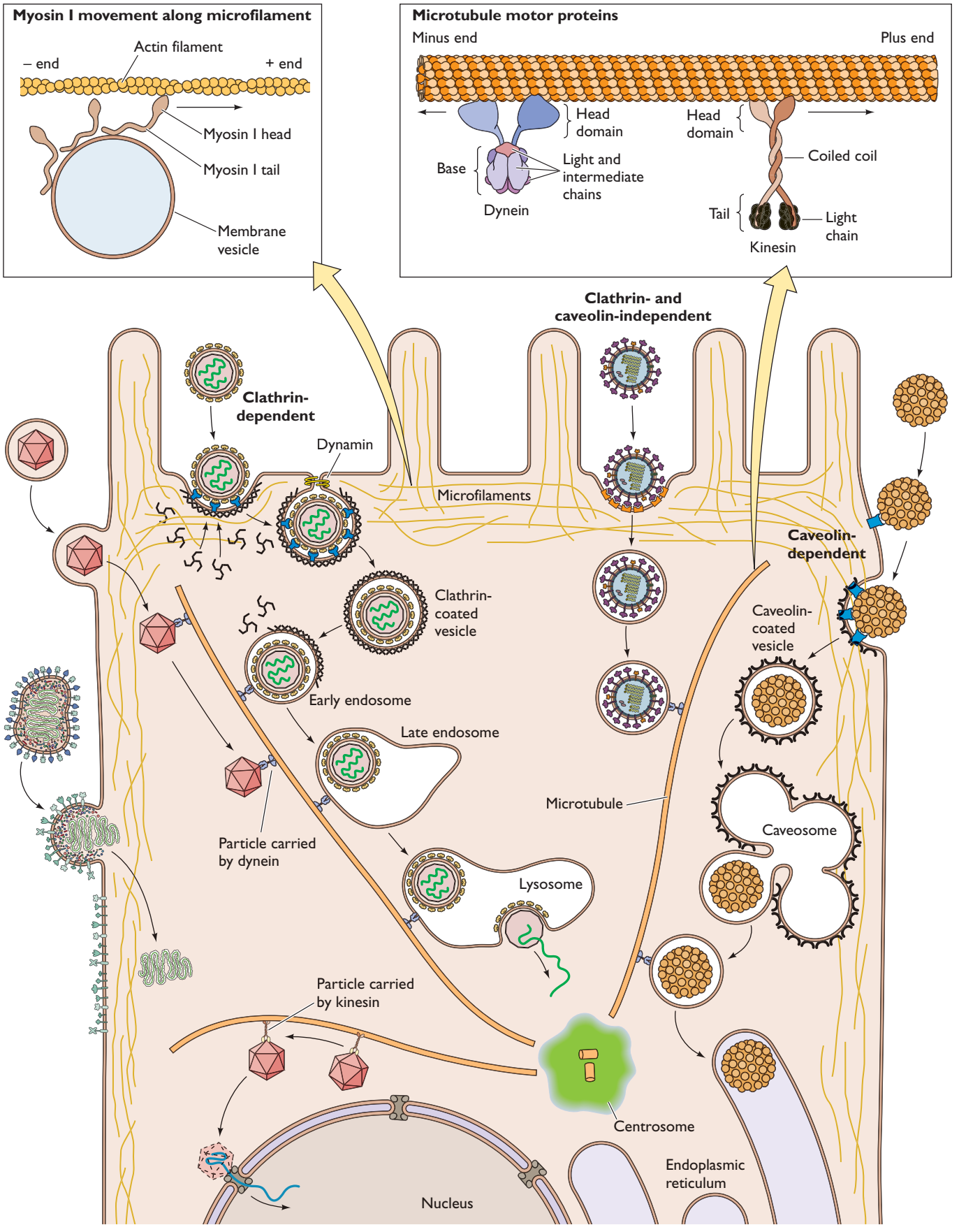

Myosin I movement along microfilament
Actin filament
– end
+ end
Myosin I head
Myosin I tail
Membrane vesicle
Microtubule motor proteins
Minus end
Plus end
Head domain
Base
Light and intermediate chains
Dynein
Head domain
Coiled coil
Tail
Light chain
Kinesin
Clathrin- and caveolin-independent
Clathrin-dependent
Dynamin
Microfilaments
Caveolin-dependent
Clathrin-coated vesicle
Caveolin-coated vesicle
Early endosome
Late endosome
Microtubule
Particle carried by dynein
Caveosome
Lysosome
Particle carried by kinesin
Centrosome
Endoplasmic reticulum
Nucleus

Figure 5.10

requires an even closer approach of the membranes, to within 1.5 nm of each other. This step depends on the removal of water molecules from the membrane surfaces, an energetically unfavorable process. A multisubunit protein complex is thought to provide the energy required for such a close approach in mammalian cells. The complex formed by targeting and docking proteins recruits additional proteins that induce fusion of the two membranes. After fusion occurs, the complex dissociates until needed once again. As individual components of the complex lack fusion activity, fusion can be regulated by assembly and disassembly.

The precise mechanism by which lipid bilayers fuse is not completely understood, but the action of fusion proteins is thought to result in the formation of an opening called a **fusion pore**, allowing exchange of material across the membranes. Much of our understanding about membrane fusion reactions comes from studies using individual viral proteins that promote membrane fusion (Box 5.4). Membrane fusion reactions catalyzed by such proteins appear to be less complex than those mediated by cellular proteins, for in most cases a single viral gene product is sufficient. This simplicity may be a consequence of the fact that abundant quantities of viral fusion proteins are produced during infection. Cell fusion proteins are far less abundant and must therefore be recycled, a requirement that is best accomplished by assembling and disassembling a multisubunit protein complex.

The membranes of enveloped viruses fuse with those of the cell as a first step in delivery of the viral nucleic acid. Viral fusion may occur either at the plasma membrane or from within an endosome or other vesicle. The membranes of the virus and the cell are first brought into close contact by interaction of a viral glycoprotein with a cell receptor. The same viral glycoprotein, or a different viral integral membrane protein, then catalyzes the fusion of the juxtaposed membranes. As described in the following sections, virus-mediated fusion must be regulated to prevent viruses from aggregating or to ensure that fusion does not occur in the incorrect cellular compartment. In some cases, fusogenic potential is masked until the fusion protein interacts with other integral membrane proteins. In others, low pH is required to expose fusion domains. The activity of fusion proteins may also be regulated by cleavage of a precursor. This requirement probably prevents premature activation of fusion potential during virus assembly. Cleavage also generates the metastable states of viral glycoproteins that can subsequently undergo the conformational rearrangements required for fusion activity.

Movement of Virions and Subviral Particles within Cells

Virions and subviral particles move within the host cell during entry and egress (Chapters 12 and 13). However, movement of molecules larger than 500 kDa does not occur by passive diffusion, because the cytoplasm is crowded with organelles, high concentrations of proteins, and the cytoskeleton (Box 5.5). Rather, viruses and their components are transported via the actin and microtubule cytoskeletons. Such movement can be visualized in live cells by using fluorescently labeled virions (Chapter 2).

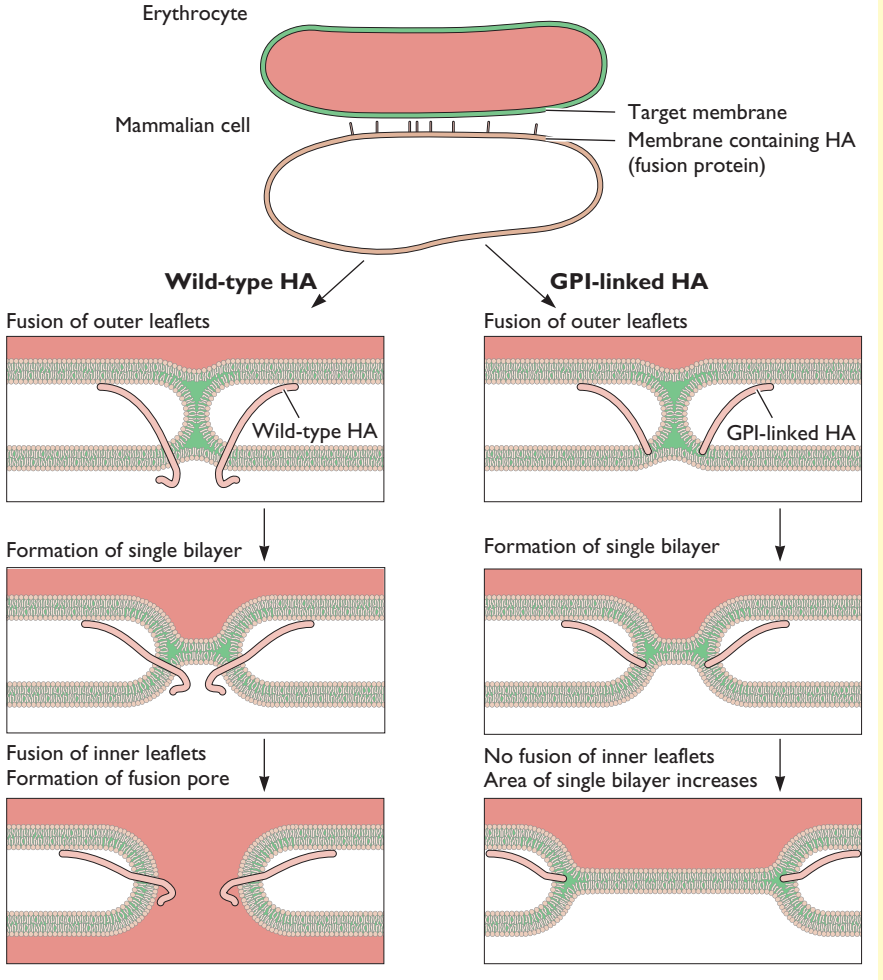
The cytoskeleton is a dynamic network of protein filaments that extends throughout the cytoplasm. It is composed of three types of filament—microtubules, intermediate filaments, and microfilaments (Fig. 5.10).

Figure 5.10 Virus entry and movement in cells. Examples of genome uncoating at the plasma membrane are shown on the left side of the cell. Fusion at the plasma membrane releases the nucleocapsid into the cytoplasm. In some cases, the subviral particle is transported on microtubules toward the nucleus, where the nucleic acid is released. Uptake of virions by clathrin-dependent endocytosis commences with binding to a specific cell surface receptor. The ligand-receptor complex diffuses into an invagination of the plasma membrane coated with the protein clathrin on the cytosolic side (clathrin-coated pits). The coated pit further invaginates and pinches off, a process that is facilitated by the GTPase dynamin. The resulting coated vesicle then fuses with an early endosome. Endosomes are acidic, as a result of the activity of vacuolar proton ATPases. Virion uncoating ususally occurs from early or late endosomes. Late endosomes then fuse with lysosomes. Virions may enter cells by a dynamin- and caveolin-dependent endocytic pathway (right side of the cell). This pathway brings virions to the endoplasmic reticulum via the caveosome, a pH-neutral compartment. Clathrin- and caveolin-independent endocytic pathways of viral entry have also been described (center of cell). Movement of endocytic vesicles within cells occurs on microfilaments (inset, top left) or microtubules (inset, top right), components of the cytoskeleton. Microfilaments are two-stranded helical polymers of the ATPase actin. They are dispersed throughout the cell but are most highly concentrated beneath the plasma membrane, where they are connected via integrins and other proteins to the extracellular matrix. Transport along microfilaments is accomplished by myosin motors. Microtubules are 25-nm hollow cylinders made of the GTPase tubulin. They radiate from the **centrosome** to the cell periphery. Movement on microtubules is carried out by kinesin and dynein motors. Insets adapted from G. M. Cooper, *The Cell: a Molecular Approach* (ASM Press, Washington, DC, and Sinauer Associates, Sunderland, MA, 1997), with permission.

BOX 5.4 EXPERIMENTS

Membrane fusion proceeds through a hemifusion intermediate

Fusion is thought to proceed through a hemifusion intermediate in which the outer leaflets of two opposing bilayers fuse (see figure), followed by fusion of the inner leaflets and the formation of a fusion pore. Direct evidence that fusion proceeds via a hemifusion intermediate has been obtained with influenza virus HA (see figure). **(Left)** Cultured mammalian cells expressing wild-type HA are fused with erythrocytes containing two different types of fluorescent dye, one in the cytoplasm and one in the lipid membrane. Upon exposure to low pH, HA undergoes conformational change and the fusion peptide is inserted into the erythrocyte membrane. The green dye is transferred from the lipid bilayer of the erythrocyte to the bilayer of the cultured cell. The HA trimers tilt, causing reorientation of the transmembrane domain and generating stress within the hemifusion diaphragm. Fusion pore formation relieves the stress. The red dye within the cytoplasm of the erythrocyte is then transferred to the cytoplasm of the cultured cell. **(Right)** An altered form of HA was produced, lacking the transmembrane and cytoplasmic domains and with membrane anchoring provided by linkage to a glycosylphosphatidylinositol (GPI) moiety. Upon exposure to low pH, the HA fusion peptide is inserted into the erythrocyte membrane, and green dye is transferred to the membranes of the mammalian cell. When the HA trimers tilt, no stress is transmitted to the hemifusion diaphragm because no transmembrane domain is present, and the diaphragm becomes larger. Fusion pores do not form, and there is no mixing of the contents of the cytoplasm, indicating that complete membrane fusion has not occurred. These results prove that hemifusion, or fusion of only the inner leaflet of the bilayer, can occur among whole cells. The findings also demonstrate that the transmembrane domain of the HA polypeptide plays a role in the fusion process.



Glycosylphosphatidylinositol-anchored influenza virus HA induces hemifusion. **(Left)** Model of the steps of fusion mediated by wild-type HA. **(Right)** Effect on fusion by an altered form of HA lacking the transmembrane and cytoplasmic domains. Adapted from G. B. Melikyan et al., *J. Cell Biol.* **131**:679–691, 1995, with permission.

Microtubules are organized in a polarized manner, with minus ends situated at the microtubule-organizing center and plus ends located at the cell periphery. This arrangement permits directed movement of cellular and viral components over long distances. Actin filaments (microfilaments) typically assist in virus movement close to the plasma membrane.

Transport along actin filaments is accomplished by myosin motors, and movement on microtubules is carried out by kinesin and dynein motors (Fig. 5.10). Hydrolysis of adenosine triphosphate (ATP) provides the energy for the motors to move their cargo along cytoskeletal tracks. Dyneins and kinesins participate in movement of viral components during both entry (see "Mechanisms of

BOX 5.5 EXPERIMENTS

Passive diffusion cannot account for intracellular movement of virion components

The crowded cytoplasm of a cell. The image shows the interior of a eukaryotic cell, starting at the cell surface, which is studded with membrane proteins. A small portion of the cytoplasm is shown, illustrating the profusion of cytoskeletal elements, ribosomes, and other small molecules. Adapted from David S. Goodsell (http://www.scripps.edu/pub/goodsell/gallery/patterson.html), with permission.

In aqueous solutions, molecules can move rapidly by diffusion, a process of random motion produced by collision with other molecules in the solution. Under ideal conditions, diffusion coefficients typically range from 10^{-6} to 10^{-8} cm^2/s. However, the intracellular milieu is far from such an ideal: the very high intracellular protein concentrations (up to 300 mg/ml), the presence of numerous organelles, and the cytoskeletal networks (see figure) severely restrict diffusion of molecules with molecular mass greater than 500 kDa. Measurements of diffusion coefficients of beads microinjected into cells, of cytoplasmic vesicles, and of DNA molecules indicate that these values are from 5- to 1,000-fold lower in the cytoplasm than in aqueous solution. As shown in the table, such estimates indicate that viral particles (or the components to be assembled into progeny virions) could not reach the appropriate intracellular destinations by passive diffusion within even a few years, let alone the few hours or days that comprise infectious cycles.

Estimated rates of transport of viral components by diffusion[a]

Viral component	Time to travel 10 μm[b,c]	
	In H_2O (s)	In cytoplasm (h)
Poliovirus capsid	3.85	0.5
Herpes simplex virus nucleocapsid	14.6	2.0
Vaccinia virus intracellular mature virion	35.0	4.9

[a]Adapted from B. Sodeik, *Trends Microbiol.* **8:**465–472, 2000, with permission.

[b]The length of a typical human cell. Note the different timescales for H_2O and cytoplasm.

[c]Diffusion constants were calculated by a formula that considers the radius of the virus particle and the viscosity of water at room temperature. The assumption was made that diffusion constants in the cytoplasm would be 500 times lower than in water.

Uncoating" below) and egress (Chapters 12 and 13). In some cases, the actin cytoskeleton is remodeled during entry and egress, for example, when viruses bud from the plasma membrane.

There are two basic ways for viruses to travel within the cell—within a membrane vesicle such as an endosome, which interacts with the cytoskeletal transport machinery, or directly in the cytoplasm (Fig. 5.10). In the latter case, some form of the virus particle must bind directly to the transport machinery. The cytoplasmic domain of CD155, the cellular receptor for poliovirus, binds the light chain of the motor protein dynein. This interaction might target endocytic vesicles containing CD155 to the microtubule network, allowing transport of the viral capsid in the cytoplasm. After leaving endosomes, the subviral particles derived from adenoviruses and parvoviruses are transported along microtubules to the nucleus. Although adenovirus particles have an overall net movement toward the nucleus, they exhibit bidirectional plus- and minus-end-directed microtubule movement. Adenovirus binding to cells activates two different signal transduction pathways that increase the net velocity of minus-end-directed capsid motility. The signaling pathways are therefore required for efficient delivery of the viral genome to the nucleus. It is not yet known how viral subviral particles are loaded onto and released from the microtubules to move to the nuclear pore complex, where the viral genomes enter the nucleus.

Some viruses move along the surfaces of cells prior to entry, often to locate a clathrin-coated pit. If the cell receptor is rare or inaccessible, virions may first bind to more abundant or accessible receptors, such as carbohydrates, and then migrate to receptors that allow entry into the cell. For example, after binding, polyomavirus particles move laterally ("surf") on the plasma membrane for 5 to 10 s and then are internalized. Virions can be visualized moving along the plasma membrane toward the cell body on **filopodia**, thin extensions of the plasma membrane (Fig. 5.10). Virions move along filopodia by an actin-dependent mechanism. Filopodial bridges mediate cell-to-cell spread of a retrovirus in cultured cells. The filopodia originate from uninfected cells and contact infected cells with their tips. The interaction of the viral envelope glycoprotein on the surface of infected cells with the receptor on uninfected cells stabilizes the interaction.

Virions move along the outside of the filipodial bridge to the uninfected cell. Virion transport is a consequence of actin-based movement of the viral receptor toward the uninfected cell.

The intricate mechanisms by which the genomes of viruses move in eukaryotic cells are in stark contrast to the simple injection of the bacterial genome into the host cell (Box 5.6). During this process, the bacteriophage particle remains on the surface of the bacterium.

Virus-Induced Signaling via Cell Receptors

Binding of virions to cell receptors not only concentrates the particles on the cell surface but also may activate signaling pathways that facilitate virus entry and movement within the cell or produce cellular responses that enhance virus propagation and/or affect pathogenesis.

Signaling triggered by binding of coxsackievirus B3 to its cellular receptor makes receptors accessible for virus entry. The coxsackievirus and adenovirus receptor, Car, is not present on the apical surface of epithelial cells that line the intestinal and respiratory tracts. This membrane protein is a component of tight junctions and is inaccessible to virions. To enter epithelial cells, group B coxsackieviruses bind a receptor, CD55, which is present on the apical surface. Virus binding to CD55 activates Abl kinase, which in turn triggers Rac-dependent actin rearrangements. These changes allow virus movement to the tight junction, where it can bind Car and enter cells.

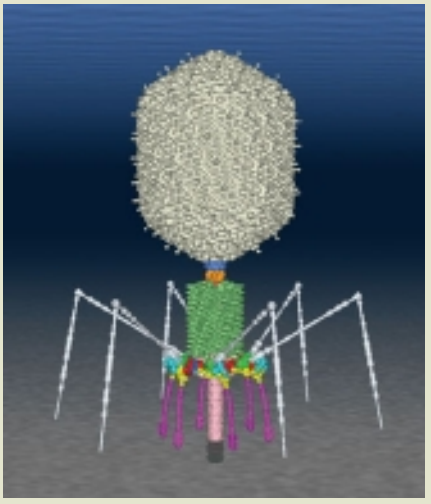
Signaling is essential for the entry of simian virus 40 into cells. Binding of this virus to its glycolipid cell receptor, GM1 ganglioside, causes activation of tyrosine kinases. The signaling that ensues causes reorganization of actin filaments, internalization of the virus in caveolae, and transport of the caveolar vesicles to the endoplasmic reticulum. The activities of nearly 80 cellular protein kinases regulate the entry of this virus into cells.

Interactions between human immunodeficiency virus type 1 SU and CD4 have been implicated in virus-induced cell killing. Both CD4 and human immunodeficiency virus type 1 coreceptor molecules are coupled via their

BOX 5.6 DISCUSSION

The bacteriophage DNA injection machine

The mechanisms by which the bacteriophage genome enters the bacterial host are unlike those for viruses of eukaryotic cells. One major difference is that the bacteriophage particle remains on the surface of the bacterium as the nucleic acid passes into the cell. The DNA genome of some bacteriophages is packaged under high pressure (up to 870 lb/in^2) in the capsid and is injected into the cell in a process that has no counterpart in the entry process of eukaryotic viruses. The complete structure of bacteriophage T4 illustrates this remarkable process (see figure). To initiate infection, the tail fibers attach to receptors (black) on the surface of *Escherichia coli*. Binding causes a conformational change in the baseplate, which leads to contraction of the sheath. This movement drives the rigid tail tube through the outer membrane, using a needle at the tip. When the needle touches the peptidoglycan layer in the periplasm, the needle dissolves and three lysozyme domains in the baseplate are activated. These disrupt the peptidoglycan layer of the bacterium, allowing DNA to enter.



Structure of bacteriophage T4. A model of the 2,000-Å bacteriophage as produced from electron microscopy and X-ray crystallography. Components of the virion are color coded: virion head (beige), tail tube (pink), contractile sheath around the tail tube (green), baseplate (multicolored), and tail fibers (white and magenta). In the illustration, the virion contacts the cell surface, and the tail sheath is contracted prior to DNA release into the cell. From P. G. Leitman et al., *Cell* **118:**419–430, 2004, with permission. Courtesy of Michael Rossmann, Purdue University.

cytoplasmic domains to intracellular signaling pathways. The normal role of CD4 is to bind to the major histocompatibility complex class II-peptide complex on antigen-presenting cells and stabilize its interaction with the T-cell receptor. This interaction leads to activation and differentiation of the T cell by means of a protein kinase ($p56^{lck}$) associated with the cytoplasmic domain of CD4 at the inner leaflet of the plasma membrane (Volume II, Fig. 4.11). The chemokine receptors also signal interaction with their ligand, affecting cellular gene expression. The binding of SU to human $CD4^+$ T cells is followed by signaling through chemokine receptors and induction of apoptosis. It has been reported that interactions between SU and chemokine receptors on neuronal cells induce apoptosis. The destruction of cytotoxic T cells by macrophages has also been attributed to such interactions. Such effects may explain the depletion of cytotoxic T cells and the neurological disorders that are symptoms of AIDS.

Mechanisms of Uncoating

Uncoating is the release of viral nucleic acid from its protective protein coat and/or lipid envelope, although in most cases the liberated nucleic acid is still associated with viral proteins. For enveloped viruses, uncoating occurs when viral and cellular membranes fuse, either at the plasma membrane or within intracellular vesicles. Nonenveloped viruses typically enter the cell by endocytosis, and the genome is released from intracellular transport vesicles or while docked at the nuclear pore complex.

Uncoating at the Plasma Membrane

The particles of many enveloped viruses, including members of the family *Paramyxoviridae* such as Sendai virus and measles virus, fuse directly with the plasma membrane at neutral pH. These virions bind to cell surface receptors via a viral integral membrane protein (Fig. 5.11). Once the viral and cell membranes have been closely juxtaposed by this receptor-ligand interaction, fusion is induced by a second viral glycoprotein known as fusion (F) protein, and the viral nucleocapsid is released into the cell cytoplasm.

F protein is a type I integral membrane glycoprotein (the N terminus lies outside the viral membrane) with similarities to influenza virus HA in its synthesis and structure. It is a homotrimer that is synthesized as a precursor called F0 and cleaved during transit to the cell surface by a host cell protease to produce two subunits, F1 and F2, held together by disulfide bonds. The newly formed N-terminal 20 amino acids of the F1 subunit, which are highly hydrophobic, form a region called the **fusion peptide** because it inserts into target membranes to initiate fusion. Viruses with the uncleaved F0 precursor can be produced in cells that lack the protease responsible for its cleavage. Such virus particles are noninfectious; they bind to target cells but the viral genome does not enter. Cleavage of the F0 precursor is necessary for fusion, not only because the fusion peptide is made available for insertion into the plasma membrane, but also to generate the metastable state of the protein that can undergo the conformational rearrangements needed for fusion.

Because cleaved F-protein-mediated fusion can occur at neutral pH, it must be controlled, both to ensure that virus particles fuse with only the appropriate cell and to prevent aggregation of newly assembled virions. The fusion peptide of F1 is buried between two subunits of the trimer in the pre-fusion protein. Conformational changes in F protein lead to refolding of the protein, assembly of an α-helical coiled coil, and movement of the fusion peptide toward the cell membrane (Fig. 5.11). Such movement of the fusion peptide has been described in atomic detail by comparing structures of the F protein before and after fusion.

The trigger that initiates conformational changes in the F protein is not known. The results of experiments in which hemagglutinin-neuraminidase (HN) and F glycoproteins are synthesized in cultured mammalian cells indicate that the fusion activity of F protein is absent or inefficient if HN is not present. It has therefore been hypothesized that an interaction between HN and F proteins is essential for fusion. It is thought that binding of HN protein to its cellular receptor induces conformational changes, which in turn trigger conformational change in the F protein, exposing the fusion peptide and making the protein fusion competent (Fig. 5.11). The requirement for HN protein in F fusion activity has been observed only with certain paramyxoviruses, including human parainfluenza virus type 3 and mumps virus.

As a result of fusion of the viral and plasma membranes, the viral nucleocapsid, which is a ribonucleoprotein (RNP) consisting of the (–) strand viral RNA genome and the viral proteins L, NP, and P, is released into the cytoplasm (Fig. 5.11). Once in the cytoplasm, the L, NP, and P proteins begin the synthesis of viral messenger RNAs (mRNAs), a process discussed in Chapter 6. Because members of the *Paramyxoviridae* replicate in the cytoplasm, fusion of the viral and plasma membranes achieves uncoating and delivery of the viral genome to this cellular compartment in a single step.

Fusion of human immunodeficiency virus type 1 with the plasma membrane requires participation not only of the cell receptor CD4 but also of an additional cellular protein. These proteins are cell surface receptors for small molecules produced by many cells to attract and stimulate cells of the immune defense system at sites of infection; hence these small molecules are called **chemotactic cytokines** or **chemokines**. The chemokine receptors on such cells

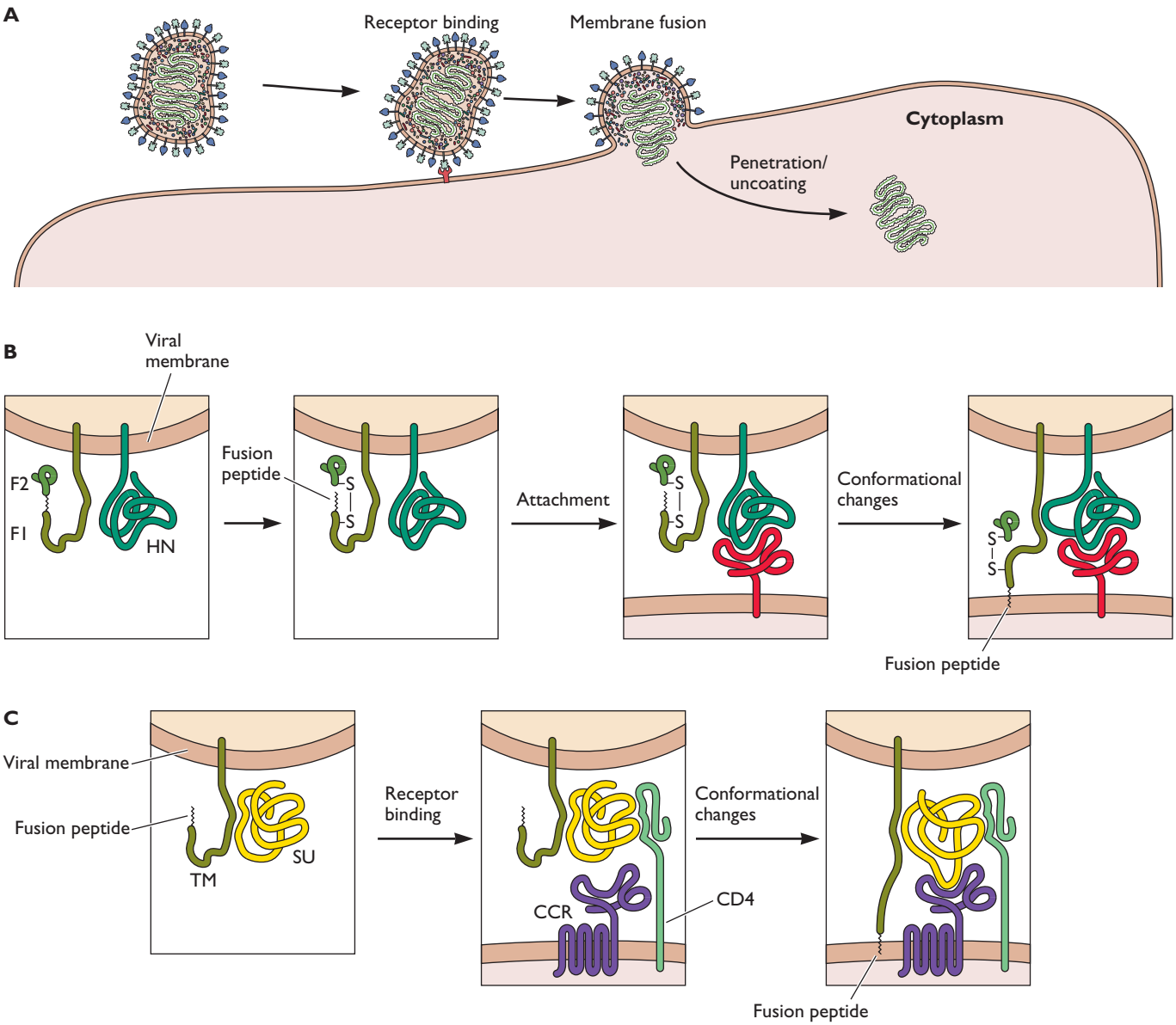


Figure 5.11 Penetration and uncoating at the plasma membrane. (A) Overview. Entry of a member of the *Paramyxoviridae*, which bind to cell surface receptors via the HN, H, or G glycoprotein. The fusion protein (F) then catalyzes membrane fusion at the cell surface at neutral pH. The viral nucleocapsid, as RNP, is released into the cytoplasm, where RNA synthesis begins. The mechanism by which contacts between the viral nucleocapsid and the M protein, which forms a shell beneath the lipid bilayer, are broken to facilitate release of the nucleocapsid is not known. **(B)** Model for F-protein-mediated membrane fusion. Binding of HN to the cell receptor (red) induces conformational changes in HN that in turn induce conformational changes in the F protein, moving the fusion peptide from a buried position nearer to the cell membrane. **(C)** Model of the role of chemokine receptors in human immunodeficiency virus type 1 fusion at the plasma membrane. For simplicity, the envelope glycoprotein is shown as a monomer, although trimer and tetramer forms have been reported. Binding of SU to CD4 exposes a high-affinity chemokine receptor-binding site on SU. The SU-chemokine receptor interaction leads to conformational changes in TM that expose the fusion peptide and permit it to insert into the cell membrane, catalyzing fusion in a manner similar to that proposed for influenza virus (cf. Fig. 5.12 and 5.13).

comprise a large family of proteins with seven membrane-spanning domains and are coupled to intracellular signal transduction pathways. There are two major coreceptors for human immunodeficiency virus type 1 infection. CXCr4 (a member of a family of chemokines characterized by having their first two cysteines separated by a single amino acid) appears to be a specific coreceptor for virus strains that infect T cells preferentially. The second is CCr5, a coreceptor for the macrophage-tropic strains of the virus. The chemokines that bind to this receptor activate both T cells and macrophages, and the receptor is found on both types of cell. Individuals who are homozygous for deletions in the CCr5 gene and produce nonfunctional coreceptors have no discernible immune function abnormality, but they appear to be resistant to infection with human immunodeficiency virus type 1. Even heterozygous individuals seem to be somewhat resistant to the virus. Other members of the CC chemokine receptor family (CCr2b and CCr3) were subsequently found to serve as coreceptors for the virus.

Attachment to CD4 appears to create a high-affinity binding site on SU for CCr5. The atomic structure of SU bound to CD4 revealed that binding of CD4 induces conformational changes that expose binding sites for chemokine receptors (Fig. 5.11). Studies of CCr5 have shown that the first N-terminal extracellular domain is crucial for coreceptor function, suggesting that this sequence might interact with SU.

Human immunodeficiency virus type 1 TM mediates envelope fusion with the cell membrane. The high-affinity SU-CCr5 interaction may induce conformational changes in TM to expose the fusion peptide, placing it near the cell membrane, where it can catalyze fusion (Fig. 5.11). Such changes are similar to those that influenza virus HA undergoes upon exposure to low pH. X-ray crystallographic analysis of fusion-active human immunodeficiency virus type 1 TM revealed that its structure is strikingly similar to that of the low-pH fusogenic form of HA (see "Acid-Catalyzed Membrane Fusion" below).

Certain isolates of human immunodeficiency virus types 1 and 2 and simian immunodeficiency virus enter cells independently of CD4 via chemokine receptors. Given the large number of members of the chemokine receptor family and the ability of human immunodeficiency virus type 1 to interact with these proteins, it is possible that CD4-independent, chemokine receptor-mediated infection may occur with some frequency.

Uncoating during Endocytosis

Acid-Catalyzed Membrane Fusion

Many enveloped viruses undergo fusion within an endosomal compartment. The entry of influenza virus from the endosomal pathway is one of the best-understood viral entry mechanisms. At the cell surface, the virus attaches to sialic acid-containing receptors via the viral HA glycoprotein (Fig. 5.12). The virus-receptor complex is then internalized by the clathrin-dependent receptor-mediated endocytic pathway. When the endosomal pH reaches approximately 5.0, HA undergoes an acid-catalyzed conformational rearrangement, exposing a fusion peptide. The viral and endosomal membranes then fuse, allowing penetration of the viral RNP (vRNP) into the cytoplasm. Because influenza virions have a low pH threshold for fusion, uncoating occurs in late endosomes. Viruses with a high pH threshold (pH 6.5 to 6) undergo fusion with the membranes of early endosomes in the periphery of the cytoplasm.

The fusion reaction mediated by the influenza virus HA protein is a remarkable event when viewed at atomic resolution (Fig. 5.13). In native HA, the fusion peptide is joined to the three-stranded coiled-coil core by which the HA monomers interact via a 28-amino-acid sequence that forms an extended loop structure buried deep inside the molecule, about 100 Å from the globular head. In contrast, in the low-pH HA structure, this loop region is transformed into a three-stranded coiled coil. In addition, the long α-helices of the coiled coil bend upward and away from the viral membrane. The result is that the fusion peptide has moved a great distance toward the endosomal membrane (Fig. 5.13). Despite these dramatic changes, HA remains trimeric and the globular heads can still bind sialic acid. In this conformation, HA holds the viral and endosome membranes 100 Å apart, too distant for the fusion reaction to occur. To bring the viral and cellular membranes closer, it is thought that the top of the acid-induced coiled coil splays apart, spreading into the lipid bilayer (Fig. 5.12). The stems of the HA tilt, further facilitating close contact of the membranes.

In contrast to cleaved HA, the precursor HA0 is stable at low pH and cannot undergo structural changes. How does cleavage of HA produce a protein capable of fusion only at acidic pH? Cleavage of the covalent bond between HA1 and HA2 might simply allow movement of the fusion peptide, which is restricted in the uncleaved molecule. Another possibility is suggested by the observation that cleavage of HA is accompanied by movement of the fusion peptide into the cavity in HA (Fig. 5.13). This movement buries ionizable residues of the fusion peptide, perhaps setting the low-pH "trigger." It should be emphasized that after cleavage, the N terminus of HA2 is tucked into the hydrophobic interior of the trimer (Fig. 5.13). This rearrangement presumably buries the fusion peptide so that newly synthesized virions do not aggregate and lose infectivity.

When the structure of influenza virus HA is compared with those of the TM proteins of two retroviruses, the

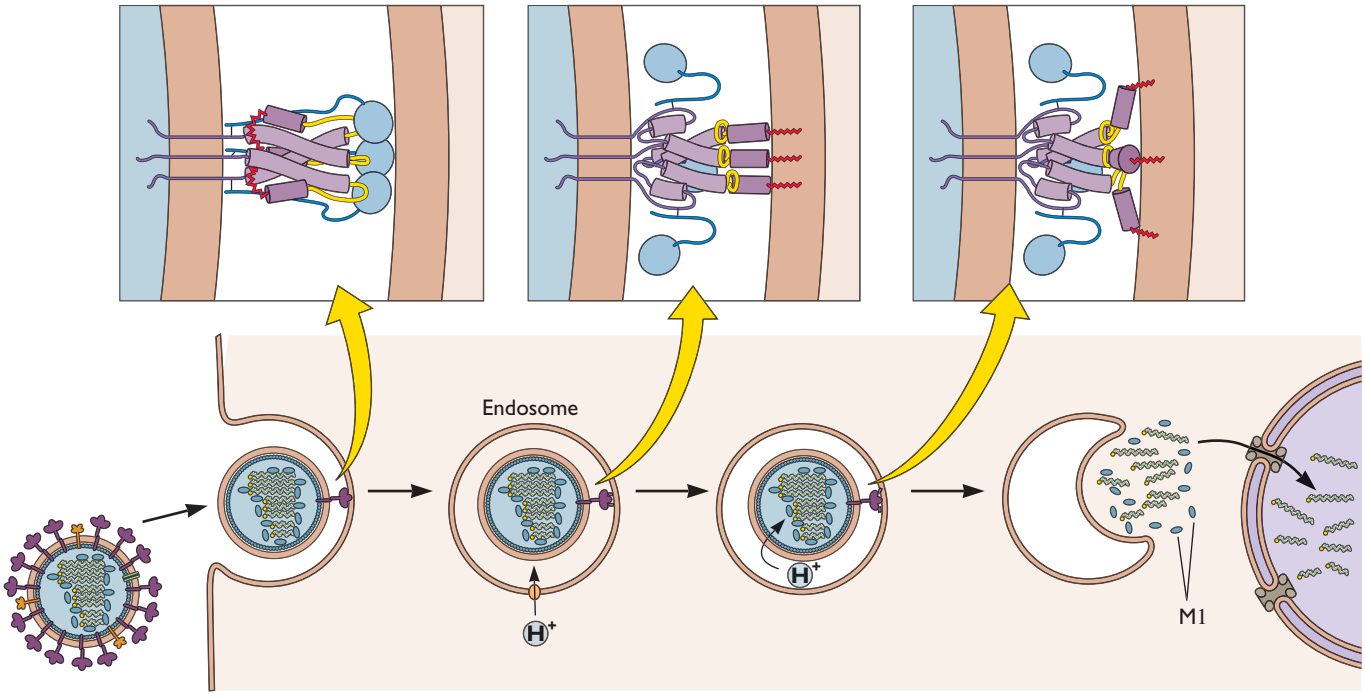


Figure 5.12 Influenza virus entry. The globular heads of native HA mediate binding of the virus to sialic acid-containing cell receptors. The virus-receptor complex is endocytosed, and import of H+ ions into the endosome acidifies the interior. Upon acidification, the viral HA undergoes a conformational rearrangement that produces a fusogenic protein. The loop region of native HA (yellow) becomes a coiled coil, moving the fusion peptides (red) to the top of the molecule near the cell membrane. At the viral membrane, the long α-helix (purple) packs against the trimer core, pulling the globular heads to the side. The long coiled coil splays into the cell membrane, bringing it closer to the viral membrane so that fusion can occur. Not shown is the tilting of HA that occurs. To allow release of vRNP into the cytoplasm, the H^+ ions in the acidic endosome are pumped into the virion interior by the M2 ion channel. As a result, vRNP is primed to dissociate from M1 after fusion of the viral and endosomal membranes. The released vRNPs are imported into the nucleus through the nuclear pore complex via a nuclear localization signal-dependent mechanism (see "Import of Influenza Virus Ribonucleoprotein" below). Adapted from C. M. Carr and P. S. Kim, *Science* **266:**234–236, 1994, with permission.

F protein of simian virus 5 and Gp2 of Ebola virus, remarkable similarities become apparent (Fig. 5.14). In all five cases, the fusion peptides are presented to membranes on top of a three-stranded coiled coil. Such a scaffold is a common feature of viral type I membrane fusion proteins: they have a region of high α-helical content and a 4-3 heptad repeat of hydrophobic amino acids, characteristic of coiled coils, next to the N-terminal fusion peptide.

The envelope proteins of alphaviruses and flaviviruses exemplify a different class of viral fusion protein (type II fusion proteins). These viral proteins contain an internal fusion peptide and are tightly associated with a second viral protein. Proteolytic cleavage of the second protein converts the fusion protein to a metastable state that can undergo structural rearrangements at low pH to promote fusion. In contrast, the fusion peptide of the influenza virus HA is adjacent to the cleavage point and becomes the N terminus of the mature fusion protein. The envelope proteins of alphaviruses and flaviviruses do not form coiled coils, as do type I fusion proteins. Rather, they contain predominantly β-barrels that are thought to tilt toward the membrane at low pH, thereby exposing the fusion peptide (Fig. 5.15).

The membrane fusion mediated by the envelope protein of the alphavirus Semliki Forest virus exhibits several unusual features. This process requires the presence of cholesterol in the cell membrane, which is not needed for fusion mediated by other viral proteins. Why cholesterol is needed for fusion is not understood. In contrast to the situation with other viruses, proteolytic cleavage of E1 is not required to produce a fusogenic protein. However, protein processing may control fusion potential in another way. In the endoplasmic reticulum, E1 protein is associated with the precursor of E2, called p62. In this heterodimeric form, p62-E1, E1 protein cannot be activated for fusion by mildly

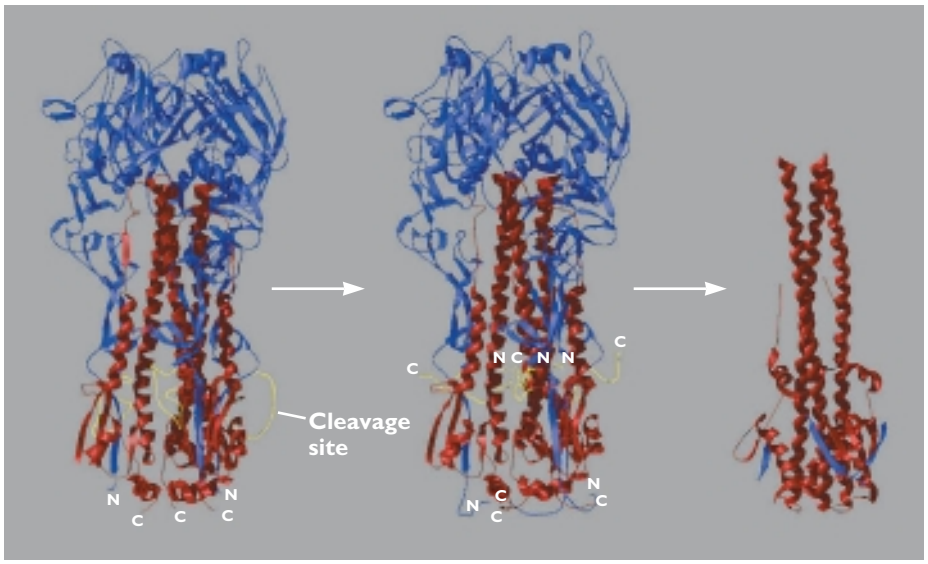


Figure 5.13 Cleavage- and low-pH-induced structural changes in the extracellular domains of influenza virus HA. (Left) Structure of the uncleaved HA0 precursor extracellular domain at neutral pH. HA1 subunits are blue, HA2 subunits are red, residues 323 of HA1 to 12 of HA2 are yellow, and the locations of some of the N and C termini are indicated. The viral membrane is at the bottom, and the globular heads are at the top. The cleavage site between HA1 and HA2 is in a loop adjacent to a deep cavity. **(Middle)** Structure of the cleaved HA trimer at neutral pH. Cleavage of HA0 generates new N and C termini, which are separated by 20 Å. The N and C termini visible in this model are labeled. The cavity is now filled with residues 1 to 10 of HA2, part of the fusion peptide. **(Right)** Structure of the low-pH trimer. The protein used for crystallization was treated with proteases, and therefore the HA1 subunit and the fusion peptide are not present. This treatment is necessary to prevent aggregation of HA at low pH. At neutral pH the fusion peptide is close to the viral membrane, linked to a short α-helix, and at acidic pH this α-helix is reoriented toward the cell membrane, carrying with it the fusion peptide. The structures are aligned on a central α-helix that is unaffected by the conformational change. Adapted from J. Chen et al., *Cell* **95:**409–417, 1998, with permission.

acidic conditions. Only after p62 has been cleaved to E2 can low pH induce disruption of E1-E2 heterodimers and formation of fusion-active E1 homotrimers.

Release of Viral Ribonucleoprotein

The genomes of many enveloped RNA viruses are present as vRNP in the virus particle. One mechanism for release of vRNP during virus entry has been identified by studies of influenza virus. Each influenza virus vRNP is composed of a segment of the RNA genome bound by nucleoprotein (NP) molecules at about 10- to 15-nucleotide intervals and the virion RNA polymerase. This complex interacts with viral M1 protein, an abundant virion protein that underlies the viral envelope and provides rigidity (Fig. 5.12). The M1 protein also contacts the internal tails of HA and neuraminidase proteins in the viral envelope. This arrangement presents two problems. Unless M1-vRNP interactions are disrupted, vRNPs might not be released into the cytoplasm. Furthermore, the vRNPs must enter the nucleus, where mRNA synthesis occurs. However, vRNP cannot enter the nucleus if M1 protein remains bound, because this protein masks a nuclear localization signal (see "Import of Influenza Virus Ribonucleoprotein" below).

The influenza virus M2 protein, the first viral protein discovered to be an ion channel, probably provides the solution to both problems. The virion envelope contains a small number (14 to 68) of molecules of M2 protein, which form a homotetramer. When purified M2 was reconstituted into synthetic lipid bilayers, ion channel activity was observed, indicating that this property requires only M2 protein. The M2 protein channel is structurally much simpler than other ion channels and is the smallest channel discovered to date.

The M2 ion channel is activated by the low pH of the endosome before HA-catalyzed membrane fusion occurs. As a result, protons enter the interior of the virus particle. It has been suggested that the reduced pH of the virion interior leads to conformational changes in the M1 protein, thereby disrupting M1-vRNP interactions. When fusion between the viral envelope and the endosomal membrane subsequently occurs, vRNPs are released into the cytoplasm free of M1 and can then be imported into

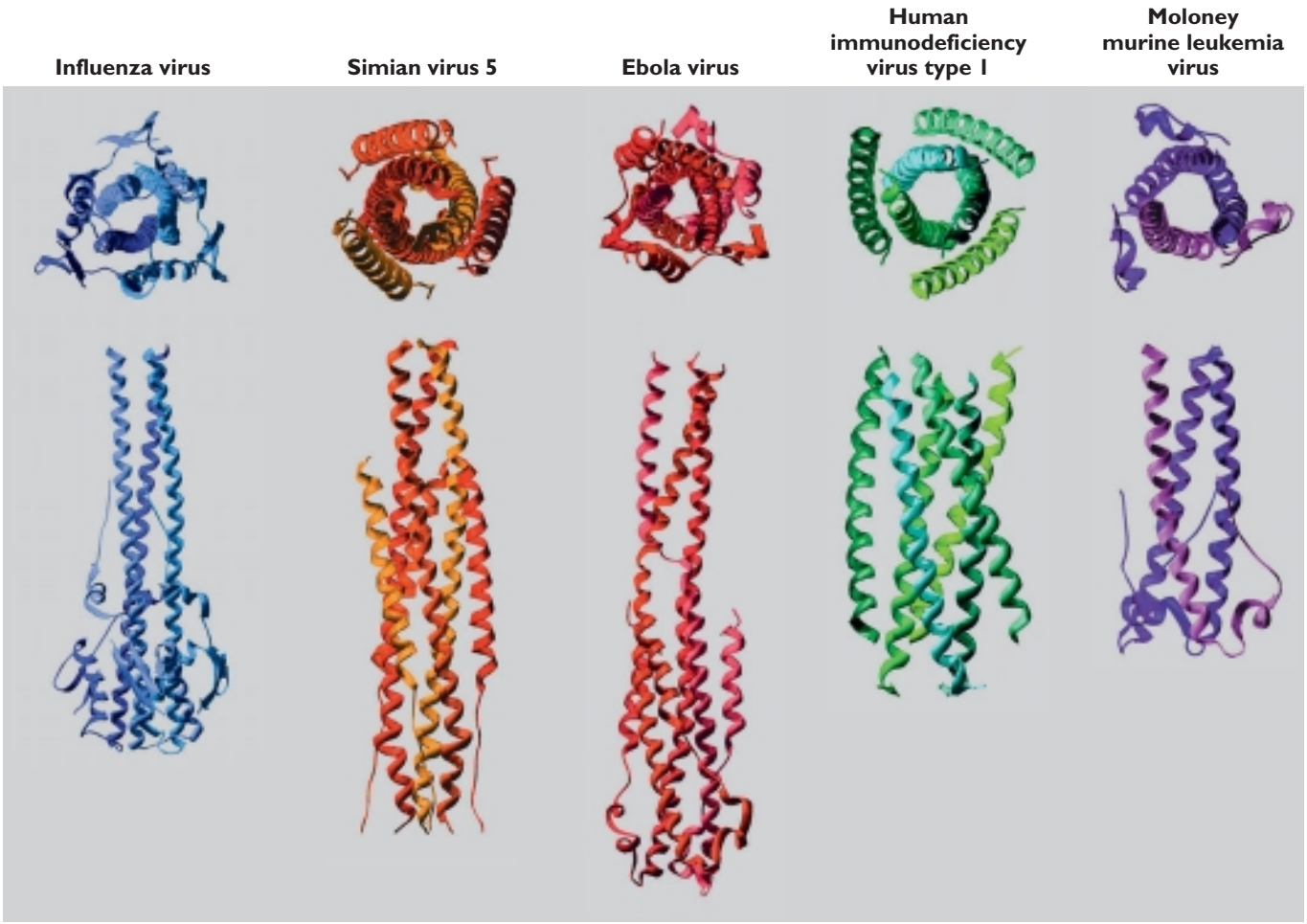


Figure 5.14 Similarities among five viral fusion proteins. (Top) View from the top of the structures. **(Bottom)** Side view. The structure shown for HA is the low-pH, or fusogenic, form. The structure of simian virus 5 F protein is of peptides from the N- and C-terminal heptad repeats. Structures of retroviral TM proteins are derived from interacting human immunodeficiency virus type 1 peptides and a peptide from Moloney murine leukemia virus and are presumed to represent the fusogenic forms because of structural similarity to HA. In all three molecules, fusion peptides would be located at the membrane-distal portion (the tops of the molecules in the bottom view). All present fusion peptides to cells on top of a central three-stranded coiled coil supported by C-terminal structures. Adapted from K. A. Baker et al., *Mol. Cell* **3:**309–319, 1999, with permission.

the nucleus (Fig. 5.12). Support for this model comes from studies with the anti-influenza virus drug **amantadine,** which specifically inhibits M2 ion channel activity (Volume II, Fig. 9.11). In the presence of this drug, influenza virus particles can bind to cells, enter endosomes, and undergo HA-mediated membrane fusion, but vRNPs are not released from the endosomal membrane.

Receptor Priming for Low-pH Fusion: Two Entry Mechanisms Combined

During the entry of avian leukosis virus into cells, virion binding to the cell receptor primes the viral fusion protein for low-pH-activated fusion. Avian leukosis virus, like many other simple retroviruses, was believed to enter cells at the plasma membrane in a pH-independent mechanism resembling that of members of the *Paramyxoviridae* (Fig. 5.11). It is now known that binding of the viral membrane glycoprotein Env-A to the cellular receptor Tva induces conformational rearrangements that convert Env-A from a native metastable state that is insensitive to low pH to a second metastable state. In this state, exposure of Env-A to low pH within the endosomal compartment leads to membrane fusion and release of the viral capsid.

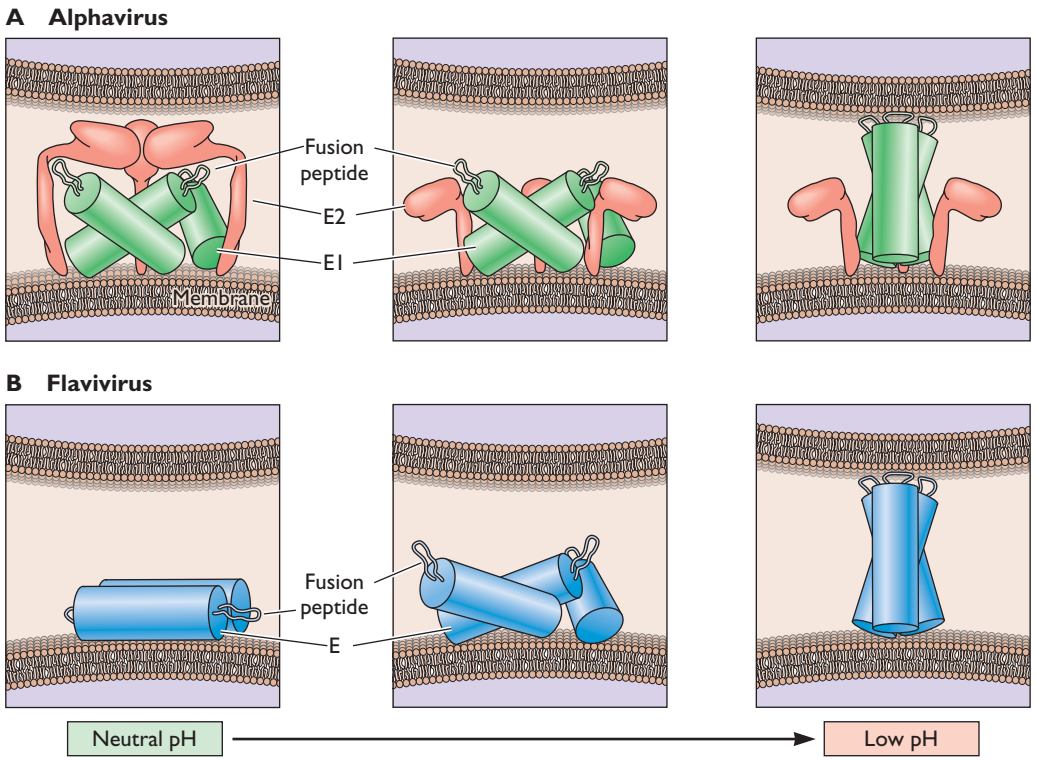


Figure 5.15 Models for low-pH-induced movement of alphavirus and flavivirus glycoproteins. Low pH causes conformational changes in the viral glycoproteins to produce the fusion-active forms. **(A)** In alphavirus virions, the fusion peptide in E1 is masked by E2. Low pH leads to disruption of E1-E2 dimers, exposing the fusion peptide. **(B)** In flavivirus virions, the fusion peptide is buried in dimers of the fusion glycoprotein E. At low pH, the dimers are disrupted, the proteins rotate to form trimers, and the fusion peptide is directed toward the cell membrane. Adapted from R. J. Kuhn et al., *Cell* **108:**717–725, 2002, with permission.

Uncoating in the Cytoplasm by Ribosomes

Some enveloped RNA-containing viruses, such as Semliki Forest virus, contain nucleocapsids that are disassembled in the cytoplasm by pH-independent mechanisms. The nucleocapsid of this virus is an icosahedral shell composed of a single viral protein, C protein, which encloses the (+) strand viral RNA. This structure is surrounded by an envelope containing viral glycoproteins called E1 and E2, which are arranged as heterodimers clustered into groups of three, each cluster forming a spike on the virus surface.

Fusion of the viral and endosomal membrane exposes the nucleocapsid to the cytoplasm (Fig. 5.16). The viral RNA within this structure is sensitive to digestion with RNase, suggesting that the nucleocapsid is permeable. Crystallographic studies of the nucleocapsid of Sindbis virus, a closely related alphavirus, confirm the presence of holes ranging in diameter from 30 to 60 Å. These holes permit the entry of small proteins such as RNase (25 to 40 Å in diameter) into the nucleocapsid. To begin translation of (+) strand viral RNA, the nucleocapsid must be disassembled, a process mediated by an abundant cellular component—the ribosome. Each ribosome binds three to six molecules of C protein, causing them to detach from the nucleocapsid. This process occurs while the nucleocapsid is attached to the cytoplasmic side of the endosomal membrane (Fig. 5.16) and ultimately results in disassembly. The uncoated viral RNA remains associated with cellular membranes, where translation and replication begin.

Disrupting the Endosomal Membrane

Adenoviruses are composed of a double-stranded DNA genome packaged in an icosahedral capsid made up of at least 10 structural proteins, as described in Chapter 4. Internalization of most adenovirus serotypes by receptor-mediated endocytosis requires attachment of fiber to an integrin or Ig-like cell surface receptor and binding of the penton base to a second cell receptor, the cellular vitronectin-binding integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Attachment is mediated by RGD sequences in each of the five subunits of

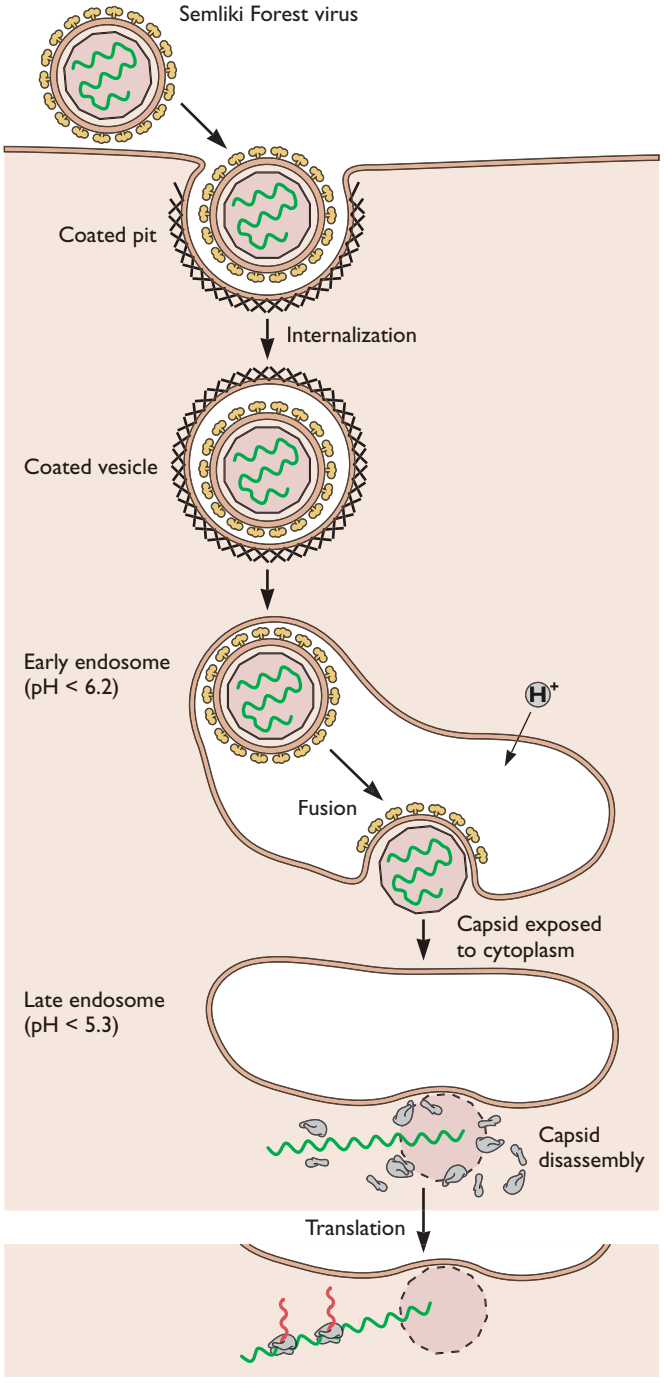


Figure 5.16 Entry of Semliki Forest virus into cells. Semliki Forest virus enters cells by clathrin-dependent receptor-mediated endocytosis, and membrane fusion is catalyzed by acidification of endosomes. Fusion results in exposure of the viral nucleocapsid to the cytoplasm, although the nucleocapsid remains attached to the cytosolic side of the endosome membrane. Cellular ribosomes then bind the capsid, disassembling it and distributing the capsid protein throughout the cytoplasm. The viral RNA is then accessible to ribosomes, which initiate translation. Adapted from M. Marsh and A. Helenius, *Adv. Virus Res.* **36:**107–151, 1989, with permission.

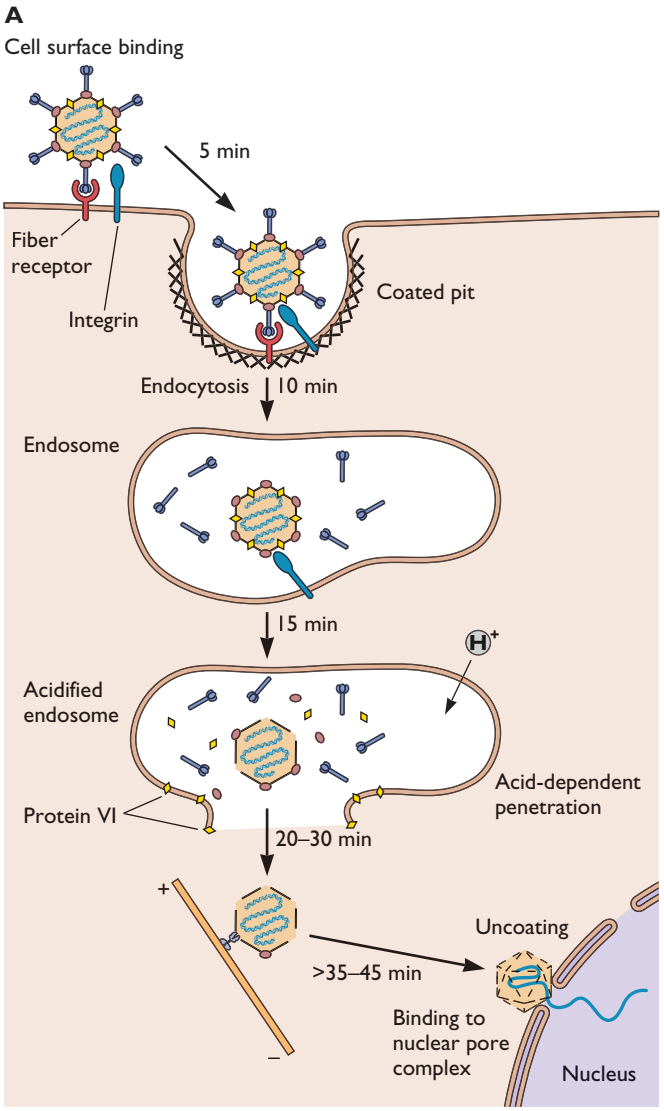
the adenovirus penton base that mimic the normal ligands of cell surface integrins. As the virus particle is transported via the endosomes from the cell surface toward the nuclear membrane, it undergoes multiple uncoating steps by which structural proteins are removed sequentially (Fig. 5.17). As the endosome becomes acidified, the viral capsid is destabilized, leading to release of proteins from the capsid. Among these is protein VI, which causes disruption of the endosomal membrane, thereby delivering the remainder of the particle into the cytoplasm. An N-terminal amphipathic α-helix of protein VI is probably responsible for its pH-dependent membrane disruption activity. This region of the protein appears to be masked in the native capsid by the hexon protein. The liberated subviral particle then docks onto the nuclear pore complex (see "Import of DNA Genomes" below).

Forming a Pore in the Endosomal Membrane

The genome of the nonenveloped picornaviruses is transferred across the cell membrane by a different mechanism, as determined by structural information at the atomic level and complementary genetic and biochemical data obtained from studies of cell entry. The interaction of poliovirus with its Ig-like cell receptor, CD155, leads to major conformational rearrangements in the virus particle (Fig. 5.18A). These altered (A) particles are missing the internal capsid protein VP4, and the N terminus of capsid protein VP1 is on the surface rather than on the interior. Because of the latter change, A particles are hydrophobic and possess an increased affinity for membranes compared to the native virus particle. It is thought that the exposed lipophilic N terminus of VP1 inserts into the cell membrane, forming a pore that allows transport of viral RNA into the cytoplasm (Fig. 5.18B). In support of this model, ion channel activity can be detected when A particles are added to lipid bilayers.

The fate of VP4 is not known, but the study of a virus with an amino acid change in VP4 indicates that this protein is required for an early stage of cell entry. Mutant virus particles can bind to target cells and convert to altered particles, but are blocked at a subsequent, unidentified step. During poliovirus assembly, VP4 and VP2 are part of the precursor VP0, which remains uncleaved until the viral RNA has been encapsidated. The cleavage of VP0 during poliovirus assembly therefore primes the capsid for uncoating by separating VP4 from VP2.

In cultured cells, release of the poliovirus genome occurs from within early endosomes located close (within 100 to 200 nm) to the plasma membrane (Fig. 5.18A). Uncoating is dependent upon actin and tyrosine kinases, possibly for movement of the capsid through the network of actin filaments (Fig. 5.10), but not on dynamin, clathrin,



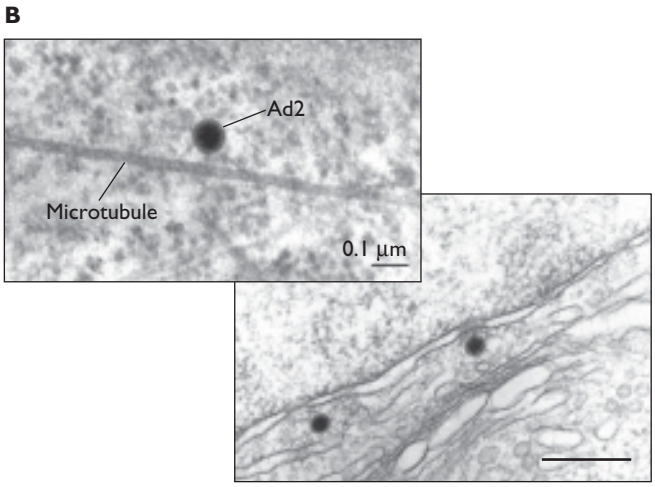


Figure 5.17 Stepwise uncoating of adenovirus. (A) Adenoviruses bind the cell receptor via the fiber protein. Interaction of the penton base with an integrin receptor leads to internalization by endocytosis. Low pH in the endosome causes destabilization of the capsid and release of protein VI. The hydrophobic N terminus of protein VI disrupts the endosome membrane, leading to release of a subviral particle into the cytoplasm. The capsid is transported in the cytoplasm along microtubules and docks onto the nuclear pore complex. **(B)** Electron micrograph of adenovirus type 2 particles bound to a microtubule (top) and bound to the cytoplasmic face of the nuclear pore complex (bottom). Bar in bottom panel = 200 nm. (A) Adapted from U. F. Greber et al., *Cell* **75:**477–486, 1993, and L. C. Trotman et al., *Nat. Cell Biol.* **3:**1092–1100, 2001, with permission. (B) Reprinted from U. F. Greber et al., *Trends Microbiol.* **2:**52–56, 1994, with permission. Courtesy of Ari Helenius, Urs Greber, and Paul Webster, University of Zurich.

caveolin, or flotillin (a marker protein for clathrin- and caveolin-independent endocytosis), endosome acidification, or microtubules. The trigger for RNA release from early endosomes is not known but is clearly dependent on prior interaction with CD155. This conclusion derives from the finding that antibody-poliovirus complexes can bind to cells that produce Fc receptors but cannot infect them. As the Fc receptor is known to be endocytosed, these results suggest that interaction of poliovirus with CD155 is required to induce conformational changes in the particle that are required for uncoating.

A critical regulator of the receptor-induced structural transitions of poliovirus appears to be a hydrophobic tunnel located below the surface of each structural unit (Fig. 5.18). The tunnel opens at the base of the canyon and extends toward the fivefold axis of symmetry. In poliovirus type 1, each tunnel is occupied by a natural ligand thought to be a molecule of sphingosine. Similar lipids have been observed in the capsids of other picornaviruses. Because of the symmetry of the capsid, each virion may contain up to 60 lipid molecules.

The lipids are thought to contribute to the stability of the native virus particle by locking the capsid in a stable conformation. Consequently, removal of the lipid is probably necessary to endow the particle with sufficient flexibility to permit the RNA to leave the shell. These conclusions come from the study of antiviral drugs known as WIN compounds (named after Sterling-Winthrop, the pharmaceutical company at which they were discovered). These compounds displace the lipid and fit tightly in the hydrophobic tunnel

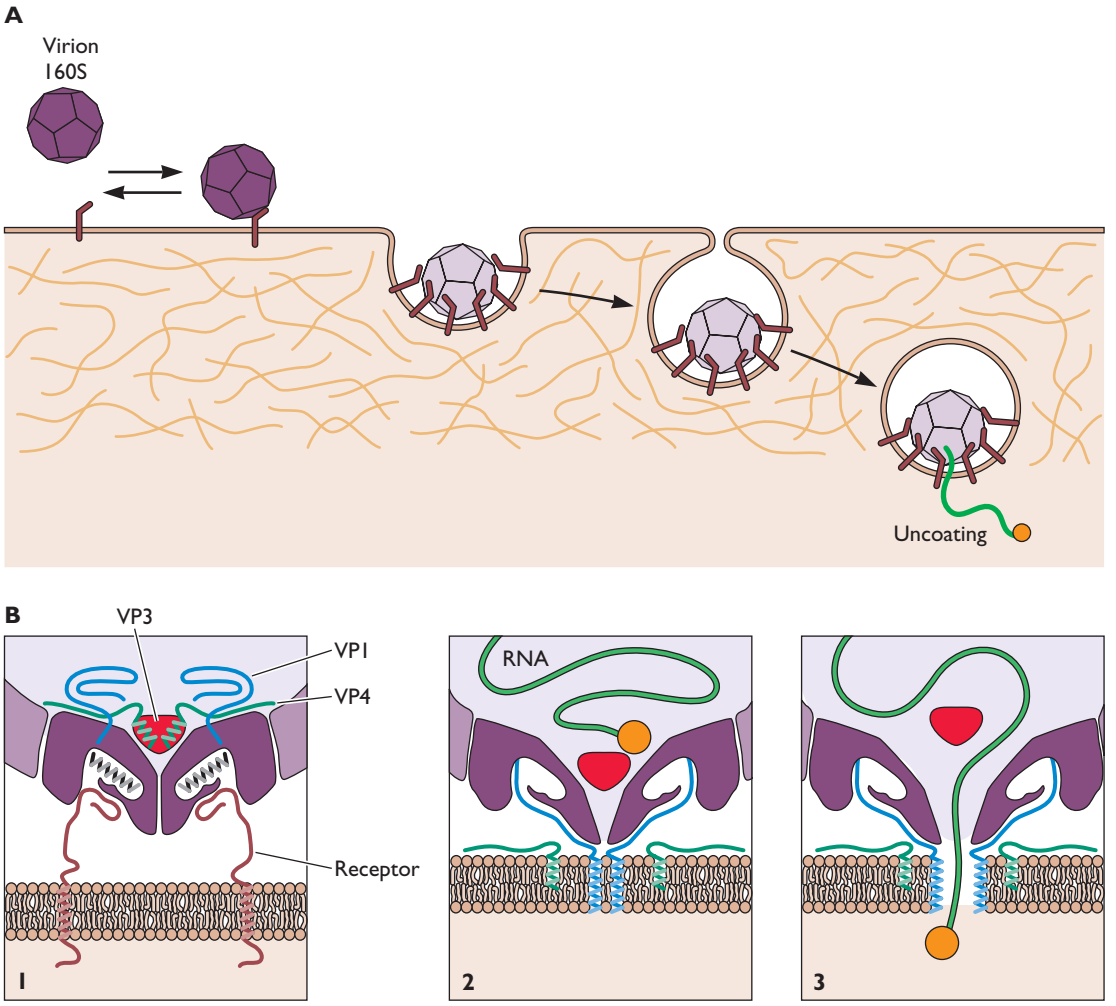


Figure 5.18 Model for poliovirus entry into cells. (A) Overview. The native virion (160S) binds to its cell receptor, CD155, and at temperatures higher than 33°C undergoes a receptor-mediated conformational transition resulting in the formation of altered (A) particles. The viral RNA, shown as a curved green line, leaves the capsid from within early endosomes close to the plasma membrane. **(B)** Model of the formation of a pore in the cell membrane after poliovirus binding. 1, Poliovirus (shown in cross section, with capsid proteins purple) binds to CD155 (brown). 2, A conformational change leads to displacement of the pocket lipid (black). The pocket may be occupied by sphingosine in the capsid of poliovirus type 1. The hydrophobic N termini of VP1 (blue) are extruded and insert into the plasma membrane. 3, A pore is formed in the membrane by the VP1 N termini, through which the RNA is released from the capsid into the cytosol. Adapted from J. M. Hogle and V. R. Racaniello, p. 71–83, *in* B. L. Semler and E. Wimmer (ed.), *Molecular Biology of Picornaviruses* (ASM Press, Washington, DC, 2002), with permission.

(Fig. 5.6). Polioviruses containing bound WIN compounds can bind to the cell receptor, but A particles are not produced. WIN compounds may therefore inhibit poliovirus infectivity by preventing the receptor-mediated conformational alterations required for uncoating. The properties of poliovirus mutants that cannot replicate in the absence of WIN compounds underscore the role of the lipids in uncoating. These drug-dependent mutants spontaneously convert to altered particles at 37°C, in the absence of the cell receptor, probably because they do not contain lipid in the hydrophobic pocket. The lipids are therefore viewed as switches, because their presence or absence determines whether the virus is stable or will be uncoated. The interaction of the virus particle with its receptor probably initiates structural changes in the virion that lead to the release of lipid. Consistent with this hypothesis is the observation that CD155 docks onto the poliovirus capsid just above the hydrophobic pocket.

Some picornaviruses enter cells by a pH-dependent pathway. For example, foot-and-mouth disease virus enters cells by receptor-mediated endocytosis. At a pH of approximately 6.5, the viral capsid dissociates to pentamers, releasing viral RNA. Dissociation of the capsid is probably a consequence of protonation of multiple histidine residues that line the pentamer interface and confer stability to the capsid at neutral pH. Consistent with this entry mechanism, antibody-coated foot-and-mouth disease virus can bind to and infect cells that carry Fc receptors, in contrast to findings with poliovirus. This result suggests that the cell receptor for foot-and-mouth disease virus does not induce uncoating-related changes in the virus particle.

Uncoating in the Lysosome

Most viruses that enter cells by receptor-mediated endocytosis leave the pathway before the vesicles reach the lysosomal compartment. This departure is not surprising, for lysosomes contain proteases and nucleases that would degrade virus particles. However, these enzymes play an important role during the uncoating of members of the *Reoviridae,* an event that takes place in lysosomes.

Orthoreoviruses are naked icosahedral viruses containing a double-stranded RNA genome of 10 segments. The viral capsid is a double-shelled structure composed of eight different structural proteins. These viruses bind to cell receptors via protein σ1 and are internalized into cells by endocytosis (Fig. 5.19A). Infection of cells by reoviruses is sensitive to bafilomycin A1, indicating that acidification of endosomes is required for entry. Low pH activates lysosomal proteases, which then modify several virion proteins, enabling the virus to cross the vesicle membrane. One viral outer capsid protein is cleaved and another is removed from the particle, producing an infectious subviral particle. These subviral particles penetrate the lysosome membrane and escape into the cytosol by a mechanism that is not yet understood. Isolated infectious subviral particles cause cell membranes to become permeable to toxins and produce pores in artificial membranes. These particles can initiate an infection by penetrating the plasma membrane, entering the cytoplasm directly. Their infectivity is not sensitive to bafilomycin A1, further supporting the idea that these particles are primed for membrane entry and do not require further acidification for this process. The core particles generated from infectious subviral particles after penetration into the cytoplasm carry out viral mRNA synthesis.

Import of Viral Genomes into the Nucleus

The replication of most DNA viruses, and some RNA viruses including retroviruses and influenza viruses, begins in the cell nucleus. The genomes of these viruses must therefore be imported from the cytoplasm into the nucleus. One way to accomplish this movement is via the cellular pathway for protein import into the nucleus. An alternative, observed in cells infected by some retroviruses, is to enter the nucleus during cell division. At this time in the cell cycle, cellular chromatin becomes accessible to virus particles. This strategy restricts infection to cells that undergo mitosis.

Many subviral particles are too large to pass through the nuclear pore complex. There are several strategies to overcome this limitation (Fig. 5.20). The influenza virus genome, which consists of eight segments that are each small enough to pass through the nuclear pore complex, is uncoated in the cytoplasm. Adenovirus subviral particles dock onto the nuclear pore complex and are disassembled by the import machinery, allowing the viral DNA to pass into the nucleus. Herpes simplex virus capsids also dock onto the nuclear pore but remain largely intact, and the nucleic acid is injected into the nucleus through a portal in the virion.

The cellular genome is highly compacted in the nucleus, and it is not understood how viral DNAs are imported against this steep gradient. The DNA of some bacteriophages is packaged in the virion at high pressure, which provides sufficient force to insert the viral DNA genome into the bacterial cell. However, no similar mechanism is known for animal viruses. Furthermore, because transport through the nuclear pore complex depends upon hydrophobic interactions with nucleoporins, the charged and hydrophilic viral nucleic acids would have difficulty passing through the pore. How the nuclear import machinery overcomes these obstacles is not known.

Nuclear Localization Signals

Proteins that reside within the nucleus are characterized by the presence of specific nuclear targeting sequences. Such **nuclear localization signals** are both necessary for nuclear localization of the proteins in which they are present and sufficient to direct heterologous, nonnuclear proteins to enter this organelle. Nuclear localization signals identified by these criteria share a number of common properties: they are generally fewer than 20 amino acids in length, they are not removed after entry of the protein into the nucleus, and they are usually rich in basic amino acids. Despite these similarities, no consensus nuclear localization sequence can be defined.

Most nuclear localization signals belong to one of two classes, simple or bipartite sequences (Fig. 5.21). A particularly well characterized example of a simple nuclear localization signal is that of simian virus 40 large T antigen, which comprises five contiguous basic residues flanked by a single hydrophobic amino acid (Fig. 5.21). This sequence is

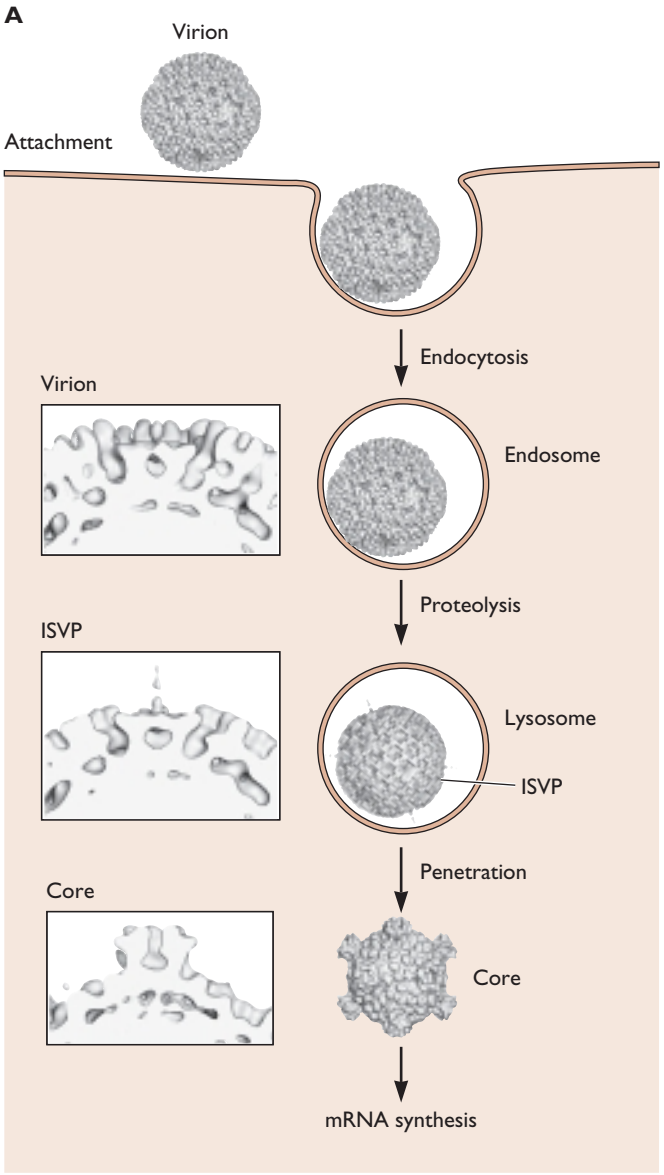


Figure 5.19 Entry of reovirus into cells. (A) The different stages in cell entry of reovirus. After the attachment of σ1 protein to the cell receptor, the virus particle enters the cell by receptor-mediated endocytosis. Proteolysis in the late endosome produces the infectious subviral particle (ISVP), which may then cross the lysosomal membrane and enter the cytoplasm as a core particle. The intact virion is composed of two concentric, icosahedrally organized protein capsids. The outer capsid is made up largely of σ3 and μ1. The dense core shell is formed mainly by λ1 and σ2. In the ISVP, 600 σ3 subunits have been released by proteolysis, and the σ1 protein changes from a compact form to an extended flexible fiber. The μ1 protein, which is thought to mediate interaction of the ISVP with membranes, is present as two cleaved fragments, μ1N and μ1C (see schematic of μ1 in panel B). The N terminus of μ1N is modified with myristate, suggesting that the protein functions in the penetration of membranes. A pair of amphipathic α-helices flank a C-terminal trypsin/chymotrypsin cleavage site at which μ1C is cleaved by lysosomal proteases. Such cleavage may release the helices to facilitate membrane penetration. The membrane-penetrating potential of μ1C in the virion may be masked by σ3; release of the σ3 in ISVPs might then allow μ1C to interact with membranes. The core is produced by the release of 12 σ1 fibers and 600 μ1 subunits. In the transition from ISVP to core, domains of λ2 rotate upward and outward to form a turretlike structure. (Insets) Close-up views of the emerging turretlike structure as the virus progresses through the ISVP and core stages. This structure may facilitate the entry of nucleotides into the core and the exit of newly synthesized viral mRNAs. **(B)** Schematic of the μ1 protein, showing locations of myristate, protease cleavage sites, and amphipathic α-helices. Virus images reprinted from K. A. Dryden et al., *J. Cell Biol.* **122:**1023–1041, 1993, with permission. Courtesy of Norm Olson and Tim Baker, Purdue University.

sufficient to relocate the enzyme pyruvate kinase, normally found in the cytoplasm, to the nucleus. Many other viral and cellular nuclear proteins contain short, basic nuclear localization signals, but these signals are not identical in primary sequence to the T-antigen signal. The presence of a nuclear localization signal is all that is needed to target a macromolecular substrate for import into the nucleus. Even gold particles with diameters as large as 26 nm are readily imported following their microinjection into the cytoplasm, as long as they are coated with proteins or peptides containing a nuclear localization signal.

The Nuclear Pore Complex

The nuclear envelope is composed of two typical lipid bilayers separated by a lumenal space (Fig. 5.22). Like all other cellular membranes, it is impermeable to macromolecules such as proteins. However, the nuclear pore complexes that stud the nuclear envelopes of all eukaryotic cells provide aqueous channels that span both the inner and outer nuclear membranes for exchange of small molecules, macromolecules, and macromolecular assemblies between nuclear and cytoplasmic compartments. Numerous experimental techniques, including direct visualization of gold particles attached to proteins or RNA molecules as they are transported, have established that nuclear proteins enter and RNA molecules exit the nucleus by transport through the nuclear pore complex. The functions of the nuclear pore complex in both protein import and RNA export are far from completely understood, not least because this important cellular machine is large (molecular mass,

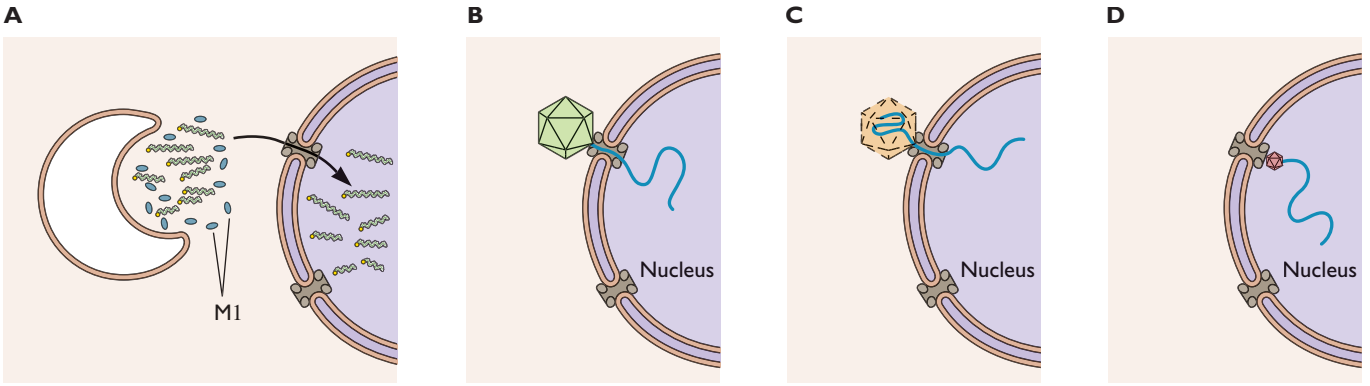


Figure 5.20 Different strategies for entering the nucleus. (A) Each segment of the influenza virus genome is small enough to be transported through the pore complex. **(B)** The herpes simplex virus type 1 capsid docks onto the nuclear pore complex and is minimally disassembled to allow transit of the viral DNA into the nucleus. **(C)** The adenovirus subviral particle is substantially dismantled by the nuclear import machinery, allowing transport of the viral DNA into the nucleus. **(D)** The capsids of some viruses (parvovirus and hepadnavirus) are small enough to enter the nuclear pore complex without disassembly.

approximately 124×10^3 kDa in vertebrates), built from many different proteins, and architecturally complex (Fig. 5.22). In comparison, ribosomes, which consist of ~82 proteins and 4 RNA molecules, have a molecular mass of 4.2×10^3 kDa.

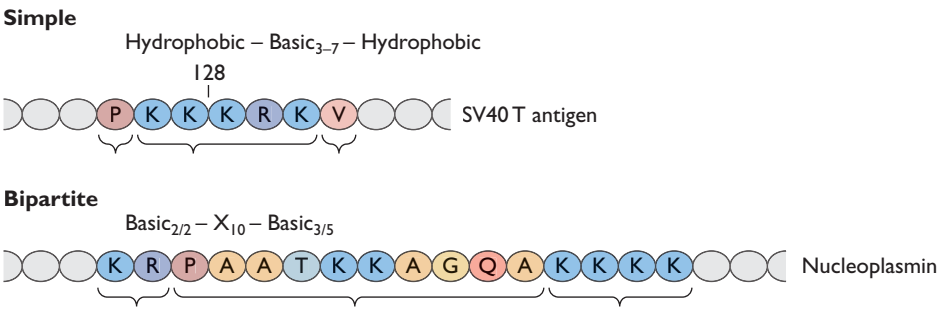
The nuclear pore complex allows passage of cargo in and out of the nucleus by either passive diffusion or facilitated translocation. Passive diffusion does not require interaction between the cargo and components of the nuclear pore complex, and becomes inefficient as molecules approach 20 to 40 kDa in mass. Objects as large as several megadaltons can pass through nuclear pore complexes by facilitated translocation. This process requires specific interactions between the cargo and components of the nuclear pore complex and is therefore selective.

The Nuclear Import Pathway

Import of a protein into the nucleus via nuclear localization signals occurs in two distinct, and experimentally separable, steps (Fig. 5.22C). A protein containing such a signal first binds to a soluble cytoplasmic receptor protein. This complex then engages with the cytoplasmic surface of the nuclear pore complex, in a reaction often called docking, and is translocated through the nuclear pore complex into the nucleus. In the nucleus, the complex is disassembled, releasing the protein cargo.

Different groups of proteins are imported into the nucleus by specific receptor systems. In what is known as the "classical system" of import, cargo proteins containing basic nuclear localization signals bind to the cytoplasmic nuclear localization signal receptor protein importin-α

Figure 5.21 Nuclear localization signals. The general form and a specific example of simple and bipartite nuclear localization signals are shown in the one-letter amino acid code, where X is any residue. Bipartite nuclear targeting signals are defined by the presence of two clusters of positively charged amino acids separated by a spacer region of variable sequence. Both clusters of basic residues, which often resemble the simple targeting sequences of proteins like simian virus 40 T antigen, are required for efficient import of the proteins in which they are found. The subscript indicates either length (3–7) or composition (e.g., 3/5 means at least 3 residues out of 5 are basic).



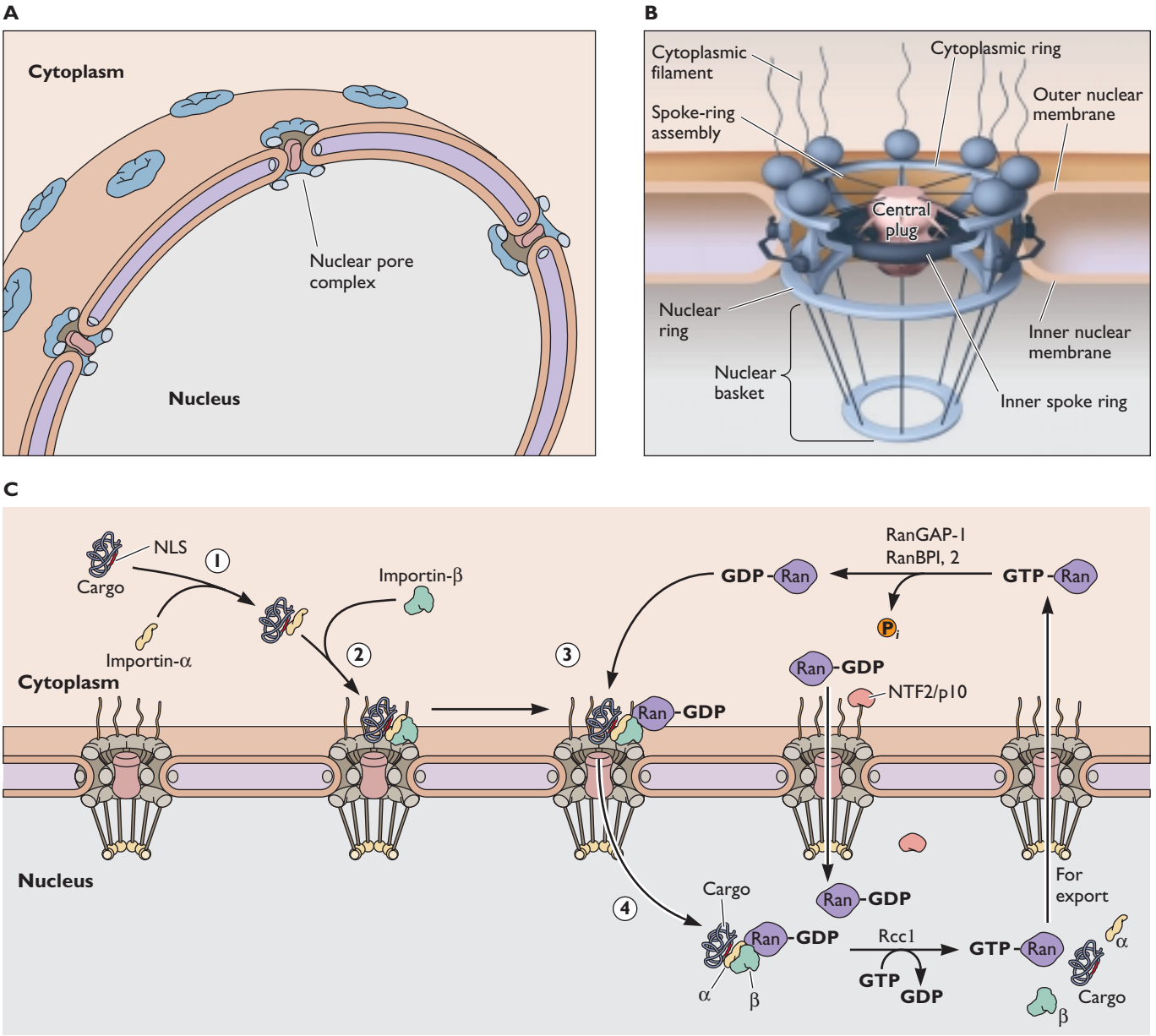


Figure 5.22 Structure and function of the nuclear pore complex. (A) Overview of the nuclear membrane, showing the topology of the nuclear pore complexes. **(B)** Schematic drawing of the nuclear pore complex, showing the spoke-ring assembly at its waist and its attachment to cytoplasmic filaments and the nuclear basket. The latter comprises eight filaments, extending 50 to 100 nm from the central structure and terminating in a distal annulus. The nuclear pore channel is shown containing the transporter. **(C)** An example of the classical protein import pathway for proteins with a simple nuclear localization signal (NLS). This pathway is illustrated schematically from left to right. Cytoplasmic and nuclear compartments are shown separated by the nuclear envelope studded with nuclear pore complexes. In step 1, a nuclear localization signal on the cargo (red) is recognized by importin-α. In step 2, importin-β binds the cargo–importin-α complex and docks onto the nucleus, probably by associating initially with nucleoporins present in the cytoplasmic filaments of the nuclear pore complex. Translocation of the substrate into the nucleus (step 4) requires additional soluble proteins, including the small guanine nucleotide-binding protein Ran (step 3). A Ran-specific guanine nucleotide exchange protein (Rcc1) and a Ran-GTPase-activating protein (RanGap-1) are localized in the nucleus and cytoplasm, respectively. The action of RanGAP-1, with the accessory proteins RanBp1 and RanBp2, maintains cytoplasmic Ran in the GDP-bound form. When Ran is in the GTP-bound form, nuclear import cannot occur. Following import, the complexes are dissociated when Ran-GDP is converted to Ran-GTP by Rcc1. Ran-GTP participates in export from the nucleus. The nuclear pool of Ran-GDP is replenished by the action of the transporter Ntf2/p10, which efficiently transports Ran-GDP from the cytoplasm to the nucleus. Hydrolysis of Ran-GTP in the cytoplasm and GTP-GDP exchange in the nucleus therefore maintain a gradient of Ran-GTP/Ran-GDP. The asymmetric distribution of RanGap-1 and Rcc1 allows for the formation of such a gradient. This gradient provides the driving force and directionality for nuclear transport. (B) Adapted from Q. Yang, M. P. Rout, and C. W. Akey, *Mol. Cell* **1:**223–234, 1998, with permission.

(Fig. 5.22C). This complex then binds importin-β, which mediates docking with the nuclear pore complex by binding to members of a family of nucleoporins. Some of these nucleoporins are found in the cytoplasmic filaments of the nuclear pore complex (Fig. 5.22), which associate with import substrates as seen by electron microscopy. The complex is translocated to the opposite side of the nuclear envelope, where the cargo is released. Other importins can bind cargo proteins directly without the need for an adapter protein. A monomeric receptor called transportin mediates the import of heterogeneous nuclear RNA-binding proteins that contain glycine- and arginine-rich nuclear localization signals. Transportin is related to importin-β, as are other monomeric receptors that mediate nuclear import of ribosomal proteins.

Release of cargo occurs when the importins associate with a small guanosine triphosphate (GTP)-binding protein termed Ran in the GTP form (Fig. 5.22). How these components work together to move the import substrate through the channel of the nuclear pore complex, a distance of more than 100 nm, is not yet well understood. It is clear that a single translocation through the nuclear pore complex does not require energy consumption. However, maintenance of a gradient of the guanosine nucleotide-bound forms of Ran, with Ran-GDP and Ran-GTP concentrated in the cytoplasm and nucleus, respectively, is absolutely essential for continued transport. For example, conversion of Ran-GDP to Ran-GTP in the nucleus, catalyzed by the guanine nucleotide exchange protein Rcc-1, promotes dissociation of imported proteins from importins (Fig. 5.22).

Import of Influenza Virus Ribonucleoprotein

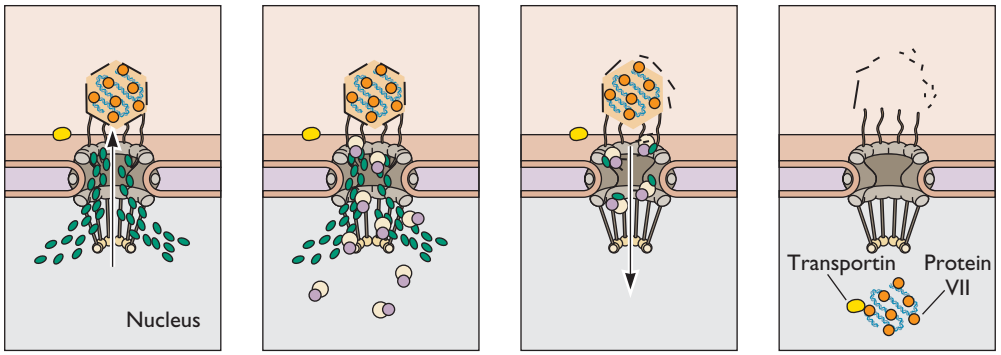
Influenza virus is among the few RNA-containing viruses that replicate in the cell nucleus. After vRNPs separate from M1 and are released into the cytosol, they are rapidly imported into the nucleus (Fig. 5.12). Such import depends on the presence of a nuclear localization signal in the NP protein, a component of vRNPs. Naked viral RNA does not dock onto the nuclear pore complex, nor is it taken up into the nucleus, but in the presence of NP the viral RNA can enter this organelle.

Import of DNA Genomes

The capsids of many DNA-containing viruses are larger than 26 nm and cannot be imported into the nucleus from the cytoplasm. One mechanism for crossing the nuclear membrane involves docking onto the nuclear pore complex, followed by delivery of the viral DNA into the nucleus. Adenoviral and herpesviral DNAs are transported into the nucleus via this mechanism. Partially disassembled adenovirus capsids dock onto the nuclear pore complex through interactions with the filament protein Can/Nup214 (Fig. 5.23). Small quantities of histone H1 from the nucleus bind to hexon proteins on the nuclear side of the viral capsid. The H1 import proteins importin-β and importin-7 recognize H1 bound to hexon, and promote further disassembly. These interactions also promote conformational changes that allow viral protein VII and the viral DNA to associate with transportin. The protein VII-viral DNA complex is imported into the nucleus, where viral transcription begins.

Herpesvirus capsids also dock onto the nuclear pore complex, but undergo only limited disassembly. The viral

Figure 5.23 Uncoating of adenovirus at the nuclear pore complex. After release from the endosome, the partially disassembled capsid docks onto the nuclear pore complex-filament protein Can/Nup214. Histone H1 from the nucleus (green ovals) binds to hexon. Importin-β and importin-7 bind histone H1, leading to further disassembly of the capsid. Once there is sufficient dismantling, the viral DNA, bound to protein VII, is delivered into the nucleus by the import protein transportin.



DNA probably exits through one of the pentameric faces of the capsid and passes through the nuclear pore complex.

Only the smallest capsids can enter the nuclear pore complex without disassembly. The capsids of parvoviruses and hepatitis B virus can be observed intact within the central channel of the complex. Uncoating takes place within the nuclear basket (Fig. 5.22B).

Import of Retroviral Genomes

Fusion of retroviral and plasma membranes releases the viral core into the cytoplasm. The retroviral core consists of the viral RNA genome, coated with NC protein, and the enzymes reverse transcriptase (RT) and integrase (IN), enclosed by CA protein. Retroviral DNA synthesis commences in the cytoplasm, within the nucleocapsid core, and after 4 to 8 h of DNA synthesis the preintegration complex, comprising viral DNA, IN, and other proteins, localizes to the nucleus. There the viral DNA is integrated into a cellular chromosome, and viral transcription begins. The mechanism of nuclear import of the preintegration complex is poorly understood, but it is quite clear that this structure is too large (~60S) to pass through the nuclear pore complex. The betaretrovirus Moloney murine leukemia virus can efficiently infect only dividing cells. These and other observations suggest that exposure of chromatin that occurs during mitosis is essential to allow efficient entry of the preintegration complex of this retrovirus into the nucleus.

In contrast to Moloney murine leukemia virus, human immunodeficiency virus type 1 can replicate in nondividing cells. The preintegration complex of this virus, and probably other lentiviruses, must therefore be transported into an intact nucleus. The exact mechanism by which the DNA of these retroviruses enter the nucleus is still unclear. There is evidence for participation of various viral proteins that contain nuclear localization signals (e.g., Vpr, MA, and IN). Others discount the role of these proteins in import and suggest that breakdown of CA is critical. Such controversy may stem from the complex nature of the import mechanism, possibly comprising more than one pathway.

Avian sarcoma and leukosis viruses, like Moloney murine leukemia virus, do not replicate in nondividing cells. However, it was recently shown that the DNA of these avian retroviruses can be integrated in cell cycle-arrested cells and during interphase in cycling cells, implying a mitosis-independent mechanism of nuclear import. IN protein of these viruses contains a nuclear localization signal in the C-terminal domain which, when fused to heterologous cytoplasmic proteins, can direct them to the nucleus. This protein may have a role in the nuclear import of the preintegration complex of these avian retroviruses.

Perspectives

Since the last edition of this textbook, it has become clear that there are many pathways for virus entry into cells. Clathrin- and dynamin-dependent endocytosis is no longer the sole entry pathway known; other routes are caveolin-dependent endocytosis and clathrin- and caveolin-independent endocytosis. The road used seems to depend on the virus, the cell type, and the conditions of infection. Do additional entry pathways exist that bring viruses into cells? What is the significance of multiple pathways used by the same virus? What pathways of viral entry operate in living animals?

The notion that endocytosis is an unregulated process has been shattered. Of particular interest has been the application of high-throughput small interfering RNA (siRNA) screens to identify host protein kinases that regulate clathrin- and caveolin-mediated endocytosis. The results indicate that vesicular stomatitis virus entry is regulated by 92 kinases while simian virus 40 entry is regulated by 80; 36 kinases are common to both virus entry pathways. Curiously, these 36 kinases have the opposite effects on entry of the two viruses studied. It will be important to determine whether such patterns are common to other virus infections and to identify other cellular genes that regulate these uptake pathways.

The development of single-particle tracking methods has advanced considerably in the past 5 years. As a consequence, our understanding of the routes that viruses travel once they are inside the cell has improved markedly. The role of cellular transport pathways in bringing viruses to the point of replication within the cell is beginning to be clarified. Yet many questions remain. How are viruses transported on the cytoskeletal network? What are the precise virus-host interactions needed? Do viral proteins regulate such transport? What are the signals for a virus to attach to and detach from microtubules and filaments?

It has become clear that virus binding to the cell surface leads to major alterations in cell activities, effects mediated by signal transduction. Virus binding induces the formation of pits, pinching off of vesicles, and rearrangement of actin filaments to facilitate vesicle movement. The precise signaling pathways required need to be elucidated. Such efforts may identify specific targets for inhibiting virus movement in cells.

The genomes of many viruses replicate in the nucleus. Incoming viral genomes enter this cellular compartment by transport through the nuclear pore complex. Studies of adenovirus import into the nucleus have revealed an active role for components of the nuclear pore complex in subviral particle disassembly. What is the molecular basis for this process? What other proteins are involved, and how general is the process? Can it be interrupted therapeutically?

How does the hydrophilic viral DNA pass through the hydrophobic pore, against a steep gradient of nucleic acid in the nucleus? Nuclear import of the lentivirus genome is barely understood. What signal allows transport of the preintegration complex through the nuclear pore?

Nearly all the conclusions discussed in this chapter were derived from studies of viral infection in cultured cells. How viruses attach to and enter cells of a living animal remains an uncharted territory. Methods are being developed to study virus entry in whole animals, and the results will be important for understanding how viruses spread and breach host defenses to reach target cells.

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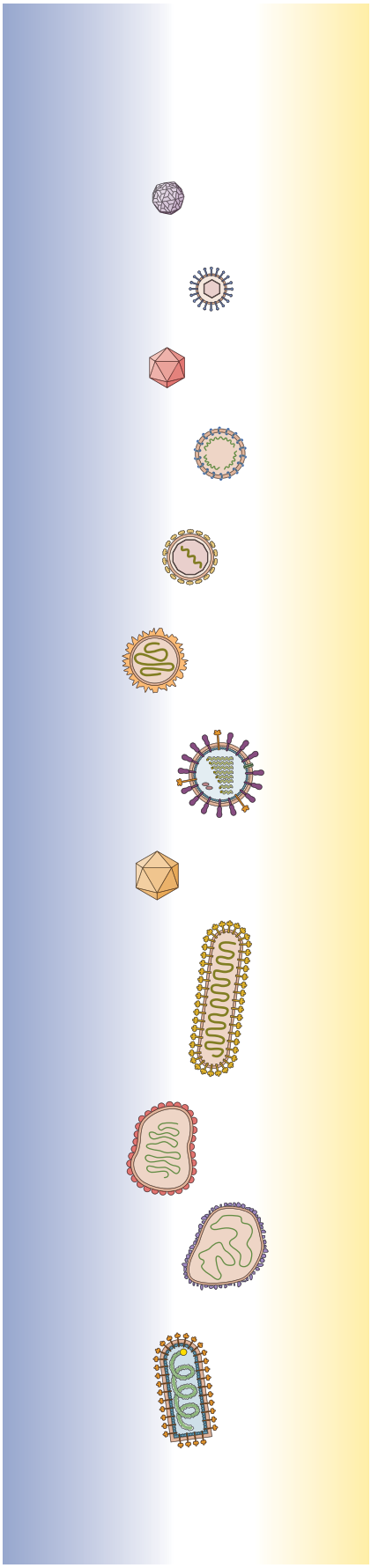


6

Synthesis of RNA from RNA Templates

Truth is ever to be found in the simplicity, and not in the multiplicity and confusion of things.
SIR ISAAC NEWTON

Introduction

The genomes of RNA viruses come in a number of conformations, including unimolecular or segmented, single stranded of (+) or (−) polarity, double stranded, and circular. These structurally diverse viral RNA genomes share a common requirement: they must be efficiently copied within the infected cell to provide both genomes for assembly into progeny virions and messenger RNAs (mRNAs) for the synthesis of viral proteins. The synthesis of these RNA molecules by RNA viruses is a unique process that has no parallel in the cell. The genomes of all RNA viruses except retroviruses (see below) encode an **RNA-dependent RNA polymerase** (Box 6.1) to catalyze the synthesis of new genomes and mRNAs.

The virions of RNA viruses with (−) strand and double-stranded RNA genomes must contain the RNA polymerase, because the incoming viral RNA can be neither translated nor copied by the cellular machinery. The deproteinized genomes of (−) strand and double-stranded RNA viruses are therefore noninfectious. In contrast, viral particles containing a (+) strand RNA genome lack a virion polymerase; the deproteinized RNAs of these viruses **are** infectious because they are translated in cells to produce, among other viral proteins, the viral RNA polymerase.

The mechanisms by which viral mRNA is made and the RNA genome is replicated in cells infected by RNA viruses appear even more diverse than the structure and organization of viral RNA genomes (Fig. 6.1). For example, the genomes of both picornaviruses and alphaviruses are single molecules of (+) strand genomic RNA, but the strategies for the production of viral RNA are quite different. Nevertheless, each mechanism of viral RNA synthesis meets two essential requirements common to the infectious cycles of all these viruses: (i) during replication the RNA genome must be copied from one end to the other with no loss of nucleotide sequence; and (ii) viral mRNAs must be produced that can be translated efficiently by the cellular protein synthetic machinery.

BOX 6.1 TERMINOLOGY

What should we call RNA polymerases and the processes they catalyze?

Historically, viral RNA-dependent RNA polymerases were given two different names depending on their activities during infection. The term **replicase** was used to describe the enzyme that copies the viral RNA to produce additional genomes, while the enzyme that produces mRNA was called **transcriptase.** In some cases this terminology indicates true differences in the enzymes that carry out synthesis of functionally different RNAs. For example, for some RNA viruses, genomic replication and mRNA synthesis are the **same** reaction. For double-stranded RNA viruses, mRNA synthesis produces templates that can also be used for genomic replication. However, these terms can also be inaccurate and misleading, and so are not used here.

The production of mRNAs from viral RNA templates is often designated **transcription.** However, this term refers to a specific process, the copying of genetic information carried in DNA into RNA. Consequently, it is not used here to describe synthesis of the mRNAs of viruses with RNA genomes. Similarly, use of the term **promoter** is reserved to designate sequences controlling transcription of DNA templates.

In this chapter we consider the mechanisms of viral RNA synthesis, the mechanism for switching from mRNA production to genome replication, and how the process of RNA-directed RNA synthesis leads to genetic diversity. Much of our understanding of viral RNA synthesis comes from experiments done with purified components. Because it is possible that events proceed differently in infected cells, the results of such *in vitro* studies are used to build hypothetical models for the different steps in RNA synthesis. While many models exist for each reaction, those presented in this chapter were selected because they are consistent with experimental results obtained in different laboratories.

The general principles of RNA synthesis are illustrated with a few viruses as examples. Members of another family of RNA viruses, the *Retroviridae,* are discussed in Chapter 7. Retroviruses encode an RNA-dependent DNA polymerase, and therefore mRNA synthesis and genome replication are very different from those of other RNA viruses.

The Nature of the RNA Template

Secondary Structures in Viral RNA

RNA molecules are not simple linear chains but can form secondary structures that have important functions. Viral RNA genomes contain secondary-structure elements such as base-paired **stem regions, hairpin loops, bulge loops, interior loops,** and **multibranched loops** (Fig. 6.2). An **RNA pseudoknot** is formed when a single-stranded loop region base pairs with a complementary sequence outside the loop. Such structures are important for RNA synthesis, translation, and assembly.

The first step in identifying a structural feature in RNA is to scan the nucleotide sequence with computer programs designed to fold the RNA into energetically stable structures. Comparative sequence analysis can provide evidence for RNA secondary structures. For example, comparison of the RNA sequences of several related viruses might establish that the structure, but not the sequence, of a stem-loop is conserved. Evidence for RNA structure comes from experiments in which RNAs are treated with enzymes or chemicals that attack single- or double-stranded regions specifically. The results of such analyses can confirm that predicted stem regions are base paired while loop regions are unpaired. Structures of RNA hairpins and pseudoknots have been determined by X-ray crystallography or nuclear magnetic resonance (Fig. 6.2C).

Naked or Nucleocapsid RNA

The genomes of (−) strand viruses are organized into nucleocapsids in which protein molecules, including the RNA-dependent RNA polymerase and accessory proteins, are bound to the genomic RNAs at regular intervals (Fig. 6.3). These tightly wound ribonucleoprotein complexes are very stable and resistant to RNase. The RNA polymerases of (−) strand viruses copy viral RNAs **only** when they are present in the nucleocapsid. For example, vesicular stomatitis virus genomic RNA is a template for RNA polymerase only when it is bound to the nucleocapsid protein N. In contrast, the genomes of (+) strand RNA viruses are not coated with viral proteins in the virion. This structural difference is consistent with the fact that mRNAs are produced from the genomes of (−) strand RNA viruses upon cell entry whereas the genomes of (+) strand RNA viruses are translated.

The viral nucleoproteins are cooperative, single-stranded RNA-binding proteins, as are the single-stranded nucleic acid-binding proteins required during DNA-directed DNA and RNA synthesis. Their function during replication is to keep the RNA single stranded and prevent base pairing between the template and product, so that additional rounds of RNA synthesis can occur. The genomes of many (+) strand RNA viruses encode helicases that serve a similar function (see "Unwinding the RNA Template" below). In addition to its enzymatic activity, polioviral $3D^{pol}$ is a cooperative single-stranded RNA-binding protein and can unwind RNA duplexes without the hydrolysis of ATP

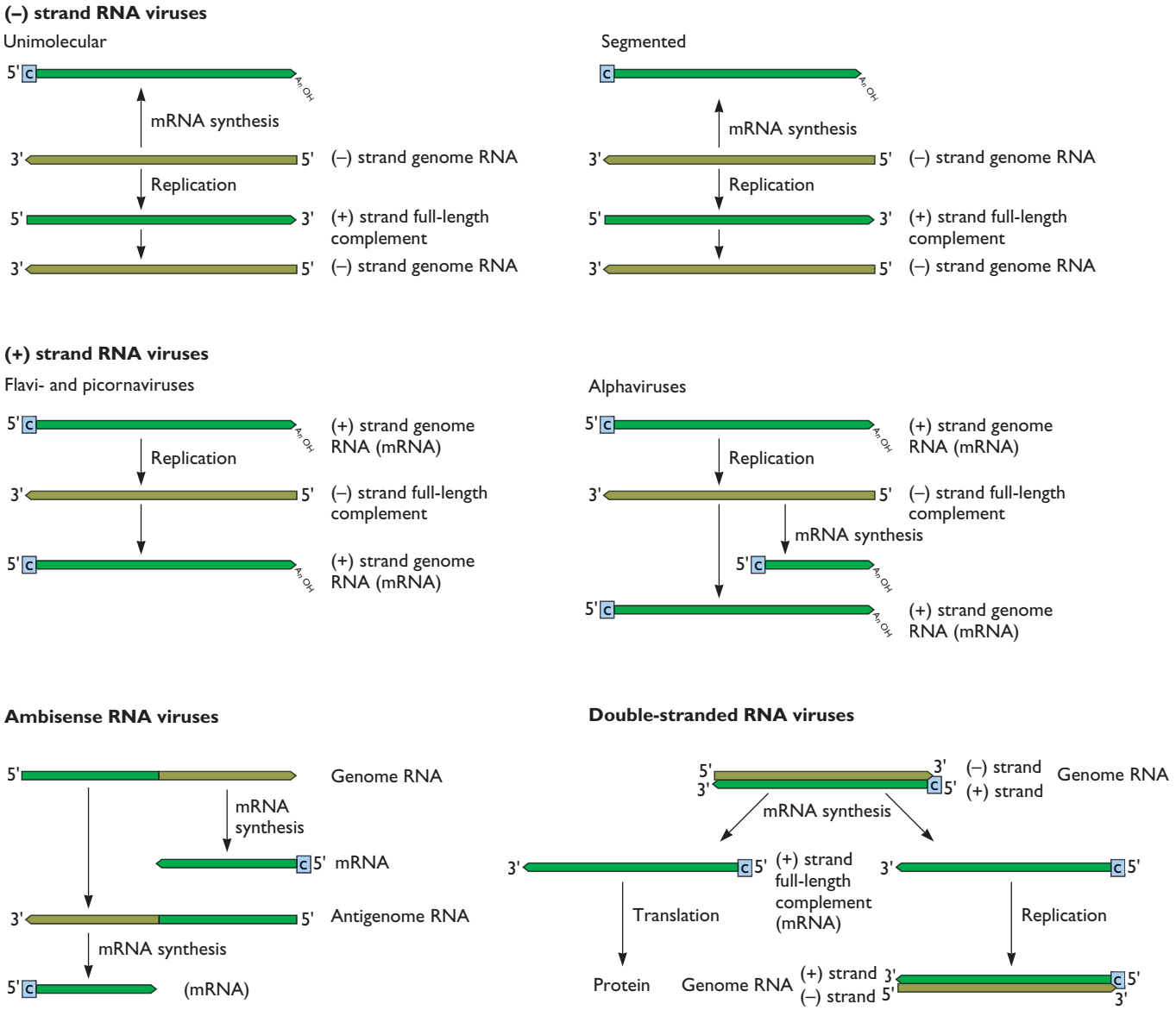


Figure 6.1 Strategies for replication and mRNA synthesis of RNA virus genomes are shown for representative virus families. Picornaviral genomic RNA is linked to VPg at the 5′ end. The (+) genomic RNA of some flaviviruses does not contain poly(A). Only one RNA segment is shown for segmented (–) strand RNA viruses.

characteristic of helicase-mediated unwinding. Polioviral RNA polymerase is therefore functionally similar to the RNA-binding nucleoproteins of (–) strand viruses.

The RNA Synthesis Machinery

Identification of RNA-Dependent RNA Polymerases

The first evidence for a viral RNA-dependent RNA polymerase emerged in the early 1960s from studies with mengovirus and poliovirus, both (+) strand RNA viruses. In these experiments, extracts were prepared from virus-infected cells and incubated with the four ribonucleoside triphosphates (adenosine triphosphate [ATP], uridine triphosphate [UTP], cytosine triphosphate [CTP], and guanosine triphosphate [GTP]), one of which was radioactively labeled. The incorporation of nucleoside monophosphate into RNA was then measured. Infection with mengovirus or poliovirus led to the appearance of a cytoplasmic enzyme that could synthesize viral RNA in the presence of actinomycin D, a drug that was known to inhibit cellular DNA-directed RNA synthesis. Lack of sensitivity to actinomycin

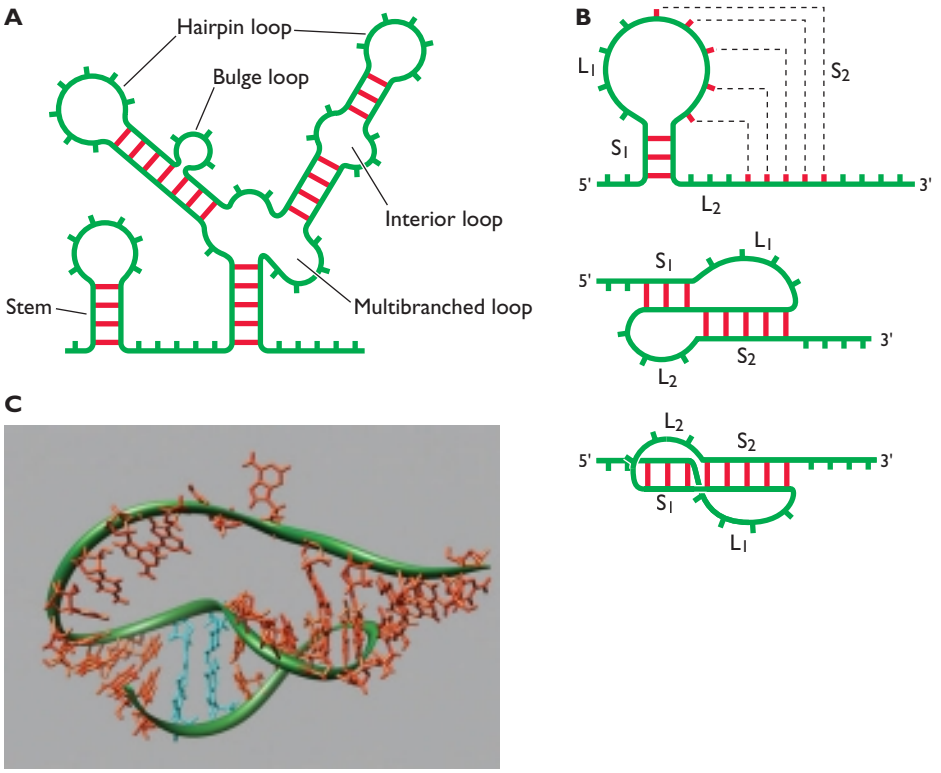


Figure 6.2 RNA secondary structure. (A) Schematic of different structural motifs in RNA. Red bars indicate base pairs; green bars indicate unpaired nucleotides. **(B)** Schematic of a pseudoknot. (Top) Stem 1 (S_1) is formed by base pairing in the stem-loop structure, and stem 2 (S_2) is formed by base pairing of nucleotides in the loop with nucleotides outside the loop. (Middle) A different view of the formation of stems S_1 and S_2. (Bottom) Coaxial stacking of S_1 and S_2 resulting in a quasicontinuous double helix. **(C)** Structure of a pseudoknot as determined by X-ray crystallography. The sugar backbone is highlighted with a green tube. Stacking of the bases in the areas of S_1 and S_2 can be seen. From Protein Data Bank file 1l2x. Adapted from C. W. Pleij, *Trends Biochem. Sci.* **15:**143–147, 1990, with permission.

D suggested that the enzyme was virus specific and could copy RNA from an RNA template and not from a DNA template. This enzyme was presumed to be an RNA-dependent RNA polymerase. Several years later, similar assays were used to demonstrate that the virions of (–) strand viruses and of double-stranded RNA viruses contain an RNA-dependent RNA polymerase that synthesizes mRNAs from the (–) strand RNA present in the virus particles.

The initial discovery of a putative RNA polymerase in poliovirus-infected cells was followed by attempts to purify the enzyme and show that it can copy viral RNA. Because polioviral genomic RNA contains a 3′ poly(A) sequence, polymerase activity was measured with a poly(A) template and an oligo(U) **primer**. After several fractionation steps, a poly(U) polymerase that could copy polioviral genomic RNA in the presence of an oligo(U) primer was purified from infected cells. Poly(U) polymerase activity coincided with a single polypeptide, now known to be the polioviral RNA polymerase $3D^{pol}$ (see Appendix, Fig. 13, for a description of this nomenclature). Purified $3D^{pol}$ RNA polymerase cannot copy polioviral genomic RNA in the absence of a primer; it is therefore a template- and primer-dependent enzyme.

Assays for RNA polymerase activity have been used to demonstrate virus-specific enzymes in virions or in extracts of cells infected with a wide variety of RNA viruses. Amino acid sequence alignments (see "Sequence Relationships among RNA Polymerases" below) can be used to identify viral proteins with motifs characteristic of RNA-dependent RNA polymerases. These approaches have been used to identify the L proteins of paramyxoviruses and bunyaviruses, the PB1 protein of influenza viruses, and the nsP4 protein of alphaviruses as candidate RNA polymerases. When the genes encoding these polymerases are expressed in cells, the proteins that are produced can copy viral RNA templates.

RNA-directed RNA synthesis follows a set of universal rules that differ slightly from those followed by DNA-dependent DNA polymerases. RNA synthesis initiates and

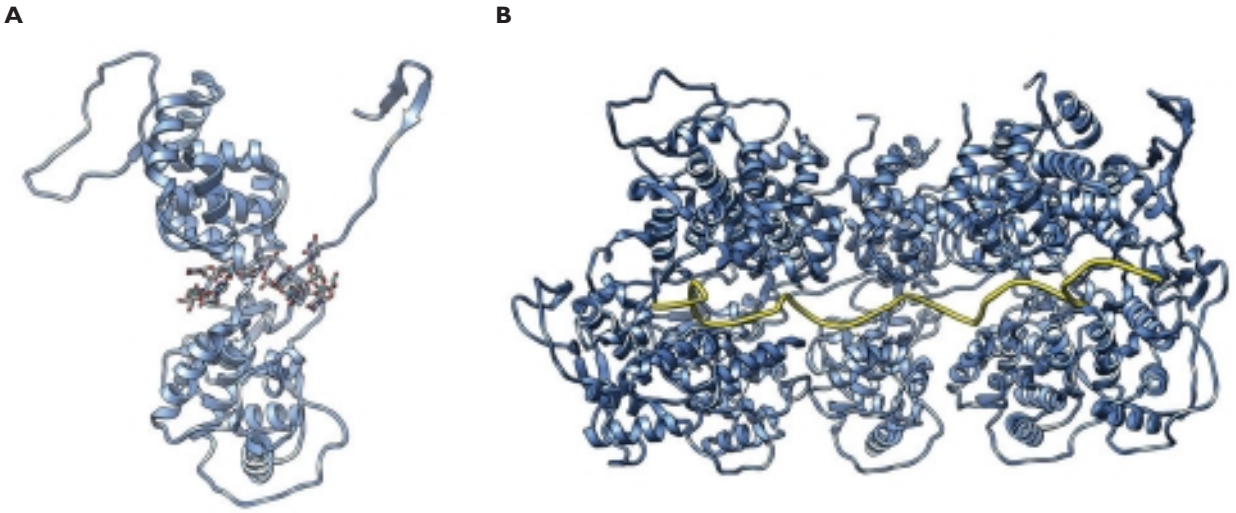


Figure 6.3 Structure of vesicular stomatitis N protein bound to RNA. (A) Ribbon diagram of the N protein monomer bound to a 9-nucleotide RNA. The RNA (stick representation) is bound in a groove located between N- and C-terminal lobes of the protein. **(B)** Structure of five N molecules bound to RNA. The ribose-phosphate backbone of the RNA is shown as a yellow tube. From Protein Data Bank file 2qvj.

terminates at specific sites in the template and is catalyzed by virus-encoded polymerases, but viral accessory proteins and even host cell proteins may also be required. Like cellular DNA-dependent RNA polymerases, some RNA-dependent RNA polymerases can initiate RNA synthesis *de novo*. Others require a primer with a free 3′-OH end to which nucleotides complementary to the template strand are added. Some RNA primers are protein linked, while others bear a 5′ cap structure (the cap structure is described in Chapter 10). A comparison of the structures and sequences of polynucleotide polymerases (see below) has led to the hypothesis that all polymerases catalyze synthesis by a mechanism that requires two metals (Box 6.2). RNA is usually synthesized by template-directed, stepwise incorporation of ribodeoxynucleoside monophosphates (NMPs) into the 3′-OH end of the growing RNA chain, which undergoes **elongation** in the 5′ → 3′ direction. Examples of nontemplated synthesis of viral RNA in cells infected with viruses of the *Paramyxoviridae* and *Filoviridae* have been described (Chapter 10).

Sequence Relationships among RNA Polymerases

The amino acid sequences of viral RNA polymerases have been compared to identify conserved regions and to provide information about the evolution of these enzymes. Although polymerases have very different amino acid sequences, four common motifs (A to D) have been identified in all polymerases (Fig. 6.3). Motif C includes a Gly-Asp-Asp sequence conserved in the RNA polymerases of most (+) strand RNA viruses. It was suggested that this sequence is part of the active site of the enzyme. In support of this hypothesis, alterations in this sequence in polioviral $3D^{pol}$ and many other viral polymerases produce an inactive enzyme. Evidence that a viral protein is an RNA polymerase is considerably strengthened when this 3-amino-acid sequence is found (Box 6.3).

Motifs A to D are also present in the sequences of polymerases that copy DNA templates (Fig. 6.4). These sequence comparisons indicate that all four classes of nucleic acid polymerases have a similar core catalytic domain (the palm domain [see below]) and most probably evolved from a common ancestor.

Three-Dimensional Structure of RNA-Dependent RNA Polymerases

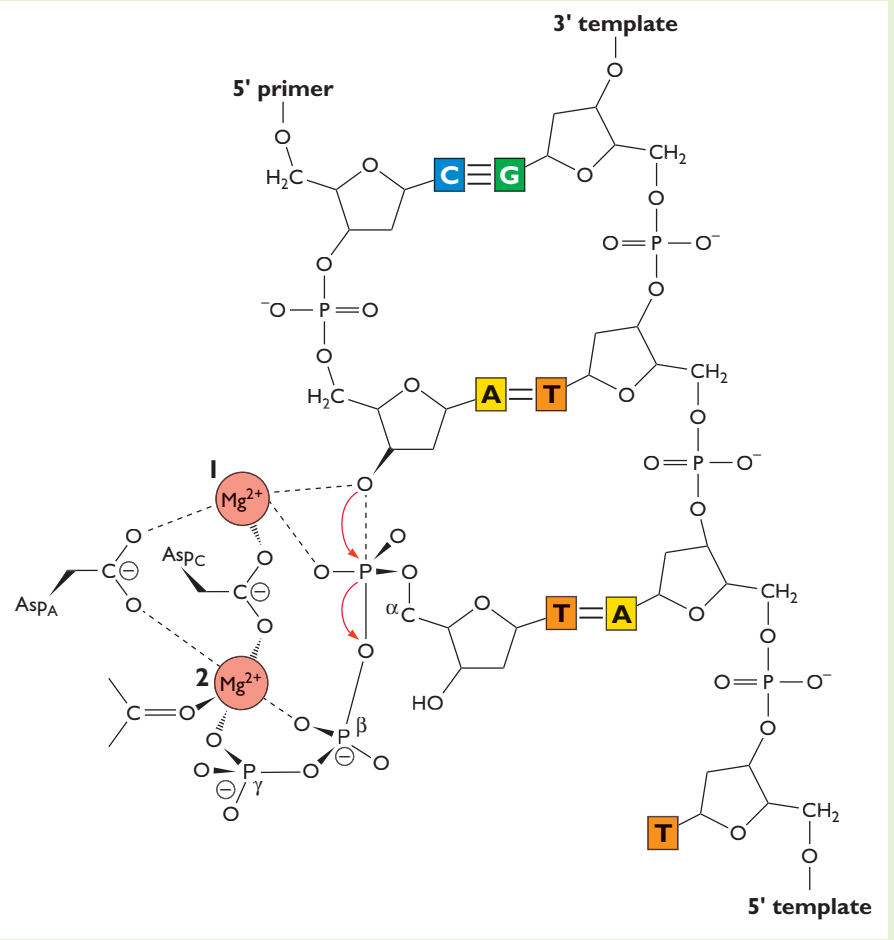
Determination of the crystal structures of more than 10 RNA-dependent RNA polymerases has confirmed the hypothesis that all polynucleotide polymerases are similar structurally. The shapes of all four types of polymerases resemble a right hand consisting of a palm, fingers, and a thumb, with the active site of the enzyme located in the palm (see Fig. 7.12). This shape provides the correct arrangement of substrates and metal ions at the catalytic site. The structures of RNA-dependent RNA polymerases differ in detail from those of other polymerases, presumably to accommodate different templates and priming mechanisms.

All the available structures of RNA-dependent RNA polymerases show that the enzymes adopt closed structures

BOX 6.2 BACKGROUND

Two-metal mechanism of catalysis by polymerases

All polynucleotide polymerases are thought to catalyze synthesis by a two-metal mechanism that requires two conserved aspartic acid residues, one in motif A and one in motif C (see figure). The carboxylate groups of these amino acids coordinate two divalent metal ions, shown as Mg^{2+} in the figure. One metal ion promotes deprotonation of the 3′-OH group of the nascent strand, and the other ion stabilizes the transition state at the α-phosphate of NTP and facilitates the release of pyrophosphate (PP_i).



Two-metal mechanism of polymerase catalysis. Red arrows indicate the net movement of electrons.

in which the active site is completely encircled (Fig. 6.5). In contrast, structures of other polynucleotide polymerases resemble an open hand (Fig. 6.6). The closed structure, which is formed by interactions between the fingers and thumb domains, creates a nucleoside triphosphate (NTP) entry tunnel at the back of the enzyme and a template-binding site at the front. Residues within motif F, a conserved region unique to RNA-dependent RNA polymerases (Fig. 6.4), form the NTP entry tunnel.

The palm domain of RNA-dependent RNA polymerases is structurally similar to that of other polymerases and contains the four motifs (A to D) that are conserved in all polymerases (Fig. 6.6). The motifs confer specific functions, such as nucleotide recognition and binding (A and B), phosphoryl transfer (A and C), and mediation of the structure of the palm domain (D). The fifth motif, E, which is present in RNA-dependent, but not in DNA-dependent, polymerases, lies between the palm and thumb domains. It binds the nucleotide primer.

RNA-dependent RNA polymerases prefer to incorporate NTPs over deoxyribonucleoside triphosphates (dNTPs). NTP recognition by poliovirus $3D^{pol}$ is regulated by Asp238, which forms a hydrogen bond with the ribose 2′-OH (Fig. 6.7). dNTPs are not bound because Asp238 cannot form a hydrogen bond with 2′-deoxyribose. An Asp is present at this position in all RNA-dependent RNA polymerases.

BOX 6.3 BACKGROUND

The Gly-Asp-Asp sequence of RNA polymerase motif C

The Asp-Asp sequence of motif C is also conserved in RNA-dependent DNA polymerases of retroviruses and in RNA polymerases of double-stranded RNA and segmented (–) strand viruses. The RNA polymerases of nonsegmented (–) strand viruses contain Gly-Asp-Asn instead of Gly-Asp-Asp. Mutational studies have shown that this sequence in the L protein of vesicular stomatitis virus is essential for RNA synthesis. The RNA polymerase of birnavirus, an insect virus with a double-stranded RNA genome, has Ala-Asp-Asn instead of Gly-Asp-Asp. Substitution of Ala-Asp-Asn with Gly-Asp-Asp produces an RNA polymerase with increased synthetic activity. This observation has led to the suggestion that Ala-Asp-Asn may have been selected during the evolution of these birnaviruses to reduce pathogenicity and facilitate virus spread.

A Tyr at this position in RNA-dependent DNA polymerase is responsible for discriminating against NTPs and selecting dNTPs. Motif C of $3D^{pol}$ contains the Asp-Asp sequence conserved in RNA-dependent polymerases; the first Asp is also conserved in DNA-dependent polymerases. The two Asp residues of motif C and the conserved Asp238 of motif A form a cluster that coordinates the triphosphate moiety of the NTP and the metal ions required for catalysis (Fig. 6.7).

The binding site on the RNA polymerase for the RNA template has been revealed by structural studies of foot-and-mouth disease virus $3D^{pol}$ complexed with a molecule of RNA that serves as both template and primer (Fig. 6.8). The phosphodiester backbone of the template RNA contacts basic amino acid residues in a channel on the polymerase. These contacts propel the template toward the active site. The template is near the fingers domain, while the phosphodiester backbone of the primer interacts with an α-helix of the thumb domain. Amino acids in motifs C and E of the palm domain stabilize the 3′ end of the primer to facilitate elongation. The double-stranded region of the nucleic acid, which mimics the double-stranded product, runs from the active site to the protein C terminus. This structure indicates that the product would exit the polymerase from the large central cavity.

Mechanisms of RNA Synthesis

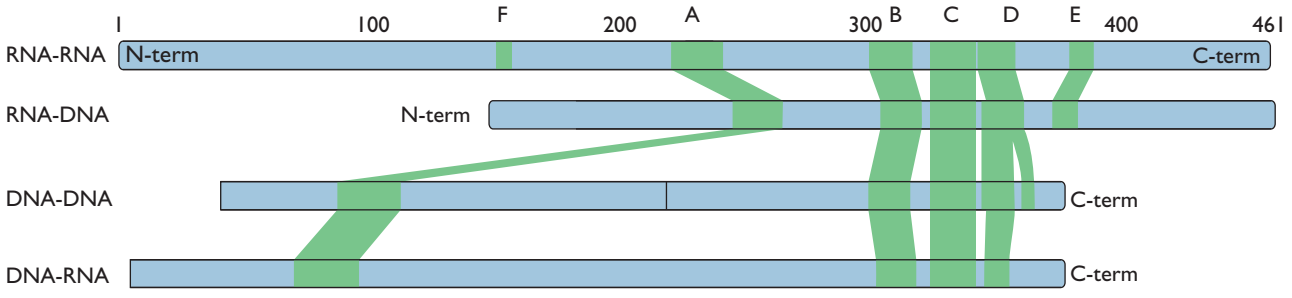
Initiation

The requirement for a primer in the initiation step of nucleic acid synthesis varies among the different classes of polymerases (Fig. 6.9). All DNA polymerases are primer-dependent enzymes, while DNA-dependent RNA polymerases initiate RNA synthesis *de novo*. Some RNA-dependent RNA polymerases can initiate RNA synthesis without a primer, while others require a primer. Nucleic acid synthesis by these RNA polymerases is initiated by a protein primer or an oligonucleotide cleaved from the 5′ end of cellular mRNA.

De Novo Initiation

The requirements for *de novo* initiation are an RNA-dependent RNA polymerase, an RNA template, the initiating NTP, and a second NTP. The first phosphodiester bond is made between the 3′-OH of the initiating NTP and the second NTP. Elongation usually follows immediately.

Figure 6.4 Protein domain alignments for the four categories of nucleic acid polymerases. Numbers at the top are from the $3D^{pol}$ amino acid sequence. Sequence and structure motifs in each polymerase category are shaded green, and the alignments between the different polymerases are shown in green. Motif F is found only in RNA-dependent RNA polymerases. Adapted from J. L. Hansen et al., *Structure* **5**:1109–1127, 1997, with permission. Courtesy of S. Schultz, University of Colorado, Boulder.



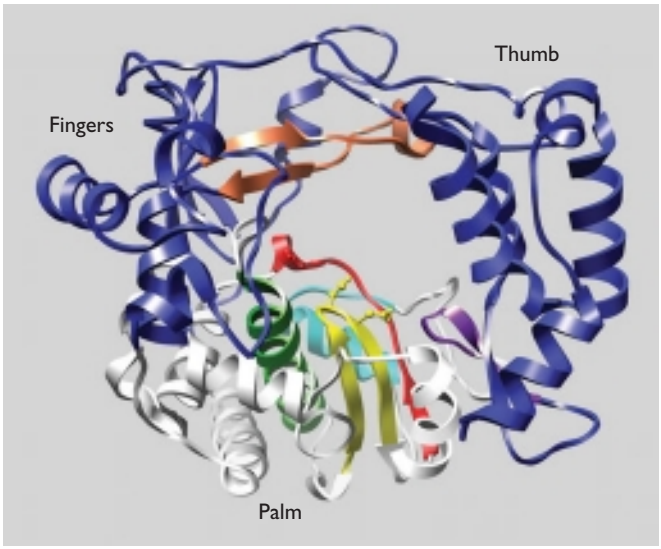


Figure 6.5 Structure of polioviral 3D^{pol}. The thumb, palm, and fingers domains are labeled. Fingers and thumb domains are blue. Conserved structure/sequence motifs are colored as follows: motif A, red; B, green; C, yellow; D, cyan; E, purple; F, orange. From Protein Data Bank file 1ra6.

In most cases initiation takes place at the exact 3′ end of the template, but it may instead occur internally, for example during replication of the genomes of some (−) strand RNA viruses, such as bunyaviruses, arenaviruses, and nairoviruses (Fig. 6.9). Initiation begins at an internal C, and after extension of a few nucleotides, the daughter strand is shifted in the 3′ direction so that the 5′-terminal G residue is not base paired with the template strand. Because the daughter strand slips, this mechanism is called "prime and realign."

The structures of RNA polymerases that initiate *de novo* have a less accessible active site compared with polymerases that initiate with a primer. The thumb domains of some RNA polymerases, such as the hepatitis C virus and bacteriophage ϕ6 RNA polymerases, are large and partially block the active site (Fig. 6.10). This conformation produces two positively charged tunnels that allow access of RNA template and NTP. The NTP tunnel is formed by the fingers and palm domains. The template tunnel, which is formed by the thumb and fingers, is wide enough to accommodate single-stranded but not double-stranded RNA. A β-hairpin that protrudes from the thumb domain toward the active site of the hepatitis C virus RNA polymerase appears to be important for correct positioning of the 3′ terminus of the viral RNA.

Primer-Dependent Initiation

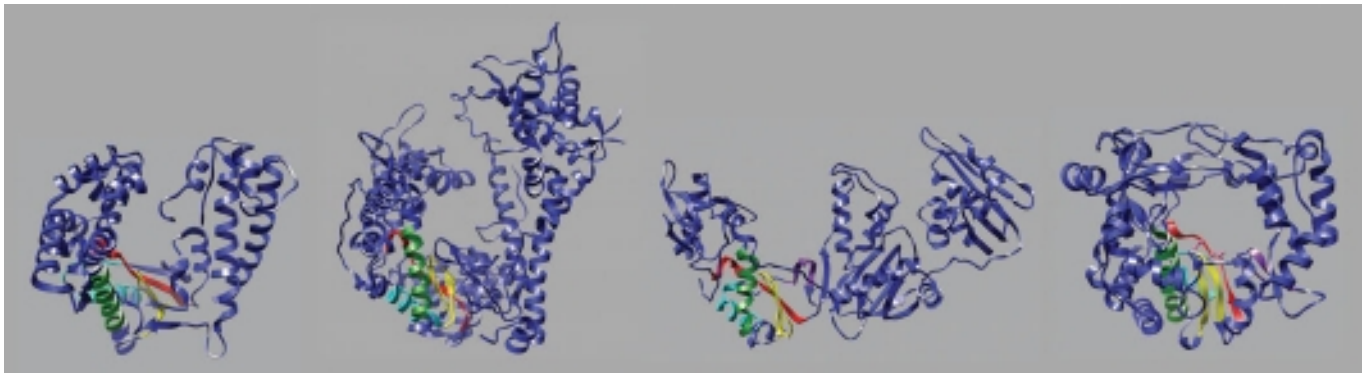
Protein priming. A protein-linked oligonucleotide serves as a primer for RNA synthesis by some RNA polymerases. Protein priming also occurs during DNA replication of adenoviruses and certain DNA-containing bacteriophages (Chapter 9). Picornaviral 3D^{pol} is a primer-dependent enzyme that does not copy viral RNA *in vitro* without an oligo(U) primer. Polioviral genomic RNA, as well as newly synthesized (+) and (−) strand RNAs, are covalently linked at their 5′ ends to the 22-amino acid protein VPg (Fig. 6.11A), suggesting that VPg might function as a primer for RNA synthesis. This hypothesis was supported by the discovery of a uridylylated form of the protein, VPg-pUpU, in infected cells. VPg can be uridylylated *in vitro* by 3D^{pol} and then can prime the synthesis of VPg-linked poly(U) from a poly(A) template RNA. The template for uridylylation of VPg is an RNA hairpin, the *cis*-acting replication element (cre), located in the coding region of picornaviruses (Fig. 6.11B and C). VPg-pUpU is likely to serve as a primer for viral RNA synthesis in infected cells (Fig. 6.12).

Structures of the RNA polymerases of different picornaviruses indicate that the active site is more accessible than in polymerases with a *de novo* mechanism of initiation. The thumb domains of picornaviral polymerases are small, which leaves a wide central cavity that can accommodate the template primer complex (Fig. 6.8) and the protein primer (Fig. 6.13). Binding of VPg in the central cavity is mediated by the interaction of the protein with motif F and portions of the fingers and thumb domains. This interaction places the third amino acid of VPg, tyrosine, which is linked to the first U, in the active site of 3D^{pol}.

Protein priming by the birnavirus RNA polymerase VP1 is unusual because the primer is the polymerase, not a separate protein. VP1 has self-guanylylation activity, which occurs in the absence of a template. The guanylylation site is a serine located approximately 23 Å from the catalytic site of the polymerase. The long distance between them suggests that guanylylation may be carried out at a second active site. After two G residues are added to VP1, it binds to the conserved CC sequence at the terminus of the viral RNA template to initiate nucleotide synthesis. The 5′ ends of mRNAs and genomic double-stranded RNAs produced by this reaction are therefore linked to a VP1 molecule.

Priming by capped RNA fragments. Influenza virus mRNA synthesis is blocked by treatment of cells with the fungal toxin α-amanitin at concentrations that inhibit cellular DNA-dependent RNA polymerase II. In contrast, the toxin does not affect viral mRNA synthesis *in vitro*. This surprising finding demonstrated that the viral RNA polymerase is dependent on a host nuclear function. Inhibition by α-amanitin is explained by the requirement for a continuous supply of newly synthesized cellular RNA polymerase II transcripts to provide primers for viral mRNA synthesis. Nuclear RNA polymerase II transcripts are cleaved by a virus-encoded, cap-dependent endonuclease that is part

A



Klenow T7 RNAP HIV-1 RT Poliovirus polymerase

B

Motif A

Poliovirus	225	M E E K L F A F **D** Y T G Y D A S L	241
HIV-1 RT	102	K K K S V T V L **D** V G D A Y F S V	118
Klenow	697	E D Y V I V S A **D** Y S Q I E L R I M A H L S R	719
T7 RNAP	529	N C S L P L A F **D** G S C S G I Q H F S A M L R	551

Motif B

Poliovirus	278	K T Y C V K G G M **P S G** C S G T S I F N S M I N N L I I R T L L L K T	312
HIV-1 RT	141	G I R Y Q Y N V L **P Q G** W K G S P A I F Q S S M T K I L E P F K K Q N	175
Klenow	833	G A R R A A A E R A A I N A P M Q G T A A D I I K R A M I A V D A W L Q A E	870
T7 RNAP	168	E I D A H K Q E S G I A P N F V H S Q D G S H I R K T V V W A H E K Y G	803

Motif C

Poliovirus	313	Y K G I D L D H L K M I A **Y G D D** V I A S Y P H E	337
HIV-1 RT	176	P D I V I Y Q **Y M D D** L Y V G S D L E	194
Klenow	871	Q P R V R M I M Q **V H D E** L V F E V H K D	891
T7 RNAP	804	I E S F A L **I H D S** F G T I	817

Motif D

Poliovirus	338	V D A S L L A Q S G K D Y **G** L T M T P A D **K** S A T	362
HIV-1 RT	195	I G Q H R T K I E E L R Q H L L R W **G** L T T P D • K **K** H Q K	223
Klenow	892	D V D A V A K Q I H Q L M E N C * L L V E V G S G E N W D Q A H	928
T7 RNAP	818	P A D A A N L F K A V R E T	831

Motif E

Poliovirus	363	F E T V T W E N V T F L K R F F R A	To thumb	380
HIV-1 RT	224	E P P F L W M G Y E L H P	To thumb	236

Figure 6.6 Polymerase structure and sequence motifs in representative structures of each of the four types of nucleic acid polymerases. (A) Ribbon diagrams of the polymerase domain of the large (Klenow) fragment of *Escherichia coli* DNA polymerase I, a DNA-dependent DNA polymerase; T7 RNA polymerase (T7 RNAP), a DNA-dependent RNA polymerase; human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT), an RNA-dependent DNA polymerase; and polioviral $3D^{pol}$, an RNA-dependent RNA polymerase. The thumb domain is at the right, and the fingers domain is at the left. The conserved structure/sequence motifs A, B, C, D, and E are red, green, yellow, cyan, and purple, respectively. From Protein Data Bank files 1qsl, 1s77, 3hvt, and 1ra6. **(B)** Sequence alignments based on crystal structures. Boxes are color coded to match the motifs in panel A. The most highly conserved amino acids are in bold type. Double lines are regions in which structures differ. Adapted from J. L. Hansen et al., *Structure* **5**:1109–1127, 1997, with permission. Courtesy of S. Schultz, University of Colorado, Boulder.

Figure 6.7 Structure of UTP bound to poliovirus 3D^{pol}. The NTP bridges the fingers (top) and palm (bottom) domains. The base is stacked with Arg174 from the fingers. Hydrogen bonds are shown as cyan lines. The Asp238 of motif A, which is conserved in all RNA-dependent RNA polymerases, hydrogen bonds with the 2′-OH of the ribose moiety; this interaction discriminates NTPs from dNTPs. Asp328 and Asp329, which coordinate Mg^{2+}, are also labeled. Produced from Protein Data Bank file 2im2.

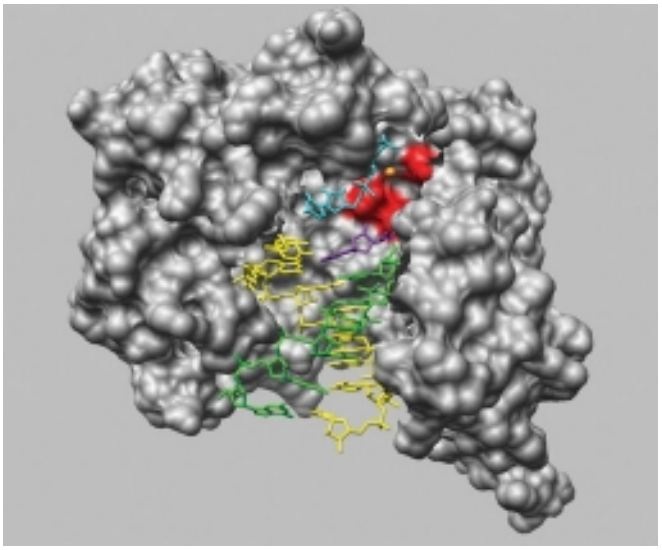


Figure 6.8 Structure of foot-and-mouth disease virus 3D^{pol} complexed with a template-primer RNA, ATP, and UTP. The enzyme is shown in a surface representation (gray). Parts of the enzyme have been removed to reveal the substrate cavities. Catalytic aspartates are red. The Mg^{2+} ion is an orange ball. The bound RNA is shown as a stick representation; the template is yellow, and the primer is green. The ATP, which is incorporated into the primer, is purple, and the UTP is cyan. From Protein Data Bank file 2e9z.

of the RNA polymerase (Fig. 6.14B). The resulting 10- to 13-nucleotide capped fragments serve as primers for the initiation of viral mRNA synthesis.

Bunyaviral mRNA synthesis is also primed with capped fragments of cellular RNAs. In contrast to that of influenza virus, bunyaviral mRNA synthesis is not inhibited by α-amanitin because it occurs in the cytoplasm, where there are many capped cellular RNAs.

Elongation

All nucleic acid synthesis begins with the formation of a complex of polymerase, template-primer, and initiating NTP, followed by a conformational change that reorients the triphosphate moiety. This change leads to phosphoryl transfer, and incorporation of the nucleoside monophosphate into the 3′ terminus of the primer or the growing chain. During synthesis by T7 RNA polymerase, the NTP first binds in a preinsertion site. The enzyme then undergoes conformational changes that push the NTP into the active site for catalysis. NTP has also been observed bound in a preinsertion site in the fingers domain of picornaviral 3D^{pol} (Fig. 6.7); no major movements of domains are observed when NTPs are bound. However, 3D^{pol} is known to undergo conformational changes after binding template RNA and the initiating NTP. Therefore, it is hypothesized that movements of the fingers domain occur that push NTPs into the catalytic site. The structures of elongation complexes of foot-and-mouth disease virus 3D^{pol} support this model. These structures were obtained by incubating cocrystals of 3D^{pol} and a template-primer RNA with different NTPs. For example, when ATP was added to the cocrystals, it was hydrolyzed and incorporated into the primer molecule, and the double-stranded RNA was translocated. The 3′ end of the template strand, the newly incorporated adenine, was positioned at the active site of the enzyme. When UTP was added to these cocrystals, it became positioned close to, but not at, the active site. Presumably this structure represents the state just before the fingers domain pushes the UTP into the active site. These structures show that Asp245 (Fig. 6.6, motif A) and Asn307 (motif B) play an important role in recognition of the NTP and positioning the ribose in its binding pocket.

Template Specificity

Viral RNA-dependent RNA polymerases must select viral templates in a vast excess of cellular mRNAs and then initiate correctly to ensure accurate RNA synthesis. Different mechanisms that contribute to template specificity have been identified. Initiation specificity may be regulated by the affinity of the RNA polymerase for the initiating nucleotide. For example, the RNA polymerase of bacteriophage ϕ6 prefers

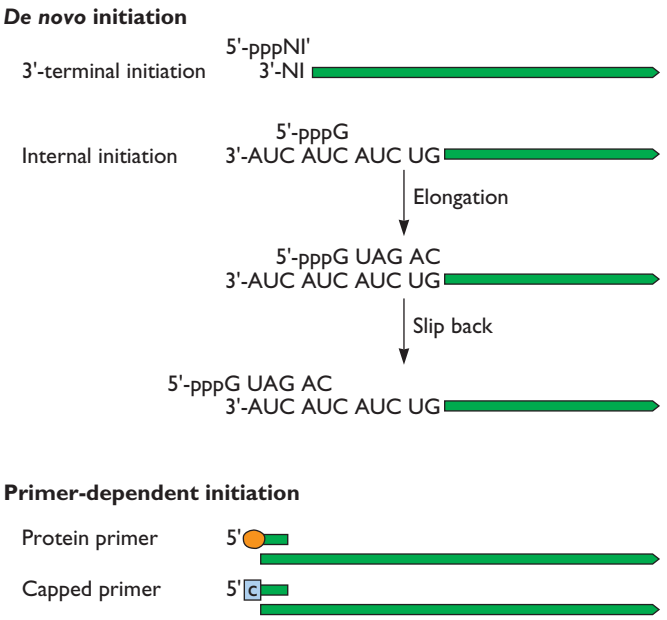


Figure 6.9 Mechanisms of initiation of RNA synthesis. *De novo* initiation may occur at the 3′ end of the viral RNA or from an internal base. When a primer is required, it may be a capped or protein-linked oligonucleotide.

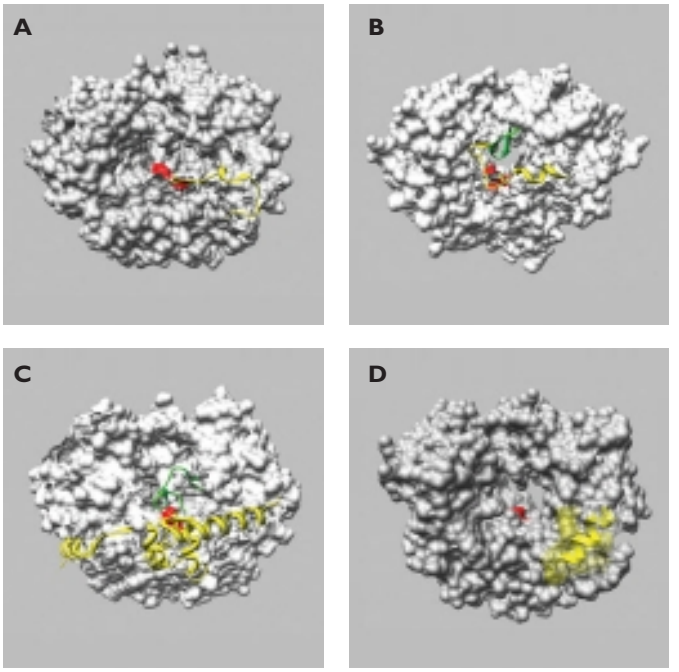


Figure 6.10 Partially blocked active site of flavivirus and bacteriophage ϕ6 RNA polymerases. RNA polymerase of Norwalk virus **(A)**, hepatitis C virus **(B)**, bacteriophage ϕ6 **(C)**, and poliovirus **(D)** are shown in surface representation. The carboxy terminus is shown in ribbon representation and colored yellow. Loop insertions of hepatitis C virus and bacteriophage ϕ6 enzymes are green. From Protein Data Bank files 1sh2, 1c2p, 1hhs, and 1ra6.

3′-terminal C, while the N3 and C4 amino groups of the first C of the template are important for bovine viral diarrhea virus RNA synthesis. Reovirus RNA polymerase prefers a G at the second position of the template RNA. This preference is controlled by hydrogen bonding of carbonyl and amino groups of the G with two amino acids of the enzyme.

Template specificity may also reside in the recognition of RNA sequences or structures at the 5′ and 3′ ends of viral RNAs by viral proteins. RNA synthesis initiates specifically within a polypyrimidine tract in the 3′ untranslated region of hepatitis C virus RNA. The 3′ noncoding region of polioviral genomic RNA contains an RNA pseudoknot structure that is conserved among picornaviruses (Fig. 6.11B). Protein 3AB-3CD binds this structure and may direct the polymerase to that site for the initiation of (–) strand RNA synthesis (Box 6.4). Polioviral $3CD^{pro}$ protein plays an important role in viral RNA synthesis by participating in the formation of a ribonucleoprotein at the 5′ end of the (+) strand RNA. The 3CD protein is a precursor of the $3C^{pro}$ protease and $3D^{pol}$. Protein 3CD, together with a cellular protein known as poly(rC)-binding protein 2, binds to a cloverleaf structure in the viral RNA (Fig. 6.12). The RNA-binding domain of 3CD is contained within the 3C portion of the protein, and alterations within this domain inhibit complex formation and RNA synthesis without affecting viral protein processing.

Internal RNA sequences may confer initiation specificity to RNA polymerases. The *cis*-acting replication elements (cre) in the coding sequence of poliovirus protein 2C and rhinovirus capsid protein VP1 contain short RNA sequences that are required for RNA synthesis. These sequences are binding sites for $3CD^{pro}$ and serve as a template for the priming of viral RNA synthesis by VPg protein (Fig. 6.12).

During mRNA synthesis by influenza virus polymerase, sequences at the RNA termini play an important role in ensuring that the 5′ ends of newly synthesized influenza virus mRNAs are not cleaved and used as primers (Fig. 6.15). If such cleavage were to occur, there would be no net synthesis of viral mRNAs. The three P proteins form a multisubunit assembly that can neither bind to capped primers nor synthesize mRNAs. Addition of a sequence corresponding to the 5′-terminal 11 nucleotides of the viral RNA, which is highly conserved in all eight genome segments, activates the cap-binding activity of the P proteins. The PB1 protein binds this RNA sequence and activates the cap-binding PB2 subunit, probably by conformational change. Concomitantly with activation of cap binding, the P proteins acquire the ability to bind to a conserved sequence at the 3′ ends of genomic RNA segments. This second interaction activates the endonuclease that cleaves

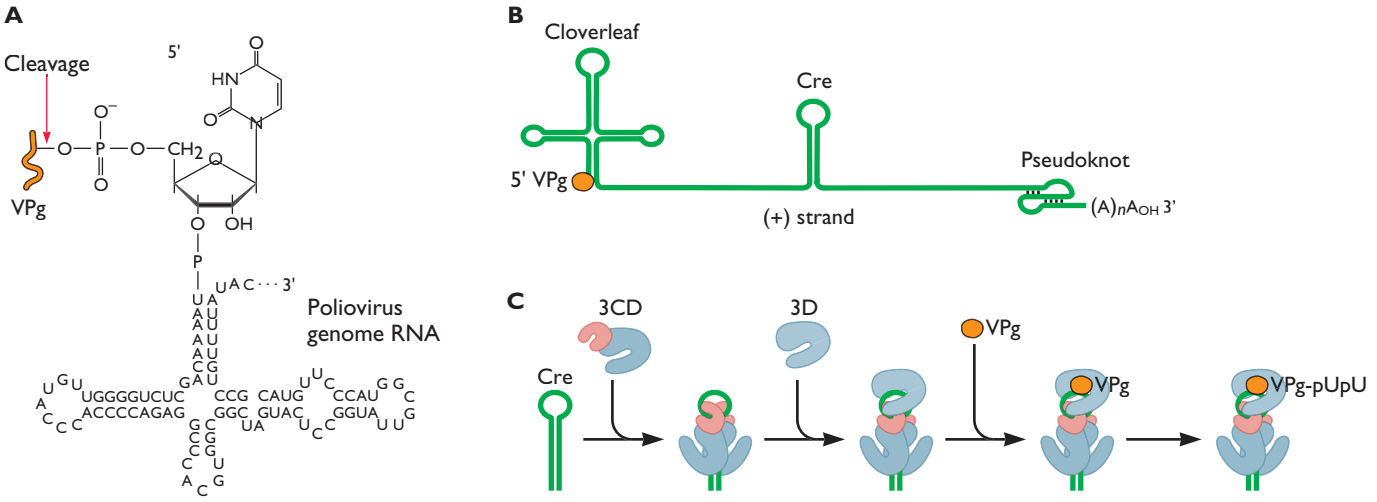


Figure 6.11 Uridylylation of VPg. (A) Linkage of VPg to polioviral genomic RNA. Polioviral RNA is linked to the 22-amino-acid VPg (orange) via an *O*4-(5′-uridylyl)-tyrosine linkage. This phosphodiester bond is cleaved at the indicated site by a cellular enzyme to produce the viral mRNA containing a 5′-terminal pU. **(B)** Structure of the poliovirus (+) strand RNA template, showing the 5′ cloverleaf structure, the internal cre (*cis*-acting replication element) sequence, and the 3′ pseudoknot. **(C)** Model for assembly of the VPg uridylylation complex. Two molecules of 3CD bind to cre. The 3C dimer melts part of the stem. $3D^{pol}$ binds to the complex by interactions between the back of the thumb domain and the surface of 3C. VPg then binds the complex and is linked to two U moieties in a reaction templated by the cre sequence.

capped host cell RNAs, producing the primers for viral mRNA synthesis. Such binding to two sites in the nascent viral mRNA blocks access of a second P protein and protects newly synthesized viral mRNA from endonucleolytic cleavage by P proteins.

Protein-protein interactions play roles in directing the RNA polymerase to the RNA template. The vesicular stomatitis virus RNA polymerase for mRNA synthesis consists of the P protein and the L protein, the catalytic subunit. The P protein binds both the L protein and the complex of N and the (−) strand RNA. In this way the P protein brings the L polymerase to the RNA template. Cellular general initiation proteins have a similar function in bringing RNA polymerase II to the correct site to initiate transcription of DNA templates.

Unwinding the RNA Template

Base-paired regions in viral RNA must be disrupted to permit copying by RNA-dependent RNA polymerase. RNA helicases, which are encoded in the genomes of many RNA viruses, are thought to unwind the genomes of double-stranded RNA viruses as well as the secondary structures in template RNAs. They also prevent extensive base pairing between template RNA and the nascent complementary strand. The RNA helicases of several viruses that are important human pathogens, including the flaviviruses hepatitis C virus and dengue virus, have been studied extensively because these proteins are potential targets for chemotherapeutic intervention. To facilitate the development of new agents that inhibit these helicases, their three-dimensional structures have been determined by X-ray crystallography. These molecules comprise three domains that mediate hydrolysis of NTPs and RNA binding (Fig. 6.16). Between the domains is a cleft that is large enough to accommodate single-stranded but not double-stranded RNA. Unwinding of double-stranded RNA probably occurs as one strand of RNA passes through the cleft and the other passes outside of the molecule.

The bacteriophage ϕ6 RNA polymerase can separate the strands of double-stranded RNA without the activity of a helicase. Examination of the structure of the enzyme suggests how such melting might be accomplished. This RNA polymerase has a plow-like protuberance around the entrance to the template channel that is thought to separate the two strands of the double-stranded RNA, allowing only one to enter the channel.

Role of Cellular Proteins

Host cell components required for viral RNA synthesis were initially called “host factors” because nothing was known about their chemical composition. Evidence that cellular proteins are essential components of a viral RNA polymerase first came from studies of the bacteriophage Qβ. The RNA-dependent RNA polymerase of this virus

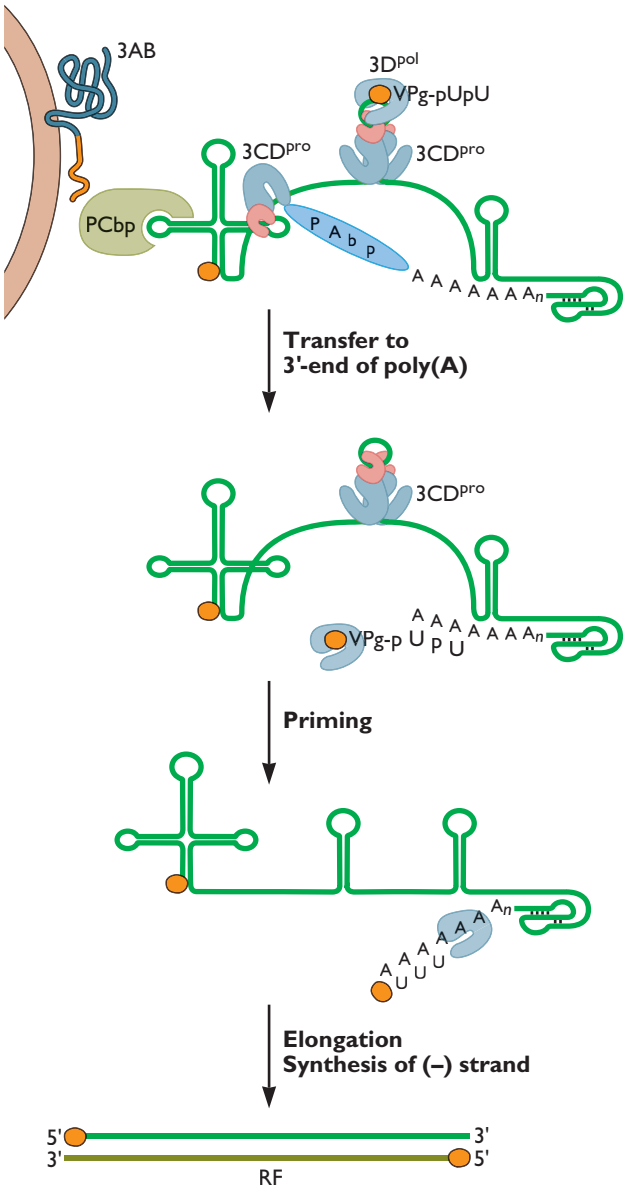


Figure 6.12 Poliovirus (−) strand RNA synthesis. The precursor of VPg, 3AB, contains a hydrophobic domain and is a membrane-bound donor of VPg. A ribonucleoprotein complex is formed when poly(rC)-binding protein 2 (PCbp2) and $3CD^{pro}$ bind the cloverleaf structure located within the first 108 nucleotides of (+) strand RNA. The ribonucleoprotein complex interacts with poly(A)-binding protein 1 (PAbp1), which is bound to the 3′ poly(A) sequence, bringing the ends of the genome into close proximity. Protease $3CD^{pro}$ cleaves membrane-bound 3AB, releasing VPg and 3A. VPg-pUpU is synthesized by $3D^{pol}$ using the sequence AAACA of cre as a template, transferred to the 3′ end of the genome, and used by $3D^{pol}$ as a primer for RNA synthesis. Modified from A. V. Paul, p. 227–246, *in* B. L. Semler and E. Wimmer (ed.), *Molecular Biology of Picornaviruses* (ASM Press, Washington, DC, 2002), with permission.

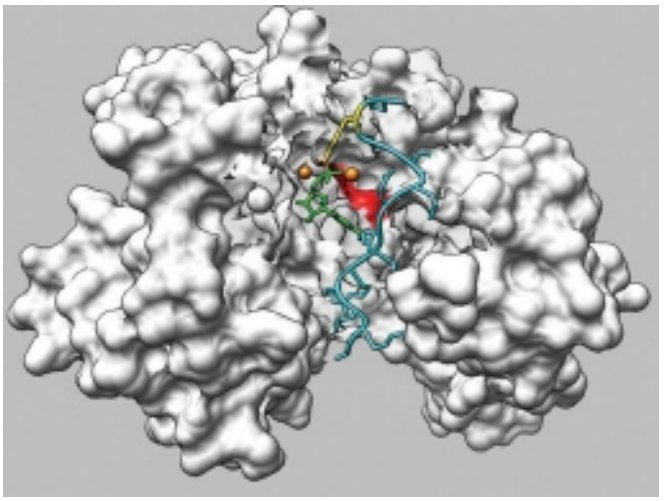


Figure 6.13 Structure of VPg bound to $3D^{pol}$ of foot-and-mouth disease virus. The polymerase is shown by surface representation. VPg protein is shown in cyan, tyrosine-3 of VPg is yellow, and the UMP linked to VPg is green. Catalytic aspartates are red, and Mg^{2+} ions are orange balls. From Protein Data Bank file 2f8e.

is a multisubunit enzyme, consisting of a 65-kDa virus-encoded protein and three host proteins, ribosomal protein S1 and the translation elongation proteins EF-Tu and EF-Ts. Proteins S1 and EF-Tu contain RNA-binding sites that enable the RNA polymerase to recognize the viral RNA template. The 65-kDa viral protein exhibits no RNA polymerase activity in the absence of the host proteins, but has sequence and structural similarity to known RNA-dependent RNA polymerases.

Polioviral RNA synthesis also requires host cell proteins. When purified polioviral RNA is incubated with a cytoplasmic extract prepared from uninfected permissive cells, the genomic RNA is translated and the viral RNA polymerase is made. If guanidine hydrochloride is included in the reaction mixture, the polymerase assembles on the viral genome but RNA synthesis is not initiated. The RNA polymerase-template assembly can be isolated free of guanidine, but RNA synthesis does not occur unless a new cytoplasmic extract is added, indicating that soluble cellular proteins are required for initiation. A similar conclusion comes from studies in which polioviral RNA was injected into oocytes derived from the African clawed toad *Xenopus laevis*. Polioviral RNA cannot replicate in *Xenopus* oocytes unless it is coinjected with a cytoplasmic extract from human cells. These observations can be explained by the requirement of the viral RNA polymerase for a mammalian protein that is absent in toad oocytes.

Host cell poly(rC)-binding protein is required for polioviral RNA synthesis. This protein binds to a cloverleaf

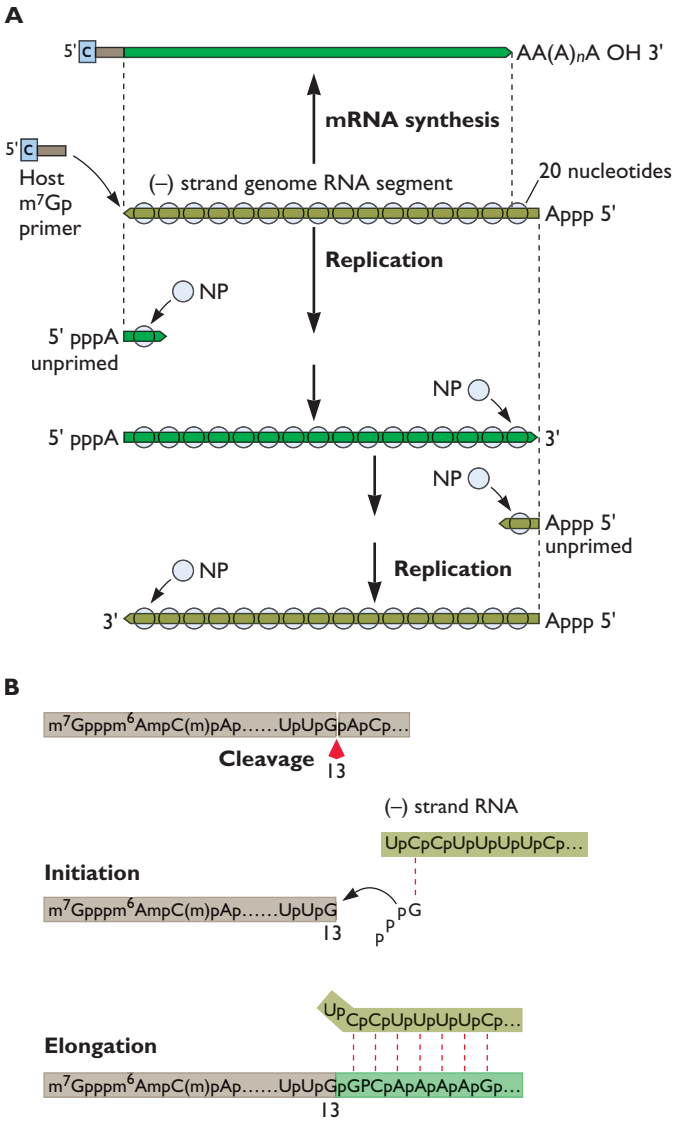


Figure 6.14 Influenza virus RNA synthesis. (A) Viral (–) strand genomes are templates for the production of either subgenomic mRNAs or full-length (+) strand RNAs. The switch from viral mRNA synthesis to genomic RNA replication is regulated by both the number of nucleocapsid (NP) protein molecules and the acquisition by the viral RNA polymerase of the ability to catalyze initiation without a primer. Binding of the NP protein to elongating (+) strands enables the polymerase to read to the 5′ end of genomic RNA. **(B)** Capped RNA-primed initiation of influenza virus mRNA synthesis. Capped RNA fragments cleaved from the 5′ ends of cellular nuclear RNAs serve as primers for viral mRNA synthesis. The 10 to 13 nucleotides in these primers do not need to hydrogen bond to the common sequence found at the 3′ ends of the influenza virus genomic RNA segments. The first nucleotide added to the primer is a G residue templated by the penultimate C residue of the genomic RNA segment; this is followed by elongation of the mRNA chains. The terminal U residue of the genomic RNA segment does not direct the incorporation of an A residue. The 5′ ends of the viral mRNAs therefore comprise 10 to 13 nucleotides plus a cap structure snatched from host nuclear pre-mRNAs. Adapted from S. J. Plotch et al., *Cell* **23:**847–858, 1981, with permission.

structure that forms in the first 108 nucleotides of (+) strand RNA (Fig. 6.12). Formation of a ribonucleoprotein composed of the 5′ cloverleaf, 3CD, and poly(rC)-binding protein is essential for the initiation of viral RNA synthesis. Interaction of poly(rC)-binding protein with the cloverleaf facilitates the binding of viral protein 3CD to the opposite side of the same cloverleaf.

Another candidate for a host protein that is essential for polioviral RNA synthesis is poly(A)-binding protein 1. This protein interacts with poly(rC)-binding protein 2, $3CD^{pro}$, and the 3′-poly(A) tail of poliovirus RNA, bringing together the ends of the viral genome (Fig. 6.12). Formation of this circular ribonucleoprotein complex is required for (–) strand RNA synthesis.

Host cell cytoskeletal proteins participate in paramyxoviral RNA synthesis. Measles virus and Sendai virus mRNA synthesis *in vitro* is stimulated by tubulin, the major structural component of microtubules of the cell's cytoskeleton, and is inhibited by anti-β-tubulin antibodies. In contrast, mRNA synthesis of human parainfluenza virus type 3 and respiratory syncytial virus requires cellular actin, which forms the microfilaments of the cytoskeletal network. Actin and tubulin might serve as anchoring sites on the cytoskeleton for the viral RNA polymerase. In support of this hypothesis, it has been shown that the incoming viral ribonucleoproteins associate with the cytoskeletal framework, where they are active in RNA synthesis. Assembling the RNA polymerase on the cytoskeleton may ensure high local concentrations of replication components and hence increase the rates or efficiencies of replication reactions.

Why Are There Unequal Amounts of (–) and (+) Strands?

Different concentrations of (+) and (–) strands are produced in infected cells. For example, in cells infected with poliovirus, genomic RNA is produced at 100-fold higher concentrations than its complement. There are different explanations for these observations. RNA genomes and their complementary strands might have different stabilities, or the two strands might be synthesized by different mechanisms that vary in efficiency.

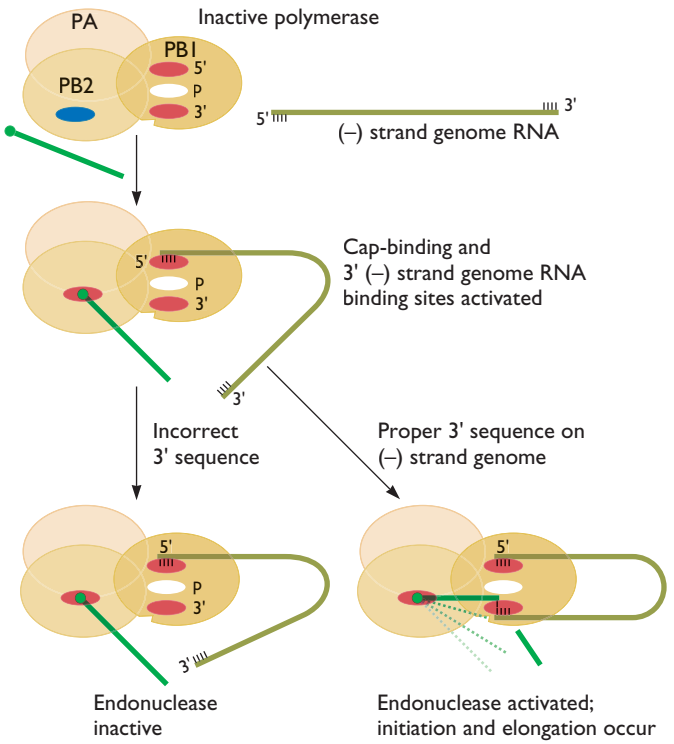
Viral (–) strand RNA is approximately 20 to 50 times more abundant than (+) strand RNA in cells infected with vesicular stomatitis virus. It was suggested that the asymmetry is a consequence of more efficient initiation of RNA synthesis at the 3′ end of (+) strand RNA than at the 3′ end of (–) strand RNA. An elegant proof of this hypothesis came from the construction and study of a rabies virus genome with identical initiation sites at the 3′ ends of both (–) and (+) strand RNAs. In cells infected with this virus, the ratio of (–) to (+) strands is 1:1.

BOX 6.4 DISCUSSION

Does the 3′ noncoding region of poliovirus RNA confer template specificity?

The isolation of infectious polioviruses without the entire 3′ noncoding region of the viral RNA has led to the suggestion that the template specificity imparted by terminal structures of RNA might be of greater importance early in infection. Early in infection, the 3′ pseudoknot structure might facilitate template selection when few viral polymerase molecules are available and membrane association has not yet provided highly concentrated areas of replication components. Later in infection, determinants of template selection by the polymerase might include the membrane association of the RNA polymerase, and the fact that translation of $3D^{pol}$, the most 3′-terminal gene, would position the polymerase at the 3′ end of the genome, ready for initiation.

Figure 6.15 Activation of the influenza virus RNA polymerase by specific virion RNA sequences. Binding of the PB1 protein to this RNA sequence induces the PB2 protein to bind to the cap of a cellular RNA. When the 3′-terminal sequence of genomic RNA binds to a second amino acid sequence in the PB1 protein, the polymerase acquires the activity to cleave the capped cellular RNA 10 to 13 nucleotides from the cap. The RNA polymerase can then carry out initiation and elongation of mRNAs. p, polymerase active site. 5′, and 3′ indicate the binding sites for the 5′ and 3′ ends, respectively, of (−) strand genomic RNA. Blue indicates an inactive site, and red indicates an active site. The polymerase is bound to both the 5′ and 3′ ends of the genomic RNA, with the capped RNA primer associated with the PB2 protein. Adapted from D. M. Knipe et al. (ed.), *Fields Virology*, 4th ed. (Lippincott Williams & Wilkins, Philadelphia, PA, 2001); P. Rao et al., *EMBO J.* **22:**1188–1198, 2003; and S. R. Shih and R. M. Krug, *Virology* **226:**430–436, 1996, with permission.

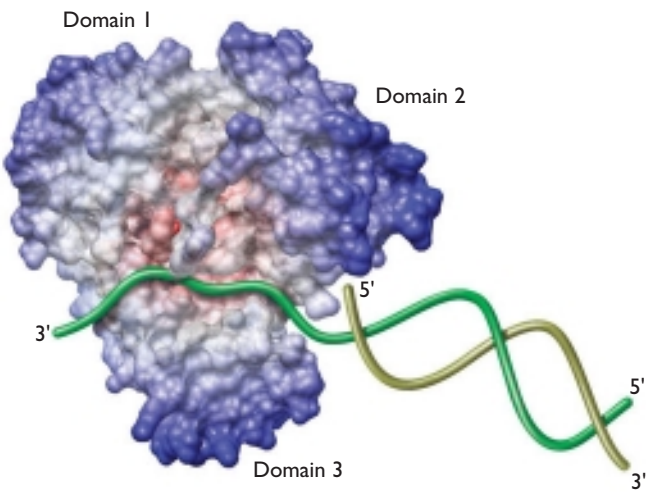


In alphavirus-infected cells, the abundance of genomic RNA is explained by the fact that (−) strand RNAs are synthesized only for a short time early in infection. The RNA polymerase that catalyzes (−) strand RNA synthesis is produced only during this period. The synthesis of (+) strands continues for much longer and leads to accumulation of mRNA and (+) strand genomic RNA.

Do Ribosomes and RNA Polymerases Collide?

The genomic RNA of (+) strand viruses can be translated in the cell, and the translation products include the viral RNA polymerase. At a certain point in infection, the RNA polymerase copies the RNA in a 3′ → 5′ direction while ribosomes traverse it in a 5′ → 3′ direction (Fig. 6.17), raising the question of whether the viral polymerase avoids

Figure 6.16 Structure of a viral RNA helicase. The RNA helicase of yellow fever virus is shown in surface representation, colored red, white, or blue depending on the distance of the amino acid from the center of the molecule. A model for melting of double-stranded RNA is shown. From Protein Data Bank file 1yks.



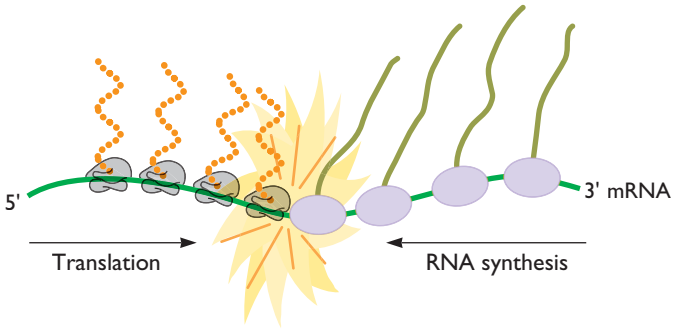


Figure 6.17 Ribosome-RNA polymerase collisions. A strand of viral RNA is shown, with ribosomes translating in the 5′ → 3′ direction and RNA polymerase copying the RNA chains in the 3′ → 5′ direction. Ribosome-polymerase collisions would occur in cells infected with (+) strand RNA viruses unless mechanisms exist to avoid simultaneous translation and replication.

collisions with ribosomes. When ribosomes are frozen on polioviral RNA by using inhibitors of protein synthesis, replication is inhibited. In contrast, when ribosomes are released, replication of the RNA increases. These results suggest that ribosomes must be cleared from viral RNA before it can serve as a template for (–) strand RNA synthesis; in other words, replication and translation cannot occur simultaneously.

The interactions of viral and cellular proteins with the cloverleaf structure in the polioviral 5′ untranslated region might determine whether the RNA genome is translated or replicated. In this model, interaction of cellular poly(rC)-binding protein 2 with the 5′ untranslated region initially stimulates translation in infected cells. Once protein $3CD^{pro}$ has been synthesized, it cleaves poly(rC)-binding protein, which prevents it from binding and leads to reduced translation. The cleaved poly(rC)-binding protein can still bind to the cloverleaf (Fig. 6.12) and promote viral RNA synthesis.

Restricting translation and RNA synthesis to distinct compartments may prevent collisions of ribosomes and polymerases. Viral mRNA synthesis takes place in the reovirus capsid, where the enzymes responsible for this process are located. The viral mRNAs are exported to the cytoplasm for translation. Synthesis of retroviral RNAs occurs in the cell nucleus, where translation does not take place. The architecture of membranous replication complexes of (+) strand viruses may favor RNA synthesis and exclude translation.

Even if mechanisms exist for controlling whether the polioviral genome is translated or replicated, some ribosome-RNA polymerase collisions are likely to occur. This conclusion is drawn from the isolation of a polioviral mutant with a genome that contains an insertion of a 15-nucleotide sequence from 28S ribosomal RNA (rRNA).

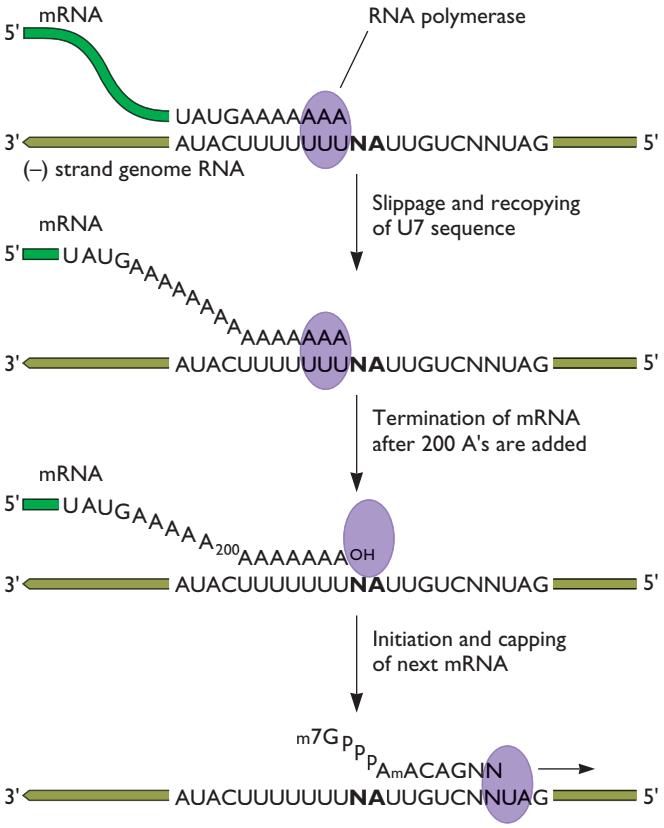


Figure 6.18 Poly(A) addition and termination at an intergenic region during vesicular stomatitis virus mRNA synthesis. Copying of the last seven U residues of an mRNA-encoding sequence is followed by slipping of the resulting seven A residues in the mRNA off the genomic sequence, which is then recopied. This process continues until approximately 200 A residues are added to the 3′ end of the mRNA. Termination then occurs, followed by initiation and capping of the next mRNA. The dinucleotide NA in the genomic RNA is not copied.

After colliding with a ribosome, the RNA polymerase apparently copied 15 nucleotides of rRNA before returning to the viral RNA template.

Synthesis of Poly(A)

The mRNAs synthesized during infection by most RNA viruses contain a 3′-poly(A) sequence, as do the vast majority of cellular mRNAs (exceptions are arenaviruses and reoviruses). The poly(A) sequence is encoded in the genome of (+) strand viruses. For example, polioviral (+) strand RNAs contain a 3′ stretch of poly(A), approximately 62 nucleotides in length, which is required for infectivity. The (–) strand RNA contains a 5′ stretch of poly(U), which is copied to form this poly(A).

Another mechanism for poly(A) addition is reiterative copying of, or "stuttering" at, a short U sequence in the (–) strand template. After initiation, vesicular stomatitis virus

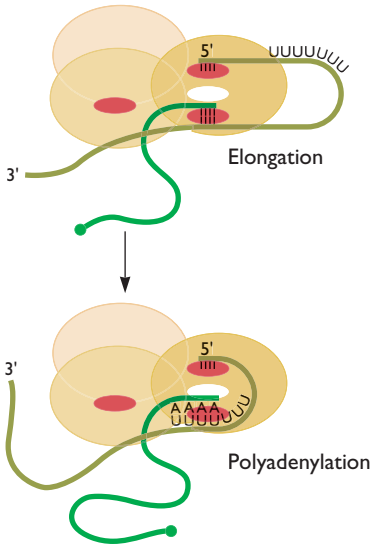


Figure 6.19 Moving-template model for influenza virus mRNA synthesis. During RNA synthesis, the polymerase remains bound to the 5′ end of the genomic RNA, and the 3′ end of the genomic RNA is threaded through (or along the surface of) the polymerase as the PB1 protein catalyzes each nucleotide addition to the growing mRNA chain. This threading process continues until the mRNA reaches a position on the genomic RNA that is close to the binding site of the polymerase. At this point the polymerase itself blocks further mRNA synthesis, and reiterative copying of the adjacent U_7 tract occurs. After about 150 A residues are added to the 3′ end of the mRNA, mRNA synthesis terminates. Adapted from D. M. Knipe et al. (ed.), *Fields Virology*, 4th ed. (Lippincott Williams & Wilkins, Philadelphia, PA, 2001); P. Rao et al., *EMBO J.* **22:**1188–1198, 2003; and S. R. Shih and R. M. Krug, *Virology* **226:**430–436, 1996, with permission.

mRNAs are elongated until the RNA polymerase reaches a conserved stop-polyadenylation signal [3′-AUACU$_7$-5′] located in each intergenic region (Fig. 6.18). Poly(A) (approximately 150 nucleotides) is added by reiterative copying of the U stretch, followed by termination. Polyadenylation is achieved by a similar mechanism during influenza virus mRNA synthesis.

Some models for RNA synthesis invoke a stationary template and moving polymerase. If instead we imagine a stationary enzyme and moving template, a mechanism becomes apparent for synthesis of poly(A) by the influenza virus polymerase (Fig. 6.19). The RNA polymerase specifically binds the 5′ end of (−) strand RNA and remains bound at this site of each genomic RNA segment throughout mRNA synthesis. The genomic RNAs would be threaded through the polymerase in a 3′ → 5′ direction as mRNA synthesis proceeds. Eventually the template would be unable to move, causing reiterative copying of the U residues.

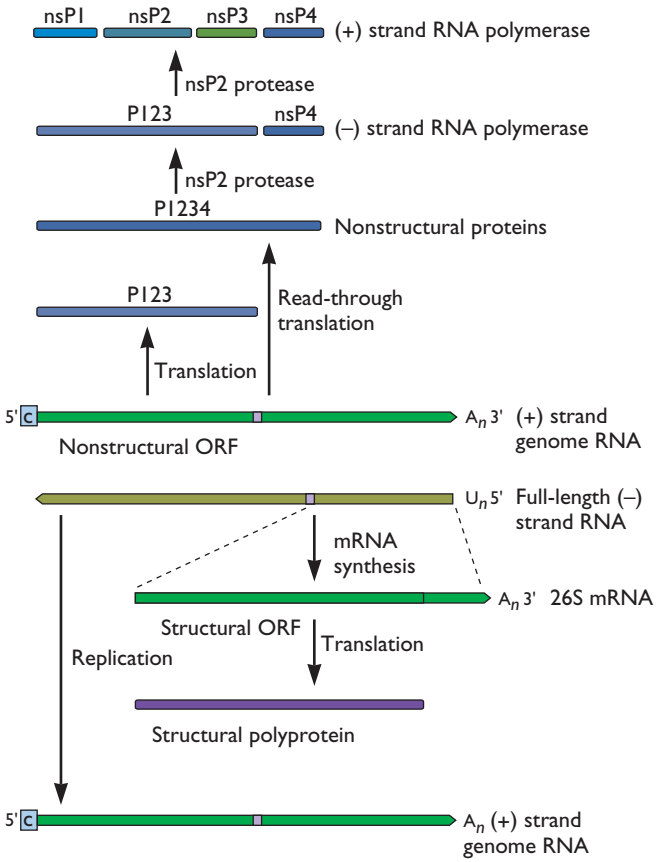


Figure 6.20 Genome structure and expression of an alphavirus, Sindbis virus. The 11,703-nucleotide Sindbis virus genome contains a 5′-terminal cap structure and a 3′-poly(A) tail. A conserved RNA secondary structure at the 3′ end of (+) strand genomic RNA is thought to control the initiation of (−) strand RNA synthesis. At early times after infection, the 5′ region of the genomic RNA (nonstructural open reading frame [ORF]) is translated to produce two nonstructural polyproteins: P123, whose synthesis is terminated at the first translational stop codon (indicated by the box), and P1234, produced by an occasional (15%) readthrough of this stop codon. The P1234 polyprotein is proteolytically cleaved to produce the enzymes that catalyze the various steps in genomic RNA replication: the synthesis of a full-length (−) strand RNA, which serves as the template for (+) strand synthesis, and either full-length genomic RNA or subgenomic 26S mRNA. The 26S mRNA, shown in expanded form, is translated into a structural polyprotein (p130) that undergoes proteolytic cleavage to produce the virion structural proteins. The 26S RNA is not copied into a (−) strand because a functional initiation site fails to form at the 3′ end.

The Switch from mRNA Production to Genome RNA Synthesis

Exact replicas of the RNA genome must be made for assembly of infectious viral particles. However, the mRNAs of most RNA viruses are **not** complete copies of the viral RNA. The replication cycle of these viruses must therefore include a switch from mRNA synthesis to the production

of full-length genomes. The majority of mechanisms for this switch regulate either the initiation or the termination of RNA synthesis.

Different RNA Polymerases for mRNA Synthesis and Genome Replication

The mechanism of mRNA synthesis of other (+) strand RNA viruses allows structural and nonstructural proteins (generally needed in greater and lesser quantities, respectively) to be produced separately. The latter are produced from full-length (+) strand (genomic) RNA, while structural proteins are translated from subgenomic mRNA(s). This strategy is a feature of the replication cycles of coronaviruses, caliciviruses, and alphaviruses. Translation of the Sindbis virus (+) strand RNA genome yields the nonstructural proteins that synthesize a full-length (–) strand (Fig. 6.20). Such RNA molecules contain not only a 3′-terminal sequence for initiation of (+) strand RNA synthesis but also an internal initiation site, used for production of a 26S subgenomic mRNA.

Alphaviral genome and mRNA synthesis is regulated by the sequential production of three RNA polymerases with different template specificities. All three enzymes are derived from the nonstructural polyprotein P1234 and contain the complete amino acid sequence of this precursor (Fig. 6.20). The covalent connections among the segments (P1, P2, P3, and P4) of the polyprotein are successively broken, with ensuing alterations in RNA polymerase specificity (Fig. 6.21). It seems likely that each proteolytic cleavage induces a conformational change in the polymerase that alters its template specificity.

The genes of RNA viruses with a nonsegmented (–) strand RNA genome are expressed by the production of subgenomic mRNAs in infected cells. The switch from subgenomic mRNA to full-length (+) strand synthesis may be accomplished by producing RNA polymerases with different specificities. For example, polymerase composed of one molecule of L protein associated with four molecules of P protein is thought to carry out vesicular stomatitis virus mRNA synthesis, while genome replication would be accomplished by an LN-(P) 4 assembly (Fig. 6.22). The ratio of the two polymerases at different times after infection is regulated by viral protein synthesis, and by the phosphorylation state of the P protein, which controls its ability to form oligomers.

Suppression of Intergenic Stop-Start Reactions by Nucleocapsid Protein

The transition from mRNA to genome RNA synthesis in cells infected with vesicular stomatitis virus requires not only different RNA polymerases but also the viral nucleocapsid (N) protein. Individual mRNAs are produced by a

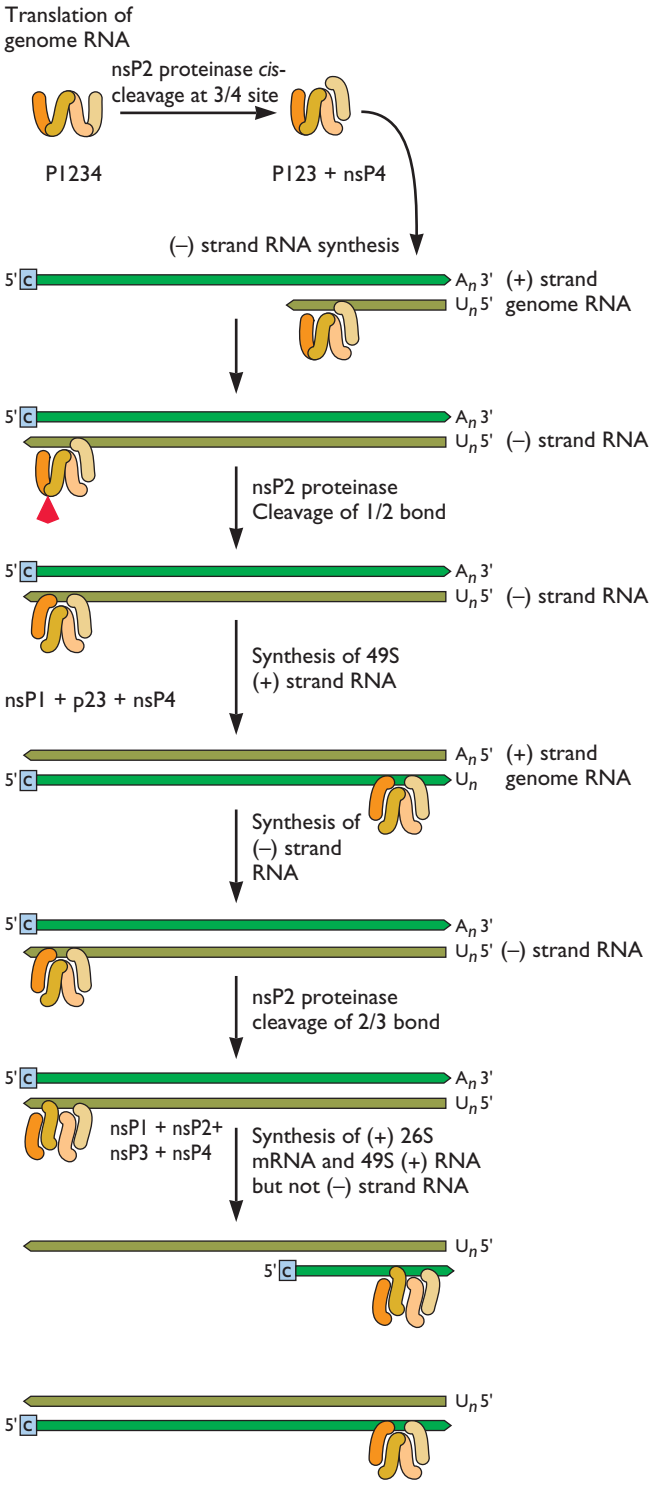


Figure 6.21 Three RNA polymerases with distinct specificities in alphavirus-infected cells. These RNA polymerases contain the entire sequence of the P1234 polyprotein and differ only in the number of proteolytic cleavages in this sequence. Adapted from J. H. Strauss and E. G. Strauss, *Microbiol. Rev.* **58:**491–562, 1994, with permission.

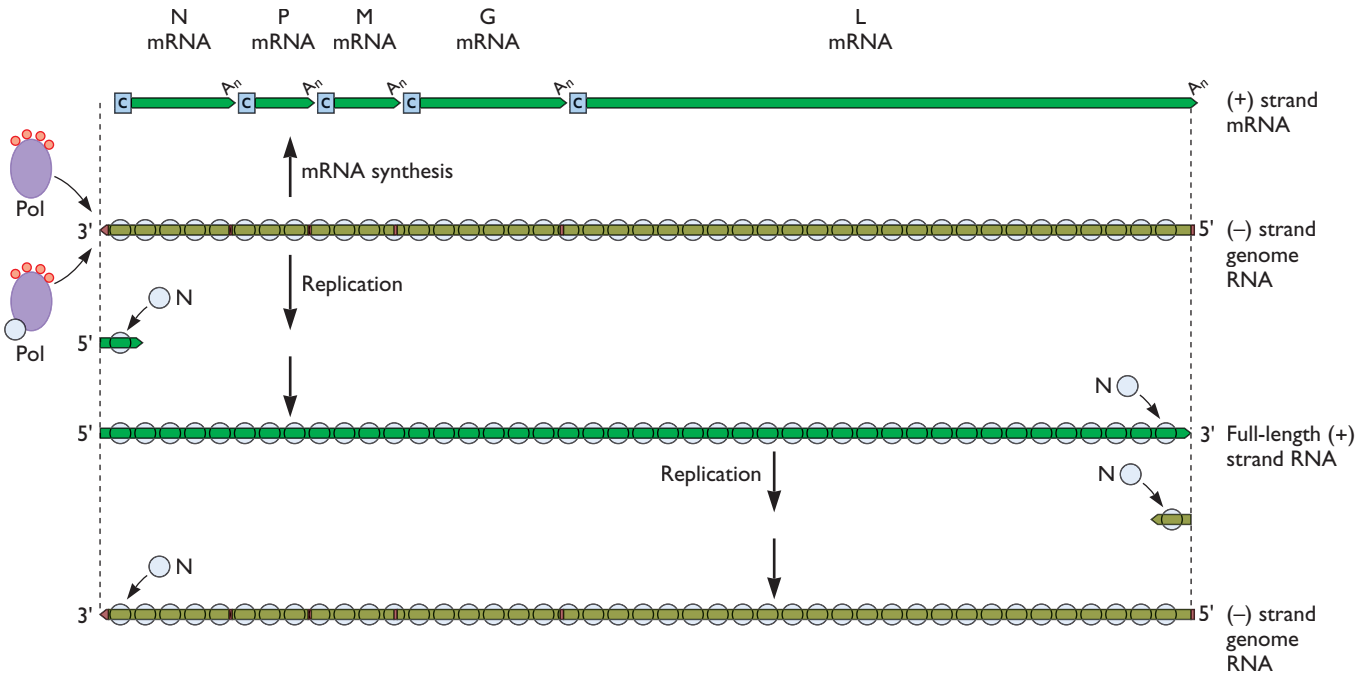


Figure 6.22 Vesicular stomatitis viral RNA synthesis. Viral (–) strand genomes are templates for the production of either subgenomic mRNAs or full-length (+) strand RNAs. The switch from mRNA synthesis to genomic RNA replication is mediated by two RNA polymerases and by the N protein. mRNA synthesis initiates at the beginning of the N gene, near the 3′ end of the viral genome. Poly(A) addition is a result of reiterative copying of a sequence of seven U residues present in each intergenic region. Chain termination and release occur after approximately 150 A residues have been added to the mRNA. The RNA polymerase then initiates synthesis of the next mRNA at the conserved start site 3′UUGUC . . . 5′. This process is repeated for all five viral genes. Synthesis of the full-length (+) strand begins at the exact 3′ end of the viral genome and is carried out by the RNA polymerase L-N-(P)4. The (+) strand RNA is bound by the viral nucleocapsid (N) protein, which is complexed with the P protein in a 1:1 molar ratio. The N-P complexes bind to the nascent (+) strand RNA, allowing the RNA polymerase to read through the intergenic junctions at which polyadenylation and termination take place during mRNA synthesis.

series of initiation and termination reactions as the RNA polymerase moves down the viral genome (Fig. 6.23). This start-stop mechanism accounts for the observation that 3′-proximal genes must be copied before downstream genes (Box 6.5); the viral RNA polymerase is unable to initiate synthesis of each mRNA independently. To produce a full-length (+) strand RNA, the stop-start reactions at intergenic regions must be suppressed. Suppression depends on the synthesis of the N and P proteins. The P protein maintains the N protein in a soluble form so that it can encapsidate the newly synthesized RNA. The N-P complexes bind to leader RNA and cause antitermination, signaling the polymerase to begin processive RNA synthesis. Additional N protein molecules then associate with the (+) strand RNA as it is elongated, and eventually bind to the seven A bases in the intergenic region. This interaction prevents the seven A bases from slipping backward along the genomic RNA template and therefore blocks reiterative copying of the seven U bases in the genome. Consequently, RNA synthesis continues through the intergenic region. The number of N-P protein complexes in infected cells therefore regulates the relative efficiencies of mRNA synthesis and genome RNA replication. The copying of full-length (+) strand RNAs to (–) strand genomic RNAs also requires the binding of N-P protein complexes to elongating RNA molecules. Newly synthesized (–) strand RNAs are produced as nucleocapsids that can be readily packaged into progeny viral particles.

The (–) strand RNA genome of paramyxoviruses is copied efficiently only when its length in nucleotides is a multiple of 6. This requirement, called the **rule of six**, is probably a consequence of the association of each N monomer with exactly six nucleotides. Assembly of the nucleocapsid begins with the first nucleotide at the 5′ end of the RNA and continues until the 3′ end is reached. If the genome length is not a multiple of 6, then the 3′ end of the genome will not be precisely aligned with the last N monomer. Such misalignment reduces the efficiency of initiation of RNA synthesis at the 3′ end. Curiously, although the

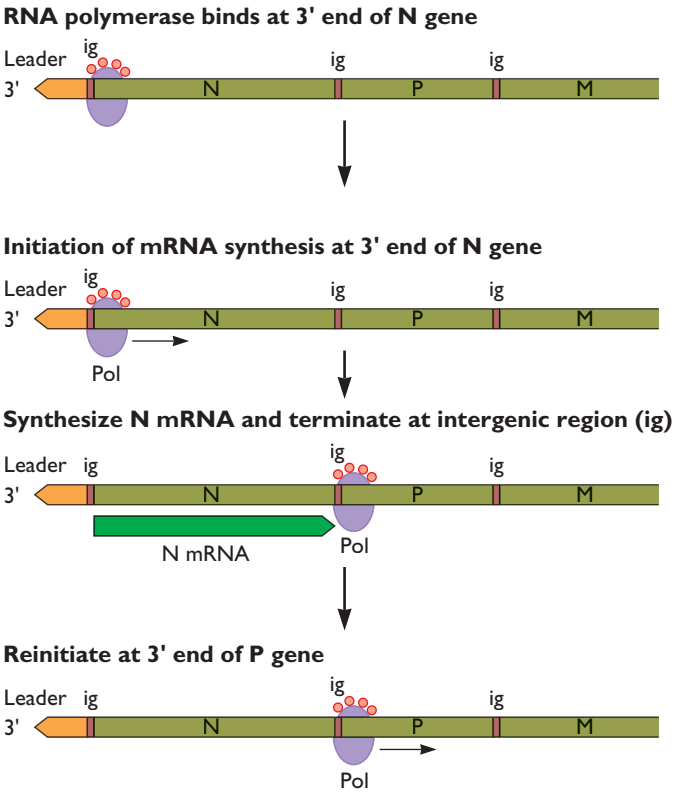


Figure 6.23 Stop-start model of vesicular stomatitis mRNA synthesis. The RNA polymerase (Pol) initiates RNA synthesis at the 3′ end of the N gene. After synthesis of the N mRNA, RNA synthesis terminates at the intergenic region, followed by reinitiation at the 3′ end of the P gene. This process continues until all five mRNAs are synthesized. Reinitiation does not occur after the last mRNA (the L mRNA) is synthesized, and as a consequence the 59 5′-terminal nucleotides of the vesicular stomatitis virus genomic RNA are not copied. Only a fraction of the polymerase molecules successfully make the transition from termination to reinitiation of mRNA synthesis at each intergenic region.

rhabdovirus N protein binds nine nucleotides of RNA, the genome length need not be a multiple of this number for efficient copying.

The influenza virus NP protein also regulates the switch from viral mRNA to full-length (+) strand synthesis (Fig. 6.14). The RNA polymerase for genome replication reads through the polyadenylation and termination signals used for mRNA production only if NP is present. This protein is thought to bind nascent (+) strand transcripts and block poly(A) addition by a mechanism analogous to that described for vesicular stomatitis virus N protein. Copying of (+) strand RNAs into (−) strand RNAs also requires NP protein. Intracellular concentrations of NP protein are therefore an important determinant of whether mRNAs or full-length (+) strands are synthesized.

Suppression of Termination Induced by a Stem-Loop Structure

The subgenomic mRNAs of certain (−) strand RNA viruses, such as arenaviruses, are produced when the RNA polymerase terminates at a stem-loop structure in the viral RNA template. Suppression of such termination results in the synthesis of full-length (+) RNAs.

Although arenaviruses are considered (−) strand RNA viruses, their genomic RNA is in fact **ambisense**: mRNAs are produced both from (−) strand genomic RNA and from complementary full-length (+) strands. The arenavirus genome comprises two RNA segments, S (small) and L (large) (Fig. 6.24). Shortly after infection, a virion-associated RNA polymerase synthesizes mRNAs from the 3′ region of the S and L genomic RNA segments. Synthesis of each mRNA terminates at a stem-loop structure. These mRNAs, which are translated to produce the nucleocapsid (NP) protein and RNA polymerase (L) protein, respectively, are the only viral RNAs made during the first several hours of infection. Later in infection, the block imposed by the stem-loop structure is overcome, permitting the synthesis of full-length S and L (+) RNAs. It was initially thought that melting of the stem-loop structure by the NP protein allowed the transcription termination signal to be bypassed. It now seems more likely that two different RNA polymerases are made in infected cells, one that produces mRNAs and a second that synthesizes full-length copies of the genome. The finding that viral mRNAs are capped while genomes are not is consistent with this hypothesis.

Different Templates Used for mRNA Synthesis and Genome Replication

A distinctive feature of the infectious cycle of double-stranded RNA viruses such as reovirus is the production of mRNAs and genomic RNAs from distinct templates in different viral particles. Because the viral genomes are double stranded, they cannot be translated by the cell. Therefore, the first step in infection is the production of mRNAs from each viral RNA segment by the virion-associated RNA polymerase λ3 (Fig. 6.25). Reoviral mRNAs contain 5′ cap structures but lack 3′ poly(A) sequences.

Although the reovirus RNA polymerase resembles a right hand, with thumb, fingers, and palm domains, it has distinctive features not observed in other enzymes. The reovirus RNA polymerase is a cube-like structure, with a catalytic site in the center that is accessible by four tunnels. One tunnel allows template entry, one serves for the exit of newly synthesized double-stranded RNA, a third permits exit of mRNA, and a fourth is for substrate entry. A priming loop is present in the palm domain that is not observed in this region of other RNA polymerases. This loop supports stacking of the initiating NTP, then retracts into the palm

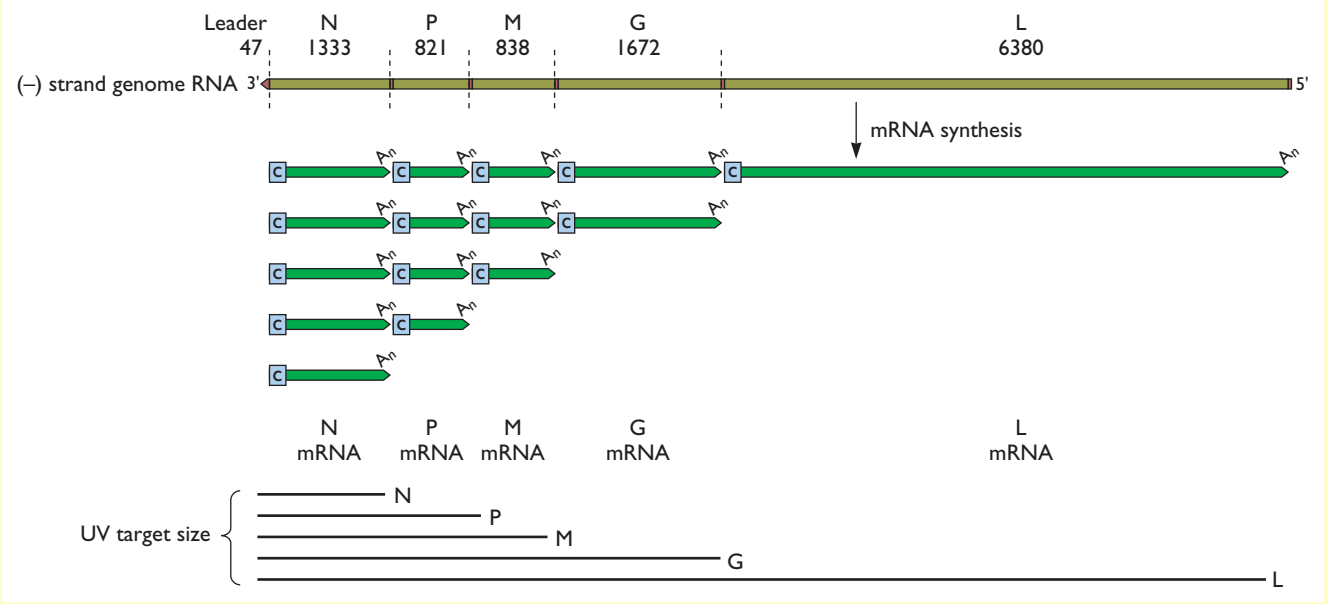
BOX 6.5 EXPERIMENTS

Mapping gene order by UV irradiation

The effects of ultraviolet (UV) irradiation provided insight into the mechanism of vesicular stomatitis virus mRNA synthesis. In these experiments, vesicular stomatitis virus particles were irradiated with UV light and the effect on the synthesis of individual mRNAs was determined. UV light causes the formation of pyrimidine dimers in RNA that block passage of the RNA polymerase. In principle, larger genes require less UV irradiation to inactivate mRNA synthesis and have a larger **target size.** The dosage of UV irradiation needed to inactivate synthesis of the N mRNA corresponded to the predicted size of the N gene, but this was not the case for the other viral mRNAs. The target size of each other mRNA was the sum of its size plus the size of other genes located 3′ to it. For example, the UV target size of the L mRNA is the size of the entire viral genome. These results indicate that these mRNAs are synthesized sequentially, in the 3′ → 5′ order in which their genes are arranged in the viral genome: N-P-M-G-L.

Ball, L. A., and C. N. White. 1976. Order of transcription of genes of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. USA* **73:**442–446.

Vesicular stomatitis virus mRNA map and UV map. The genome is shown as a dark green line at the top, and the N, P, M, G, and L genes and their relative sizes are indicated. The 47-nucleotide leader RNA is encoded at the 3′ end of the genomic RNA. The leader and intergenic regions are shown in orange. The RNAs encoded at the 3′ end of the genome are made in larger quantities than the RNAs encoded at the 5′ end of the genome. UV irradiation experiments determined the size of the vesicular stomatitis virus genome (UV target size) required for synthesis of each of the viral mRNAs. The UV target size of each viral mRNA corresponded to the size of the genomic RNA sequence encoding the mRNA plus all of the genomic sequence 3′ to this coding sequence. The transition from reiterative copying and termination to initiation is not perfect, and only about 70 to 80% of the polymerase molecules accomplish this transition at each intergenic region. Such inefficiency accounts for the observation that 3′-proximal mRNAs are more abundant than 5′-proximal mRNAs.



and fits into the minor groove of the double-stranded RNA product. This movement assists in the transition between initiation and elongation, and also allows the newly synthesized RNA to exit the polymerase.

In the reovirus core, the λ3 polymerase molecules are attached to the inner shell at each fivefold axis, below an RNA exit pore. Viral mRNAs are produced by the polymerase inside the viral particle and then extruded into the cytoplasm through this pore. Attachment of the polymerase molecules to the pores ensures that the mRNA can leave the particle, without depending upon diffusion, which would be very inefficient. Examination of the structure of actively transcribing rotavirus, a member of the *Reoviridae*, has allowed a three-dimensional visualization of how mRNAs are released from the particle (Box 6.6). Viral (+) strand RNAs that will serve as templates for (–) strand RNA synthesis are first packaged into newly assembled subviral particles (Fig. 6.25). Each (+) strand RNA is then copied just once within the subviral particle to produce double-stranded RNA.

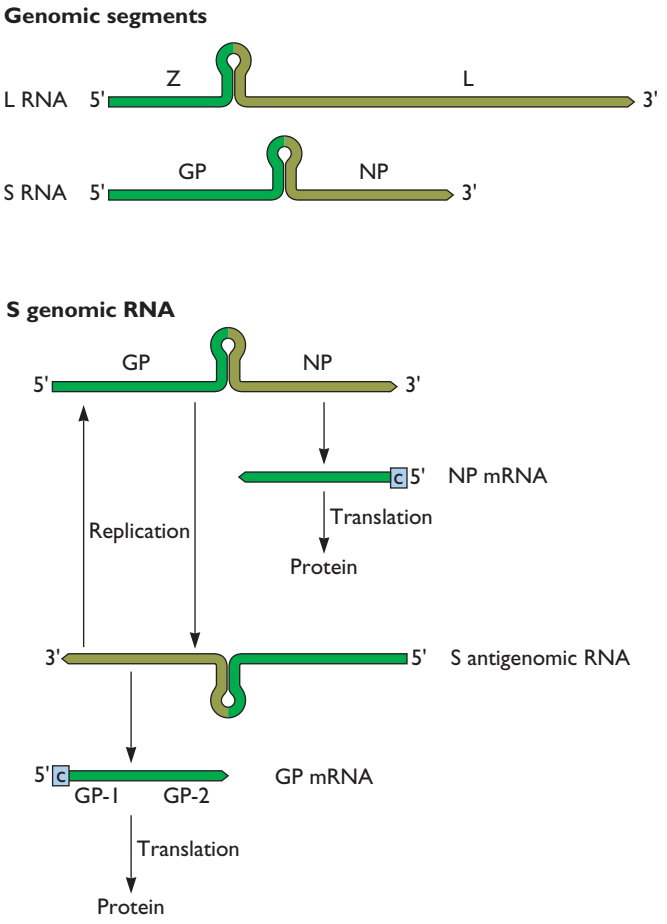


Figure 6.24 Arenavirus RNA synthesis. Arenaviruses contain two genomic RNA segments, L (large) and S (small) (top). At early times after infection, only the 3′ region of each of these segments is copied to form mRNA: the N mRNA from the S genomic RNA and the L mRNA from the L genomic RNA. Copying of the remainder of the S and L genomic RNAs may be blocked by a stem-loop structure in the genomic RNAs. After the S and L genomic RNAs are copied into full-length strands, their 3′ regions are copied to produce mRNAs: the glycoprotein precursor (GP) mRNA from S RNA and the Z mRNA (encoding an inhibitor of viral RNA synthesis) from the L RNA. Only RNA synthesis from the S RNA is shown in detail. Adapted from D. M. Knipe et al. (ed.), *Fields Virology*, 4th ed. (Lippincott Williams & Wilkins, Philadelphia, PA, 2001), with permission.

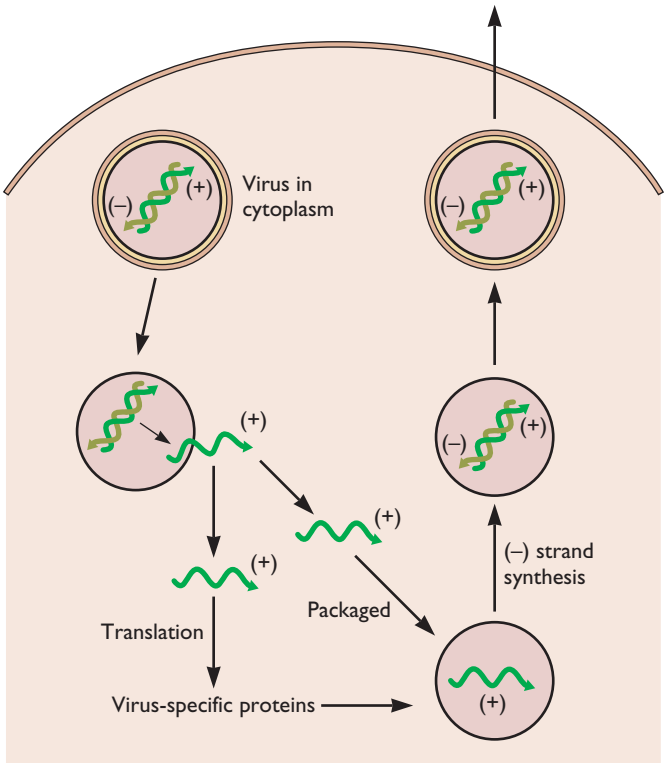


Figure 6.25 mRNA synthesis and replication of double-stranded RNA genomes. These processes occur in subviral particles containing the RNA templates and necessary enzymes. During cell entry, the virion passes through the lysosomal compartment, and proteolysis of viral capsid proteins activates the RNA synthetic machinery. Single-stranded (+) viral mRNAs, which are synthesized in parental subviral particles, are extruded into the cytoplasm, where they serve either as mRNAs or as templates for the synthesis of (–) RNA strands. In the latter case, viral mRNAs are first packaged into newly assembled subviral particles in which the synthesis of (–) RNAs to produce double-stranded RNAs occurs. These subviral particles become infectious particles. Only 1 of the 10 to 12 double-stranded RNA segments of the reoviral genome is shown.

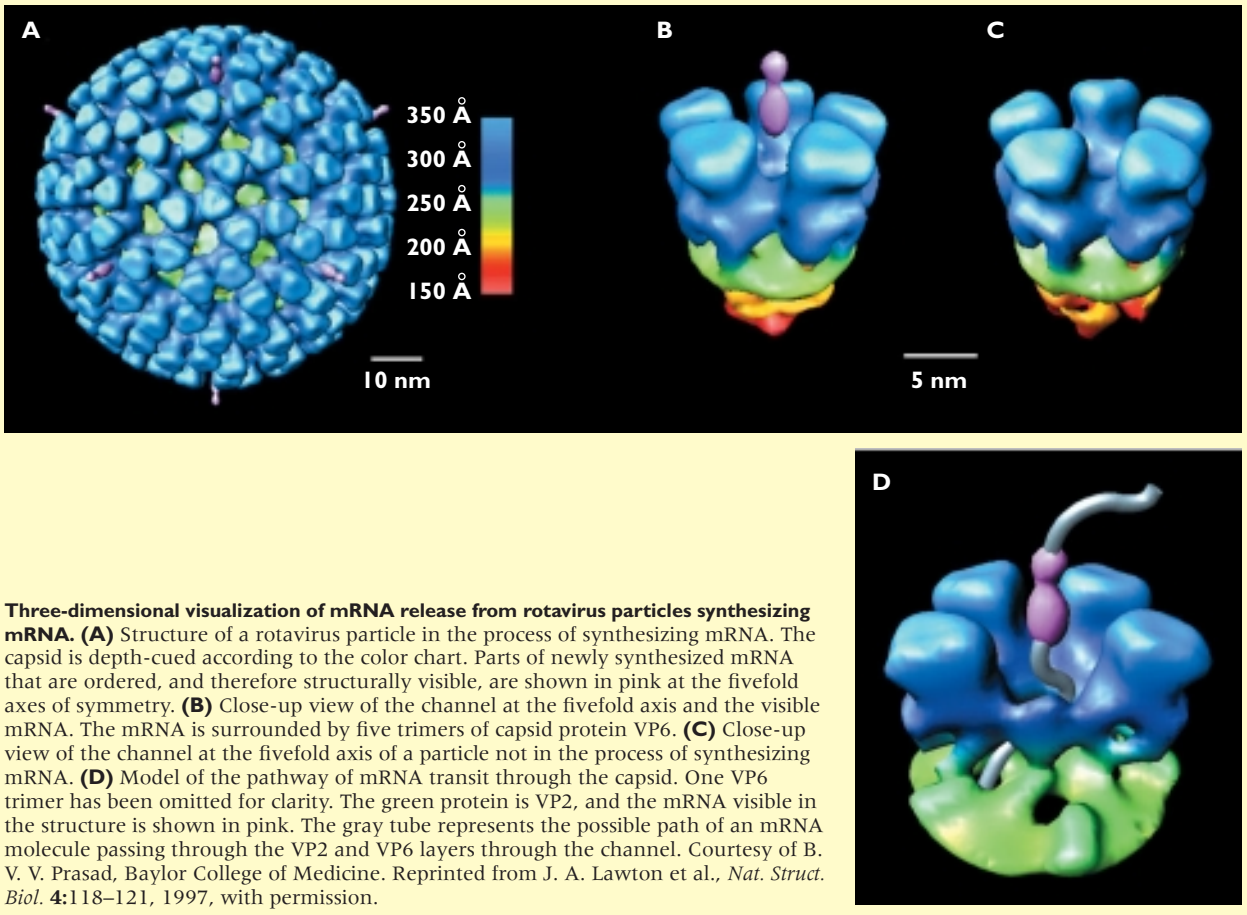
Members of different families of double-stranded RNA viruses carry out RNA synthesis in diverse ways. Replication of the genome of bacteriophage ϕ6 (3 double-stranded RNA segments) and birnaviruses (2 double-stranded RNA segments) is semiconservative, whereas that of reoviruses (10 to 12 double-stranded RNA segments) is conservative (Fig. 6.26). During conservative replication, the double-stranded RNA that exits the polymerase must be melted, so that the newly synthesized (+) strand is released and the template (–) strand reanneals with the original (+) strand. In reovirions, each double-stranded RNA segment is attached to a polymerase complex, by interaction of the 5′ cap structure with a cap-binding site on the RNA polymerase. Attachment of the 5′ cap to the polymerase facilitates insertion of the 3′ end of the (–) strand into the template channel. This arrangement allows very efficient reinitiation of RNA synthesis in the crowded core of the virion. The RNA polymerase of bacteriophage ϕ6 and birnaviruses do not have such a cap-binding site, as would be expected for enzymes that copy both strands of the double-stranded RNA segments. This strategy appears less efficient, but may be acceptable when the genome consists of only two or three double-stranded RNA segments.

BOX 6.6 EXPERIMENTS

Release of mRNA from rotavirus particles

Rotaviruses, the most important cause of gastroenteritis in children, are large icosahedral viruses made of a three-shelled capsid containing 11 double-stranded RNA segments. The structure of this virus reveals that a large portion of the viral genome (~25%) is ordered within the particle and forms a dodecahedral structure (see Fig. 4.17). In this structure, the RNAs interact with the inner capsid layer and pack around the RNA polymerase located at the fivefold axis of symmetry. Further analysis of rotavirus particles in the process of synthesizing mRNA has shown that newly synthesized molecules are extruded through the capsid through several channels located at the fivefold axes (see figure). Multiple mRNAs are released at the same time from such particles. On the basis of these observations, it has been suggested that each double-stranded genomic RNA segment is copied by an RNA polymerase located at a fivefold axis of symmetry. This model may explain why no double-stranded RNA virus with more than 12 genomic segments—the maximum number of fivefold axes—has ever been identified.

Lawton, J. A., M. K. Estes, and B. V. Prasad. 1997. Three-dimensional visualization of mRNA release from actively transcribing rotavirus particles. *Nat. Struct. Biol.* **4:**118–121.



Three-dimensional visualization of mRNA release from rotavirus particles synthesizing mRNA. (A) Structure of a rotavirus particle in the process of synthesizing mRNA. The capsid is depth-cued according to the color chart. Parts of newly synthesized mRNA that are ordered, and therefore structurally visible, are shown in pink at the fivefold axes of symmetry. **(B)** Close-up view of the channel at the fivefold axis and the visible mRNA. The mRNA is surrounded by five trimers of capsid protein VP6. **(C)** Close-up view of the channel at the fivefold axis of a particle not in the process of synthesizing mRNA. **(D)** Model of the pathway of mRNA transit through the capsid. One VP6 trimer has been omitted for clarity. The green protein is VP2, and the mRNA visible in the structure is shown in pink. The gray tube represents the possible path of an mRNA molecule passing through the VP2 and VP6 layers through the channel. Courtesy of B. V. V. Prasad, Baylor College of Medicine. Reprinted from J. A. Lawton et al., *Nat. Struct. Biol.* **4:**118–121, 1997, with permission.

Suppression of Polyadenylation

Another mechanism for switching from mRNA synthesis to genomic replication is to suppress a poly(A) addition signal; an example occurs in hepatitis delta satellite virus-infected cells. Host RNA polymerase II initiates viral mRNA synthesis at a position on the genome near the beginning of the delta antigen-coding region (Boxes 6.7 and 6.8). Once the polymerase has moved past a polyadenylation signal and the self-cleavage domain (Box 6.9), the 3′-poly(A) end of the mRNA is produced by host cell enzymes. The

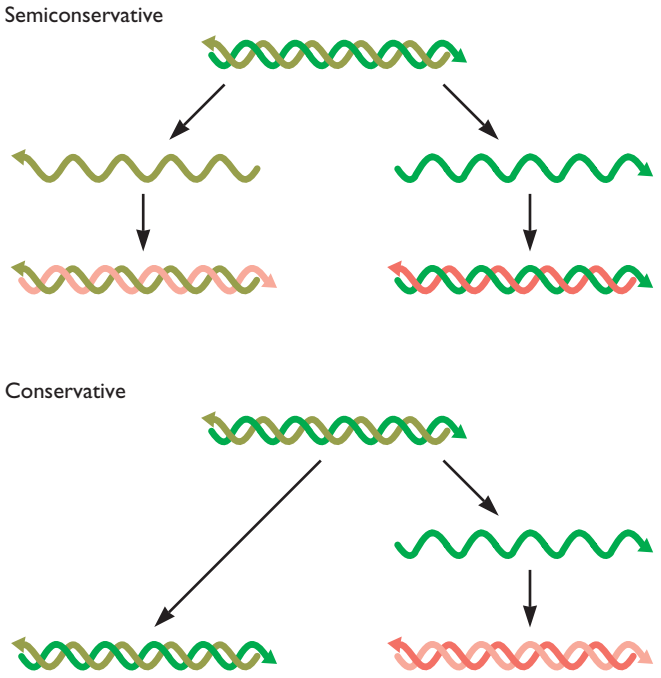


Figure 6.26 Two mechanisms for copying nucleic acids. During semiconservative replication, both strands of nucleic acid serve as templates for the synthesis of new strands (shown in red). In contrast, only one strand is copied during conservative replication.

RNA downstream of the poly(A) site is not degraded, in contrast to that of other mRNA precursors made by RNA polymerase II, but is elongated until a complete full-length (+) strand is made. The poly(A) addition site in this full-length (+) RNA is not used. The delta antigen bound to the rodlike RNA may block access of cellular enzymes to the poly(A) signal, thereby inhibiting polyadenylation.

The Same Template Used for mRNA Synthesis and Genome Replication

No switch from mRNA to genomic RNA synthesis is needed when the only viral mRNA made in infected cells is identical to the (+) strand genome, as occurs in cells infected with picornaviruses (Fig. 6.1). Newly synthesized poliviral (+) strand RNA molecules can serve as RNA templates for further genomic RNA replication, as mRNAs for the synthesis of viral proteins, or as genomic RNAs to be packaged into progeny virions. Because picornaviral mRNA is identical in sequence to the viral RNA genome, all RNAs needed for the reproduction of these viruses can be made by a simple set of RNA synthesis reactions (Fig. 6.1). Such simplicity comes at a price, because there can be no regulation of individual gene products at the level of mRNA synthesis. However, polioviral gene expression can be controlled by the rate and extent of polyprotein processing. For example, $3CD^{pro}$, the precursor of the viral polymerase $3D^{pol}$, cannot polymerize RNA. Rather, $3CD^{pro}$ is a protease that cleaves at certain Gln-Gly amino acid pairs in the polyprotein. Regulating the processing of $3CD^{pro}$ controls the concentration of RNA polymerase.

Cellular Sites of Viral RNA Synthesis

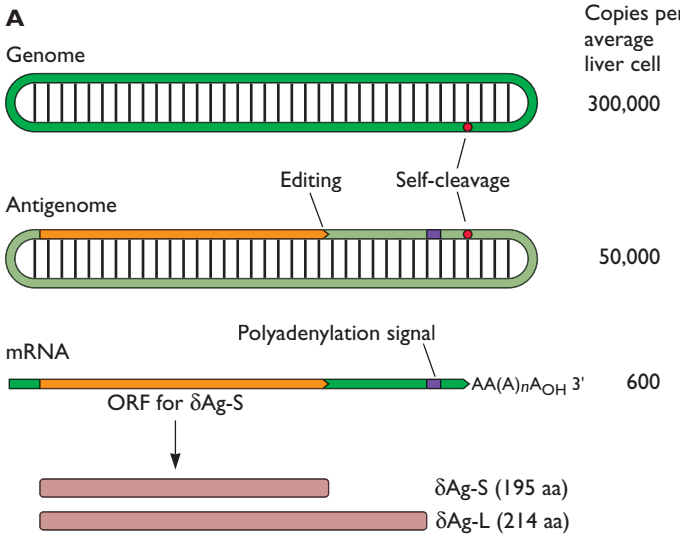
Genome and mRNA synthesis of most RNA viruses occurs in the cytoplasm of the cell, invariably in specific structures such as the nucleocapsids of (–) strand RNA viruses, subviral particles of double-stranded RNA viruses, and membrane-bound replication complexes for (+) strand RNA viruses. Membrane-bound replication complexes of different viruses are morphologically diverse, and the membranes originate from various cellular compartments. Alphaviral RNA synthesis occurs on the cytoplasmic surface of endosomes and lysosomes, and polioviral RNA polymerase is located on the surfaces of small, membranous vesicles that assemble into higher-order structures.

The membrane vesicles observed early in poliovirus-infected cells are thought to originate from two different cellular pathways. One source appears to be the endoplasmic reticulum, specifically vesicles whose production is regulated by proteins of coat protein complex II (CopII) (Chapter 12). Unlike vesicles produced from the endoplasmic reticulum in uninfected cells, those in poliovirus-infected cells do not fuse with the Golgi and therefore accumulate in the cytoplasm. The vesicles produced during poliovirus infection bear several hallmarks of autophagosomes, including a double-membrane morphology and colocalization with protein markers of these vesicles. Synthesis of poliovirus 2BC and 3A proteins in uninfected cells leads to production of such autophagosomes. Similar double-membrane vesicles are observed during infection with a variety of (+) strand RNA viruses, indicating that they may serve as a general replication platform. However, neither the production of CopII-coated vesicles nor the formation of autophagosomes is blocked by the fungal metabolite brefeldin A, which inhibits poliovirus RNA synthesis. The cellular target of this drug is the Arf GTPases, proteins that regulate the formation of secretory vesicles. The structures upon which poliovirus RNA is produced may therefore also require membrane vesicles that depend upon Arf for their formation.

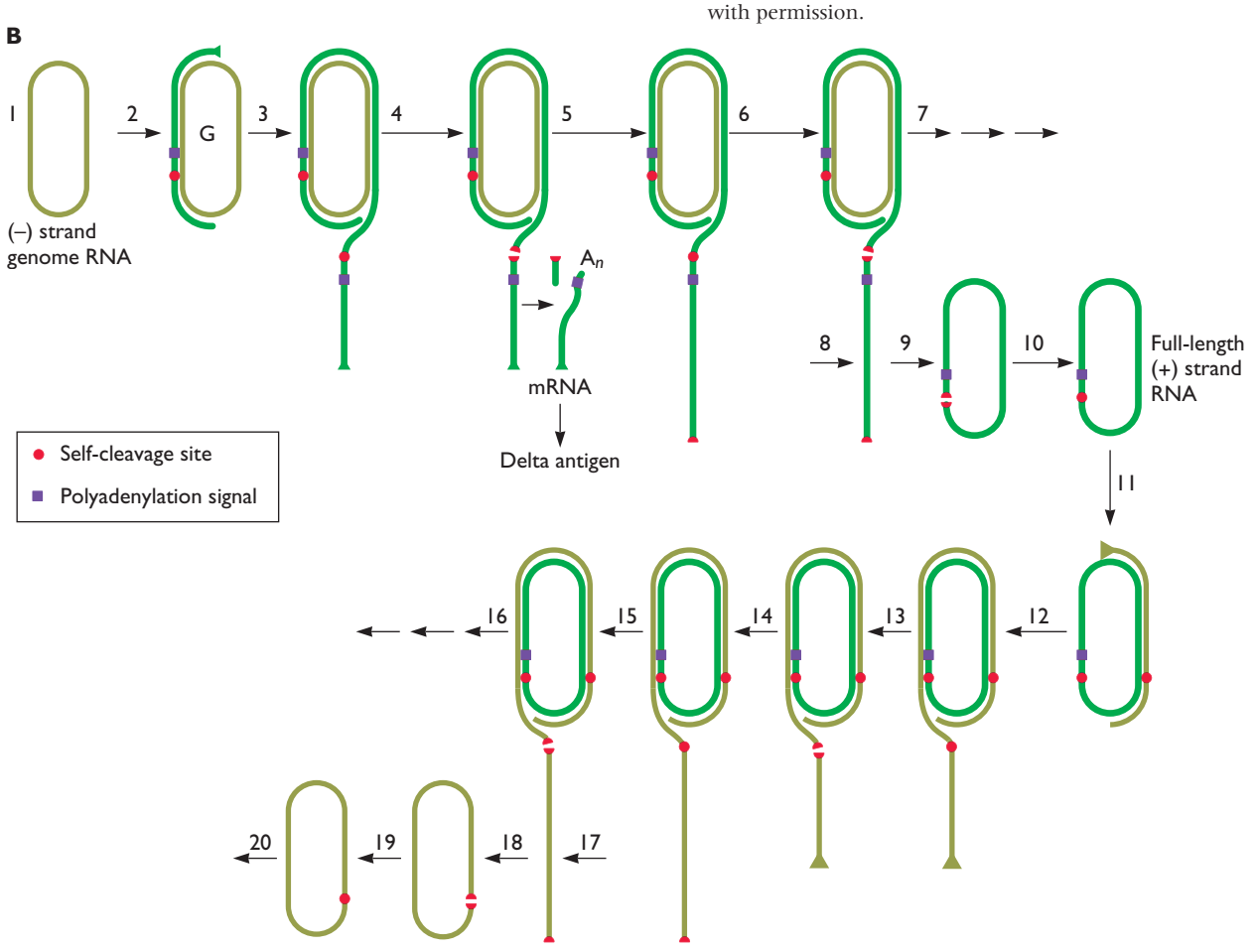
It is thought that membrane association of viral replication complexes ensures high local concentrations of replication components and hence increases the rates or efficiencies of replication reactions. As we have seen, this property may contribute to the specificity of polioviral $3D^{pol}$ for viral RNA templates. Membrane association may also have other functions, such as allowing efficient packaging of progeny RNA into virions, or providing lipid components

BOX 6.7 DISCUSSION

Unique mechanisms of mRNA and genome synthesis of the hepatitis delta satellite virus



Hepatitis delta satellite virus RNA synthesis. (A) Schematic of the forms of hepatitis delta virus RNA and δ antigen found in infected cells. aa, amino acids; ORF, open reading frame. **(B)** Overview of hepatitis delta virus mRNA and genomic RNA synthesis. In steps 1 to 3, RNA synthesis is initiated, most probably by host RNA polymerase II, at the indicated position on the (–) strand genomic RNA. The polymerase passes the poly(A) signal (purple box) and the self-cleavage domain (red circle). In steps 4 and 5, the 5′ portion of this RNA is processed by cellular enzymes to produce delta mRNA with a 3′-poly(A) tail, while RNA synthesis continues beyond the cleavage site and the RNA undergoes self-cleavage (step 6). RNA synthesis continues until at least one unit of the (–) strand genomic RNA template is copied. The poly(A) signal is ignored in this full-length (+) strand. In steps 7 to 10, after self-cleavage to release a full-length (+) strand, self-ligation produces a (+) strand circular RNA. In steps 11 to 20, mRNA synthesis initiates on the full-length (+) strand to produce (–) strands by a rolling-circle mechanism. Unit-length genomes are released by the viral ribozyme (step 15) and self-ligated to form (–) strand circular genomic RNAs. Adapted from J. M. Taylor, *Curr. Top. Microbiol. Immunol.* **239:**107–122, 1999, with permission.



(continued)

BOX 6.7 DISCUSSION

Unique mechanisms of mRNA and genome synthesis of the hepatitis delta satellite virus (continued)

The strategy for synthesis of the hepatitis delta satellite virus genome is apparently unique among animal viruses. The viral RNAs are synthesized by host cell DNA-dependent RNA polymerase II (Box 6.8), and the hepatitis delta virus RNAs are RNA catalysts, or **ribozymes** (Box 6.9). The genome of hepatitis delta virus is a 1,700-nucleotide (–) strand circular RNA, the only RNA with this structure that has been found in animal cells. As approximately 70% of the nucleotides are base paired, the viral RNA is folded into a rodlike structure (see figure). Surprisingly, all hepatitis delta virus-specific RNA synthesis is catalyzed by cellular DNA-dependent RNA polymerase II, which can also utilize RNA as a template. Genomic RNA replication requires reactions catalyzed by ribozymes (Box 6.9) residing in both the hepatitis delta virus genomic (–) strand RNA and its full-length (+) strand RNA copy. Both these hepatitis delta virus RNAs catalyze self-cleavage in the absence of any protein and may also catalyze self-ligation.

All hepatitis delta satellite virus RNAs are synthesized in the nucleus by RNA polymerase II (see figure). The switch from mRNA synthesis to the production of full-length (+) RNA is controlled by suppression of a poly(A) signal (see "Suppression of Polyadenylation"). Full-length (–) and (+) strand RNAs are copied by a rolling-circle mechanism, and ribozyme self-cleavage releases linear monomers. Subsequent ligation of the two termini by the same ribozyme produces a monomeric circular RNA. The hepatitis delta virus ribozymes are therefore needed to process the intermediates of rolling-circle RNA replication.

or physical support to the replication complex. The surfaces of membranous replication complexes isolated from poliovirus-infected cells appear to be coated with two-dimensional arrays of polymerase. These arrays are formed by interaction of $3D^{pol}$ molecules in a head-to-tail fashion. Surface catalysis is known to have several advantages, including a higher probability of collision among reactants, an increase in substrate affinity from clustering of multiple binding sites, and retention of reaction products. The last property would facilitate multiple rounds of copying (+) and (–) strand RNA templates.

Viral RNA polymerases are recruited to membranous replication complexes in different ways. Polioviral $3D^{pol}$ is targeted to membrane vesicles by binding to the viral membrane protein 3AB (Fig. 6.12). In contrast, a C-terminal, transmembrane segment of the hepatitis C virus RNA polymerase, NS5b, is responsible for attachment of the enzyme to cellular membrane replication complexes.

BOX 6.8 DISCUSSION

RNA-dependent RNA polymerase II

The mRNAs produced during hepatitis delta satellite virus infection of cells have typical properties of DNA-dependent RNA polymerase II products, including a 5′ cap and 3′-poly(A) tail. Production of these satellite mRNAs is also sensitive to α-amanitin, an inhibitor of DNA-dependent RNA polymerase II. Furthermore, the RNA genome of plant viroids can be copied by plant DNA-dependent RNA polymerase II. Based on these observations, it was suggested that the RNA genome of hepatitis delta satellite virus is copied by RNA polymerase II. Recently, experimental support for this hypothesis has been obtained. When purified mammalian RNA polymerase II was incubated with NTPs and an RNA template-primer, an RNA product was produced. Similar results were obtained when the antigenome of hepatitis delta satellite virus was used in the reaction. Structural studies revealed that the RNA template-product duplex occupies the same site on the enzyme as the DNA-RNA hybrid during transcription. When transcription protein IIS was added to the reaction mixture, the satellite genome was cleaved, and the new 3′ end was used as a primer. Compared with DNA-dependent RNA synthesis, RNA-dependent RNA synthesis by RNA polymerase II was slower and less processive. These properties may explain why the enzyme can copy only short RNA templates.

The ability of DNA-dependent RNA polymerase II to copy an RNA template provides a missing link in molecular evolution. This activity supports the hypothesis that an ancestor of RNA polymerase II copied RNA genomes that are thought to have existed during the ancient RNA world. During the transition from RNA to DNA genomes, this enzyme evolved to copy DNA templates. Today these enzymes can still copy small RNAs such as the genome of hepatitis delta satellite virus.

Chang, J., X. Nie, H. E. Chang, Z. Han, and J. Taylor. 2008. Transcription of hepatitis delta virus RNA by RNA polymerase II. *J. Virol.* **82:**1118–1127.

Lehmann, E., F. Brueckner, and P. Cramer. 2007. Molecular basis of RNA-dependent RNA polymerase II activity. *Nature* **450:**445–449.

Rackwitz, H. R., W. Rohde, and H. L. Sanger. 1981. DNA-dependent RNA polymerase II of plant origin transcribes viroid RNA into full-length copies. *Nature* **291:**297–301.

BOX 6.9 BACKGROUND

Ribozymes

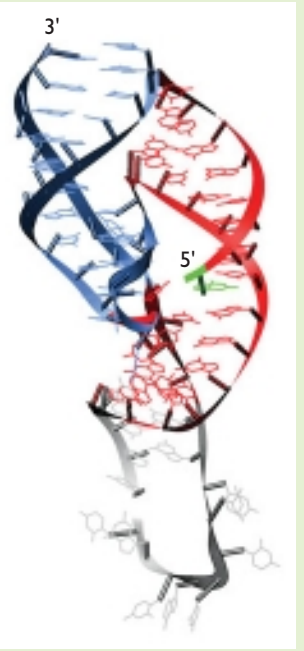
A **ribozyme** is an enzyme in which RNA, not protein, carries out catalysis. The first ribozyme discovered was the group I intron of the ciliate *Tetrahymena thermophila*. Other ribozymes have since been discovered, including RNase P of bacteria, group II self-splicing introns, hammerhead RNAs of viroids and satellite RNAs, and the ribozyme of hepatitis delta virus. Ribozymes are very diverse in size, sequence, and the mechanism of catalysis. For example, the hepatitis delta satellite virus ribozyme (see figure) catalyzes a transesterification reaction that produces products with 2′,3′-cyclic phosphate and 5′-OH termini. Only an 85-nucleotide sequence is required for activity of this ribozyme, which can cleave optimally with as little as a single nucleotide 5′ to the site of cleavage.

By joining the 85-nucleotide fragment to other upstream sequences, accurate 3′ ends of heterologous RNA transcripts synthesized *in vitro* can be obtained. This property proved crucial for producing infectious RNAs from cloned DNA copies of the genomes of (–) strand RNA viruses. Ribozymes can also be used to target the cleavage of other RNAs and are therefore being tested as antiviral and antitumor agents.

Kruger, K., P. J. Grabowski, A. J. Zaug, J. Sands, D. E. Gottschling, and T. R. Cech. 1982. Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of Tetrahymena. *Cell* **31:**147–157.

Westhof, E., and F. Michel. 1998. Ribozyme architectural diversity made visible. *Science* **282:**251–252.

Whelan, S. P., L. A. Ball, J. N. Barr, and G. T. Wertz. 1995. Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. *Proc. Natl. Acad. Sci. USA* **92:**8388–8392.



Crystal structure of the hepatitis delta satellite virus ribozyme. The RNA backbone is shown as a ribbon. The two helical stacks are shown in red and blue, and unpaired nucleotides are grey. The 5′ nucleotide, which marks the active site, is green. Produced from Protein Data Bank file 1cx0.

Polioviral 3D^{pol} cannot by itself associate with membranes but is brought to the replication complex by binding to protein 3AB. When the membrane association of this protein is disrupted by amino acid changes, viral RNA synthesis is inhibited. The hydrophobic domain of 3AB can be substituted for the C-terminal transmembrane segment of NS5b with little effect on RNA polymerase activity, indicating that membrane association is the sole function of this sequence.

Origins of Diversity in RNA Virus Genomes

Misincorporation of Nucleotides

All nucleic acid polymerases insert incorrect nucleotides during chain elongation. DNA-directed DNA polymerases have **proofreading** capabilities in the form of exonuclease activities that can correct such mistakes. RNA-dependent RNA polymerases do not possess proofreading ability. The result is that error frequencies in RNA replication can be as high as one misincorporation per 10^3 to 10^5 nucleotides polymerized, whereas the frequency of errors in DNA replication is about 10,000-fold lower. Many of these polymerization errors cause lethal amino acid changes, while other mutations may appear in the genomes of infectious virions. This phenomenon has led to the realization that RNA virus populations are **quasispecies**, or mixtures of many different genome sequences. The errors introduced during RNA replication have important consequences for viral pathogenesis and evolution, as discussed in Volume II, Chapter 10. Because RNA virus stocks are mixtures of genotypically different viruses, specific viral mutants may be isolated directly from such stocks. For example, live attenuated poliovirus vaccine strains are viral mutants that were isolated from an unmutagenized stock of wild-type virus.

Fidelity of copying by RNA-dependent RNA polymerases is determined by how the template, primer, and NTP interact at the active site. The results of structural and genetic studies of picornaviral 3D^{pol} have led to a hypothesis about how fidelity is ensured during RNA synthesis. Nucleotide binding occurs in two steps: first, the NTP is bound in such a way that the ribose cannot interact properly with the Asp of motif A and the Asn of motif B (Fig. 6.6). Next, if the NTP is correctly base paired with the template strand, there is a conformational change in the enzyme which

reorients the triphosphate and allows phosphoryl transfer to occur. This conformational change requires reorientation of the Asp and Asn residues, which would stabilize the position of the ribose in the binding pocket. This conformational change is thought to be a key fidelity checkpoint for the picornaviral RNA polymerase. This model derives from the structures of $3D^{pol}$ bound to a template primer and NTP (Fig. 6.7) and the study of an altered poliovirus $3D^{pol}$ with higher fidelity than the wild-type enzyme. The increased fidelity of this enzyme, which has a single amino acid change in the fingers domain, is due to a change in the equilibrium constant for the conformational change. Although this amino acid is remote from the active site, it is involved in hydrogen bonding to motif A, which, as discussed above, is important in holding the NTP in a catalytically appropriate conformation. Of great interest is the observation that a similar interaction between fingers and motif A can be observed in RNA polymerases from a wide variety of viruses. This mechanism of regulating fidelity may therefore be conserved in all RNA-dependent RNA polymerases.

These studies also provide mechanistic information on how ribavirin, an antiviral compound, causes lethal mutagenesis of picornaviruses. The structure of foot-and-mouth disease virus $3D^{pol}$ bound to ribavirin shows the compound positioned in the active site of the enzyme, adjacent to the 3′ end of the primer. The ribose of ribavirin is bound in the pocket, indicating that it has bypassed the fidelity checkpoint and has caused the conformational change that holds the analog in a position ready for catalysis. Therefore, ribavirin is a mutagen because it can bypass the fidelity checkpoint and be inserted into incorrect positions in newly synthesized RNA molecules.

The RNA polymerase of members of the *Nidovirales* (Box 6.10) may have evolved to allow faithful replication of the large (up to 32 kb) RNA genomes. The RNA synthesis machinery includes proteins not found in other RNA viruses, such as a U-specific endonuclease. It has been suggested that these proteins are part of a repair mechanism similar to the proofreading activity associated with DNA replication.

Segment Reassortment and RNA Recombination

Reassortment is the exchange of entire RNA molecules between genetically related viruses with segmented genomes. In cells coinfected with two different influenza viruses, the eight genome segments of each virus replicate. When new progeny viruses are assembled, they can package RNA segments from **either** parental virus. Because reassortment involves the simple exchange of RNA segments, it can occur at high frequencies.

In contrast to reassortment, recombination is the exchange of nucleotide sequences among different genomic RNA molecules (Fig. 6.27A). Recombination, a feature of many RNA viruses, is an important mechanism for producing new genomes with selective growth advantages. This process has shaped the RNA virus world by rearranging genomes or moving functional parts of RNA molecules among different viruses. RNA recombination was first discovered in cells infected with poliovirus and was subsequently found to occur with other (+) and (−) strand RNA viruses. The frequency of recombination can be relatively high: it has been estimated that 10 to 20% of polioviral genomic RNA molecules recombine in one growth cycle. RNA recombination is not limited to the research laboratory. For example, recombinants among the three serotypes of poliovirus are readily isolated from the feces of individuals immunized with the live (Sabin) vaccine. Such recombinants may possess an improved ability to replicate in the human alimentary tract and have a selective advantage over the parental viruses. Recombination can also lead to the production of pathogenic viruses (Box 6.11).

Polioviral recombination is predominantly **base pairing dependent**: it occurs between nucleotide sequences that have a high percentage of nucleotide identity (Fig. 6.27B). Other viral genomes undergo **base-pairing-independent** recombination between very different nucleotide sequences. RNA recombination is coupled with the process of genomic RNA replication: it occurs by template exchange during (−) strand synthesis, as first demonstrated in poliovirus-infected cells. The RNA polymerase first copies the 3′ end of one parental (+) strand and then exchanges one template for another at the corresponding position on a second parental (+) strand (Fig. 6.27B). Template exchange in poliovirus-infected cells occurs predominantly during (−) strand synthesis, presumably because the concentration of (+) strands is 100-fold higher than that of (−) strands. This template exchange mechanism of recombination is also known as **copy choice**. The exact mechanism of template exchange is not known, but it might be triggered by pausing of the polymerase during chain elongation (Fig. 6.27).

If the RNA polymerase skips sequences during template switching, deletions may occur. These RNAs can replicate if they contain the appropriate signals for the initiation of RNA synthesis. Subgenomic RNAs replicate more rapidly than full-length RNA and therefore compete for the components of the RNA synthesis machinery. Because of these properties, they are called **defective interfering RNAs**. Such RNAs can be packaged into viral particles only in the presence of a **helper virus** that provides viral proteins.

Defective interfering particles accumulate during the replication of both (+) and (−) strand RNA viruses.

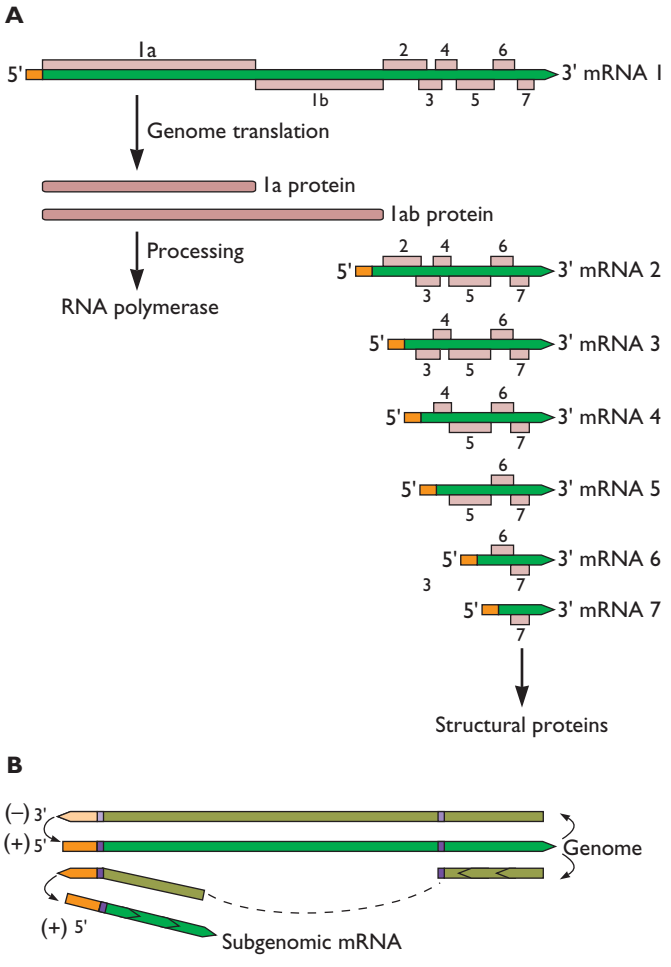
BOX 6.10 BACKGROUND

Synthesis of nested subgenomic mRNAs

An unusual pattern of mRNA synthesis occurs in cells infected with members of the families *Coronaviridae* and *Arteriviridae*, in which subgenomic mRNAs that form a 3′-coterminal nested set with the viral genome are synthesized (see figure). These viral families were combined into the order *Nidovirales* to reflect this property (*nidus* is Latin for nest).

The subgenomic mRNAs of these viruses are composed of a leader and a body that are synthesized from noncontiguous sequences at the 5′ and 3′ ends, respectively, of the viral (+) strand genome. The leader and body are separated by a conserved junction sequence encoded both at the 3′ end of the leader and at the 5′ end of the mRNA body.

Subgenome-length (–) strands are produced when the template loops out as the polymerase completes synthesis of the leader RNA (see figure). These (–) strand subgenome-length RNAs then serve as templates for mRNA synthesis.



Nidoviral genome organization and expression. (A) Organization of open reading frames. The (+) strand viral RNA is shown at the top, with open reading frames as boxes. The genomic RNA is translated to form polyproteins 1a and 1ab, which are processed to form the RNA polymerase. Structural proteins are encoded by six nested mRNAs that share a common 5′ leader sequence (orange box). **(B)** Model of the synthesis of nested mRNAs. Discontinuous transcription occurs during (–) strand RNA synthesis. Most of the (+) strand template is not copied, probably because it loops out as the polymerase completes synthesis of the leader RNA. The resulting (–) strand RNAs, with leader sequences at the 3′ ends, are then copied to form mRNAs. Adapted from E. J. Snijder et al., *J. Gen. Virol.* **79:**961–979, 1998, with permission.

Production of these viruses requires either a high multiplicity of infection or serial passaging, conditions that are achieved readily in the laboratory but rarely in nature. It is not known whether defective interfering viruses generally play a role in viral pathogenesis. However, some recombination reactions that lead to the appearance of cytopathic bovine viral diarrhea viruses delete viral RNA sequences rather than inserting cellular RNA sequences (Box 6.11). Such deletions create a new protease cleavage site at the N terminus of the NS3 protein, and the defective

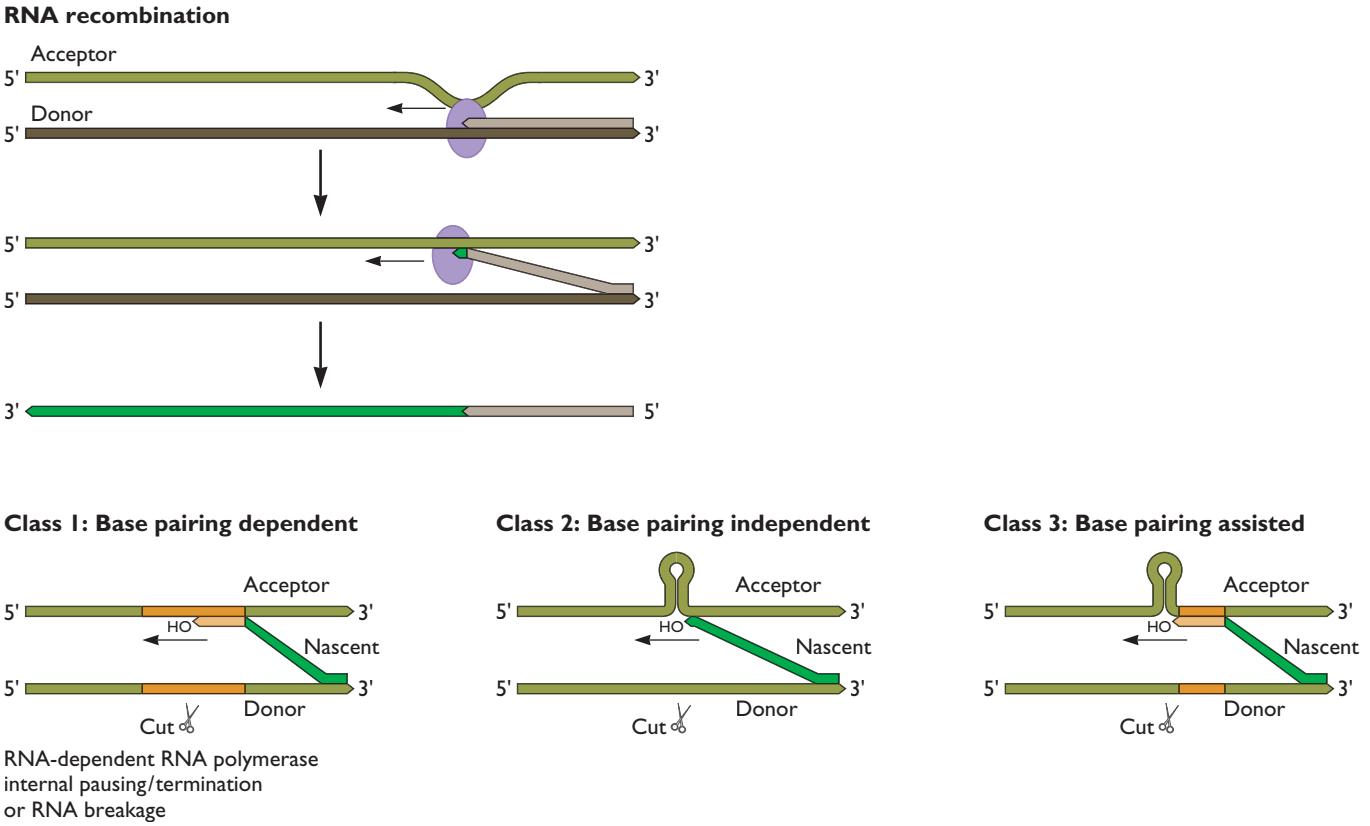


Figure 6.27 RNA recombination. (Top) Schematic representation of RNA recombination occurring during template switching by RNA polymerase, or copy choice. Two parental genomes are shown. The RNA polymerase (purple oval) has copied the 3′ end of the donor genome and is switching to the acceptor genome. The resulting recombinant molecule is shown. **(Bottom)** Three classes of RNA recombination. All three classes require RNA polymerase-mediated template exchange. Events that occur after the exchange are shown by an arrow. The hairpin symbolizes various RNA features required for class 2 and 3 recombination. In base-pairing-dependent recombination, substantial sequence similarity between parental RNAs is required and is the major determinant of recombination. In base-pairing-independent recombination, sequence similarity is not required but may be present. Recombination may be determined by other RNA features, such as RNA polymerase-binding sites, secondary structures, and heteroduplex formation between parental RNAs. Base-pairing-assisted recombination combines features of class 1 and class 2 recombination. Sequence similarity influences the frequency or site of recombination, but additional RNA features are required. Adapted from P. D. Nagy and A. E. Simon, *Virology* **235:**1–9, 1997, with permission.

interfering viruses also cause severe gastrointestinal disease in livestock.

RNA Editing

Diversity in RNA viral genomes is also achieved by RNA editing. Viral mRNAs can be edited either by insertion of a nontemplated nucleotide during synthesis or by alteration of the base after synthesis. Examples of RNA editing have been documented in members of the *Paramyxoviridae* and *Filoviridae* and in hepatitis delta satellite virus. This process is described in Chapter 10.

Perspectives

In a previous edition of this textbook, we wrote, "The crystal structure of polioviral $3D^{pol}$ hints at the beautiful pictures of other viral RNA polymerases and their associated proteins that will be forthcoming." The structural biologists have made us appear clairvoyant: since then, the three-dimensional structures of many RNA-dependent RNA polymerases have been resolved. These structures underscore the relationship of these enzymes to other nucleic acid polymerases, but also underscore structural differences that have evolved to accommodate the wide diversity of

BOX 6.11 DISCUSSION

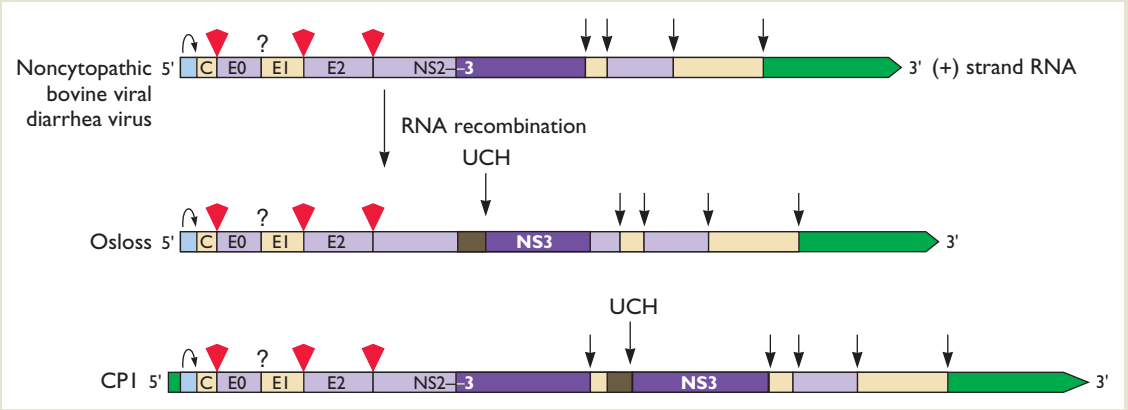
RNA recombination leading to the production of pathogenic viruses

A remarkable property of pestiviruses, members of the *Flaviviridae*, is that RNA recombination produces viruses that cause disease. Bovine viral diarrhea virus causes a usually fatal gastrointestinal disease. Infection of a fetus with this virus during the first trimester is noncytopathic, but RNA recombination produces a cytopathic virus that causes severe gastrointestinal disease after the animal is born.

Pathogenicity of bovine viral diarrhea virus is associated with the synthesis of a nonstructural protein, NS3, encoded by the recombinant cytopathic virus (see figure). The NS3 protein cannot be produced in cells infected by the noncytopathic parental virus because its precursor, the NS2-3 protein, is not proteolytically processed. In contrast, NS3 **is** synthesized in cells infected by cytopathic bovine viral diarrhea virus because base-pairing-independent RNA recombination reactions add an extra protease cleavage site in the viral polyprotein, precisely at the N terminus of the NS3 protein (see figure). This cleavage site can be created in several ways. One of the most frequent is insertion of a cellular RNA sequence coding for ubiquitin, which targets cellular proteins to a degradative pathway. Insertion of ubiquitin at the N terminus of NS3 permits cleavage of NS2-3 by any member of a widespread family of cellular proteases. This recombination event provides a selective advantage because pathogenic viruses outgrow nonpathogenic viruses. Why cytopathogenicity is associated with release of the NS3 protein, which is thought to be part of the enzymatic system for genomic RNA replication, is not known.

Retroviruses acquire cellular genes by recombination, and the resulting viruses can have lethal disease potential, as described in Volume II, Chapters 7 and 10.

Pathogenicity of bovine viral diarrhea virus is associated with production of the NS3 protein. Two cytopathic viruses, Osloss and CP1, in which the ubiquitin sequence (UCH) has been inserted at different sites, are shown. In Osloss, UCH has been inserted into the NS2-3 precursor, and NS3 is produced. In CP1, a duplication has also occurred such that an additional copy of NS3 is present after the UCH sequence. Adapted from D. M. Knipe et al. (ed.), *Fields Virology*, 4th ed. (Lippincott Williams & Wilkins, Philadelphia, PA, 2001), with permission.



RNA genome configurations. We expect that forthcoming structures will detail the conformational movements that occur during the switch between initiation and elongation, highlighting the changes that occur as the polymerase moves from an open to a closed conformation. Our understanding of how RNA polymerases alter between synthesis of mRNA and genome replication is largely hypothetical; additional structures should provide insight into the mechanism of this crucial aspect of the infectious cycle.

The nature and function of host proteins that are required for viral RNA synthesis remain obscure, as does the reason why RNA synthesis occurs in certain cellular compartments. Some kinds of membranes are the sites of RNA replication for different viruses, but the reasons for the specificity are unknown. Is the membrane simply a platform for RNA synthesis? Viral RNA-directed RNA synthesis is a unique process and therefore an excellent target for antiviral intervention. As our understanding of RNA catalysis improves, new ways of limiting viral infections will be revealed.

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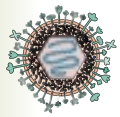


7

Reverse Transcription and Integration

"One can't believe impossible things," said Alice.

"I dare say you haven't had much practice," said the Queen. "Why, sometimes I've believed as many as six impossible things before breakfast."

Lewis Carroll
Alice in Wonderland

Retroviral Reverse Transcription

Discovery

In 1970, back-to-back reports in the scientific journal *Nature* from the laboratories of Howard Temin and David Baltimore provided the first concrete evidence for the existence of an RNA-directed DNA polymerase activity in retrovirus particles. The pathways to this discovery were quite different in the two laboratories. In Temin's case, the discovery came about through attempts to understand how this group of RNA-containing viruses could permanently alter the heredity of cells, as they do in the process of oncogenic transformation. Temin proposed that retroviral RNA genomes become integrated into the host cell's chromatin in a DNA form, an idea supported by the observation that purified cellular DNA polymerases can use RNA as a template under certain reaction conditions in vitro. Furthermore, studies of bacterial viruses such as bacteriophage lambda had established a precedent for viral DNA integration into host DNA (Box 7.1). However, with the technology available at the time, it was a difficult hypothesis to test, and attempts by Temin and others to demonstrate the existence of such a phenomenon in infected cells were generally met with skepticism. Baltimore's entrée into the problem of reverse transcription came from his interest in virion-associated polymerases, in particular one that he had just discovered to be present in vesicular stomatitis virions, a virus with a (−) strand RNA genome. It occurred to Baltimore and Temin independently that retrovirus particles might also contain the sought-after RNA-dependent DNA polymerase. As subsequent experiments showed, this was indeed the case, and the retrovirus particles themselves yielded up the enzyme activity that had earlier eluded Temin. In 1975, Temin and Baltimore were awarded the Nobel Prize in physiology or medicine for their independent discoveries of retroviral reverse transcriptase (RT).

BOX 7.1 DISCUSSION

Bacteriophage lambda, a paradigm for the joining of viral and host DNAs

In 1962, Allan Campbell proposed an elegant, but at the time revolutionary, model for site-specific integration of DNA of the bacteriophage lambda into chromosome of its host, *Escherichia coli*. The model was deduced from the fact that different linkage maps could be constructed for viral genomes at different stages in its life cycle. One linkage map, that of the prophage, was obtained from the study of lysogenic bacteria. A different linkage map was obtained by measuring recombination frequencies in the phage yield from infected cells (see part A of figure).

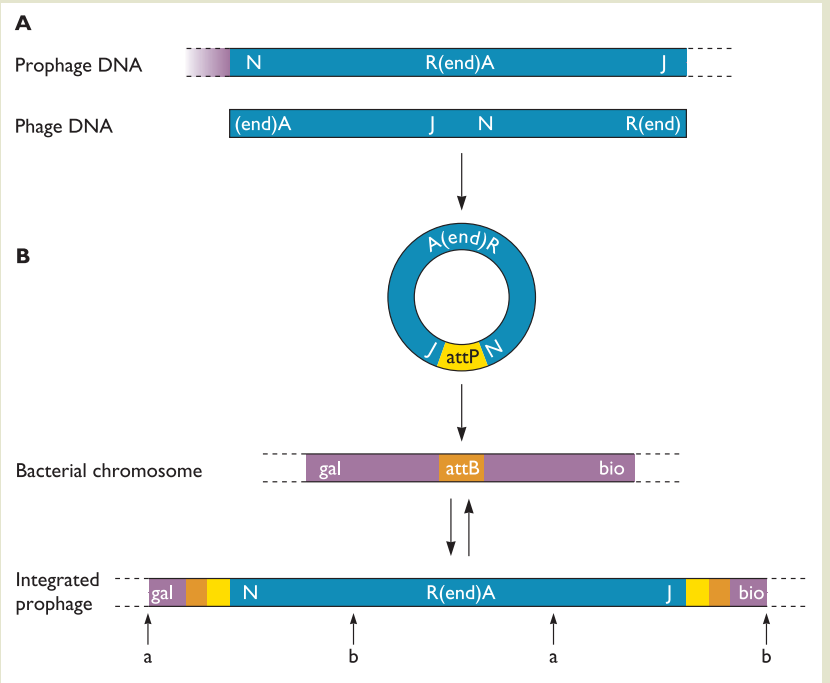
Campbell proposed that these unique features could be explained by a model for integration in which the incoming, linear double-stranded DNA phage genome must first circularize. Subsequent recombination between a specific, internal sequence in the phage genome (called *attP*) and a specific sequence in the bacterial chromosome (called *attB*) would produce an integrated viral genome, with a linkage map that was a circular permutation of that of the linear phage genome, as had been observed (see part B of figure).

Induction of the integrated provirus and subsequent viral DNA replication would proceed by a reversal of this site-specific integration reaction, a process called excision. The model explained how abnormal excision (arrows a and b) could give rise to the observed rare, specialized transducing phages that lacked certain viral genes and carried either the *gal* or *bio* genes of the host. These were the first cellular "cloned" DNAs to be identified.

Although this model seems obvious today, it was not obvious in the 1960s. An alternative model in which the linear viral DNA was attached by a partial binding or "synapse" with the bacterial chromosome was favored by a number of investigators. However, shortly after Campbell's elaboration of his model, circular molecules of lambda phage DNA were detected in infected cells, and the linear DNA extracted from purified virus particles was found to possess short complementary single-strand extensions, "cohesive ends," that could promote circle formation. Other predictions of the model were also validated in several laboratories, and viral and cellular proteins that catalyzed integration and excision were identified.

Lambda DNA integration remains an important paradigm for understanding the molecular mechanisms of DNA recombination and the parameters that influence the joining of viral and host DNAs.

Campbell, A. M. 1962. Episomes. *Adv. Genet.* **11:**101–145.



Impact

The immediate impact of the discovery of RT was to amend the then-accepted central dogma of molecular biology, that the transfer of genetic information is unidirectional: DNA → RNA → protein. It was now apparent that there could also be a "retrograde" flow of information from RNA to DNA, and the name **retroviruses** eventually came to replace the earlier designation of RNA tumor viruses. In the years following this revision of dogma, many additional reverse transcription reactions have been discovered.

Furthermore, as Temin hypothesized, study of RT has contributed to our understanding of cancer. As described in Volume II, Chapter 7, the study of oncogenic retroviruses has provided a framework for current concepts of the genetic basis of this disease. Study of reverse transcription (and integration) has also allowed us to understand the persistence of retroviral infections and aspects of the pathogenesis of acquired immunodeficiency syndrome (AIDS) that is caused by the human immunodeficiency virus. Finally, RT itself, first purified from virions and now produced in bacteria, has become an indispensable tool in molecular biology, for example allowing experimentalists to capture cellular messenger RNAs (mRNAs) as complementary DNAs (cDNAs), which can then be amplified, cloned, and expressed by well-established methodologies. For such reasons, we devote an entire chapter to these very important reactions.

The Pathways of Reverse Transcription

Significant insight into the mechanism of reverse transcription can be obtained by comparing the amino acid sequences of RTs with those of other enzymes that catalyze similar reactions. For example, RTs share (with the RNA and DNA polymerases of both prokaryotes and eukaryotes) certain sequence motifs in regions known to include critical active-site residues (see Fig. 6.4). It is hypothesized, therefore, that these enzymes employ similar catalytic mechanisms for nucleic acid polymerization reactions. Like DNA polymerases, viral RTs cannot initiate DNA synthesis *de novo*, but require a specific primer. In this chapter, we provide a detailed description of priming and reverse transcription for retroviral and hepadnaviral enzymes. But it should be noted that even as arcane and distinct from each other as these two systems may appear, they do not exhaust the repertoire for reverse transcription reactions that have evolved in nature. A wide variety of primers, as well as sites and modes of initiation, are used by the RTs of other retroelements.

Much of what has been learned about reverse transcription in retroviruses comes from the analysis of intermediates in the reaction pathway that have been identified in extracts from infected cells. Reverse transcription intermediates have also been detected in **endogenous reactions**, which take place within purified virus particles, using the encapsidated viral RNA template. It was amazing to discover that intermediates and products virtually identical to those made in infected cells can actually be synthesized in purified virions; all that is required is treatment with a mild detergent to permeabilize the envelope and addition of the metal cofactor and deoxyribonucleoside triphosphate (dNTP) substrates. The fidelity and robustness of the endogenous reaction suggests that the reverse transcription system is poised for action as soon as the virus enters the cell. Indeed, small amounts of viral DNA can be detected in purified human immunodeficiency virus type 1 particles, presumably having been synthesized using substrates picked up from the infected cell during budding. Retroviral reverse transcription intermediates have also been analyzed in totally reconstituted reactions with purified enzymes and model RNA templates.

Retroviral RT is the only protein required to accomplish all the diverse steps in the pathway described below. However, as the reactions that take place inside cells are significantly more efficient than those observed in either endogenous or reconstituted systems, it is unlikely that all the essential features have been reproduced.

Essential Components

Genomic RNA. Retrovirus particles contain two copies of the RNA genome held together by multiple regions of base pairing. (See Box 7.2 for labeling conventions.) This RNA sediments in a 70S complex, as expected for a dimer of 35S genomes. Partial denaturation and electron microscopic analyses of the 70S complex indicate that the most stable pairing is via sequences located near the 5′ ends of the two genomes (Fig. 7.1). Retroviral genomes can be thought of as being annealed head to head, an arrangement that may discourage the encapsidation of multiples larger than two (i.e., concatemers). The 70S RNA complex also includes two molecules of a specific cellular transfer RNA (tRNA) that serves as a primer for the initiation of reverse transcription (discussed below).

Despite the fact that two genomes are encapsidated, only one integrated copy of the viral DNA typically is detected after infection with single virion. Therefore, retroviral virions are said to be **pseudodiploid**. Why should such a feature have been selected during evolution? One popular notion is that the availability of two RNA templates can help retroviruses survive extensive damage to their genomes. At least parts of both genomes can, and typically are, used as templates during the reverse transcription process, accounting for the high rates of genetic recombination in these viruses. Presumably, being able to patch together

BOX 7.2 TERMINOLOGY

Conventions for designating sequences in nucleic acids

For clarity, lowercase designations are used throughout this chapter to refer to RNA sequences; uppercase designations identify the same or complementary sequences in DNA (e.g. pbr in RNA; PBR in DNA).

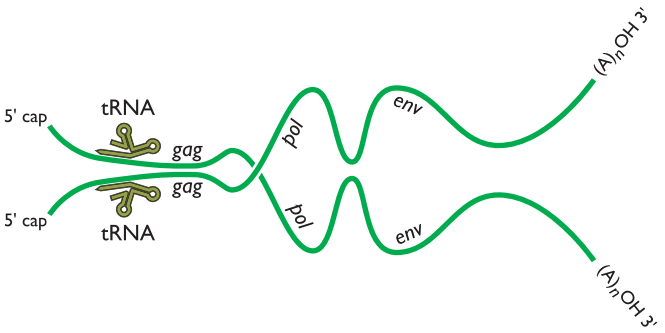


Figure 7.1 The diploid retroviral genome. The diploid genome includes the following, from 5′ to 3′: the m^7Gppp capping group; the coding regions for viral structural proteins and enzymes; *gag*, *pol*, and *env*; and the 3′-poly(A) sequence. The cell-derived primer tRNA is also shown. Points of contact represent multiple short regions of complementary base pairing. From J. M. Coffin, p. 1767–1848, *in* B. N. Fields et al. (ed.), *Fields Virology*, 3rd ed. (Lippincott-Raven, Philadelphia, PA, 1996), with permission.

one complete DNA copy from two randomly damaged RNA genomes would provide survival value; the genetic recombination produced during the process may also contribute to survival. Nevertheless, genetic experiments have shown that the use of two RNA templates is not an essential feature of the reverse transcription process. Therefore, all of the known steps in the reverse transcription pathway can take place on a single genome.

Like the genomes of (–) strand RNA viruses, the retroviral genome is coated along its length by a viral nucleocapsid protein (NC), with approximately one molecule for every 10 nucleotides. This small basic protein can bind nonspecifically to both RNA and DNA and promote the annealing of nucleic acids. Biochemical experiments suggest that NC may facilitate template exchanges and perform a role in reverse transcription similar to that of prokaryotic single-stranded-DNA-binding (SSB) proteins. In the synthesis of DNA catalyzed by bacterial DNA polymerases, the single-stranded-DNA-binding proteins enhance **processivity** (the efficiency of elongation). The ability of NC first to organize RNA genomes within the virion, and then to facilitate reverse transcription within the infected cell, may account for some of the differences in efficiency observed when comparing reactions reconstituted *in vitro* with those that take place in a natural infection.

Primer tRNA. In addition to the viral genome, retroviral virions contain a collection of cellular RNAs. These include approximately 100 copies of a nonrandom sampling of tRNAs, some 5S rRNA, 7S RNA, and traces of cellular mRNAs. We do not know how most of these cellular RNAs become incorporated into virus particles, and most have no obvious function. However, one particular tRNA molecule does have a critical role, that of serving as primer for the initiation of reverse transcription. The tRNA primer is positioned on the template genome during virus assembly via interactions with both RT and viral RNA, probably facilitated by NC. The primer tRNA is partially unwound and hydrogen-bonded to complementary sequences near the 5′ end of each RNA genome in a region called the **primer-binding site (pbs)** (Fig. 7.2). The reverse transcriptases of all retroviruses studied to date are primed by one of only a few classes of cellular tRNAs. Most mammalian retroviral RTs rely on either $tRNA^{Pro}$, $tRNA^{Lys3}$, or $tRNA^{Lys1,2}$ for this function.

In addition to the 3′-terminal 18 nucleotides that anneal to the pbs, other regions in the tRNA primer contact the RNA template and modulate reverse transcription. The template-primer interaction has been studied extensively in reconstituted reactions with RNA and RT of avian sarcoma/leukosis virus. In these *in vitro* analyses, the ability of the viral RNA to form stem-loop structures, and specific interactions between the primer $tRNA^{Trp}$ and one of these loops, appears to be critical for reverse transcription (Fig. 7.2). Similar interactions have been reported for human immunodeficiency virus RNA and its primer. Although the interactions are likely to be significant biologically, we do not yet know how RTs recognize structural features in these template-primer complexes.

Reverse transcriptase. Each retrovirus particle contains 50 to 100 molecules of RT. Retroviral RTs probably function as dimers, but the number of dimers in each virion that are actually engaged in reverse transcription is not known. As noted previously, results from studies with purified virions suggest that viral DNA synthesis can begin as soon as the viral envelope is removed. With the three orthoretroviruses studied most extensively (avian sarcoma/leukosis virus, murine leukemia virus, and human immunodeficiency virus type 1), DNA synthesis takes place mainly in the cytoplasm, in a subviral structure called the RT complex. Enzymes of these three retroviruses are used as examples throughout this chapter.

Retroviral RTs are complex molecular machines with moving parts and multiple activities. The distinct catalytic activities brought into play at various stages in the pathway of reverse transcription include RNA-directed and DNA-directed DNA polymerization, DNA unwinding, and the hydrolysis of RNA in RNA-DNA hybrid by RNase H. The first three activities reside within the polymerase domain, with RNase H activity comprising a separate domain. In digesting RNA-DNA hybrids, the RNase H functions primarily as an endonuclease, producing oligoribonucleotides of 2 to 15 nucleotides. The activities of the RNase H domain

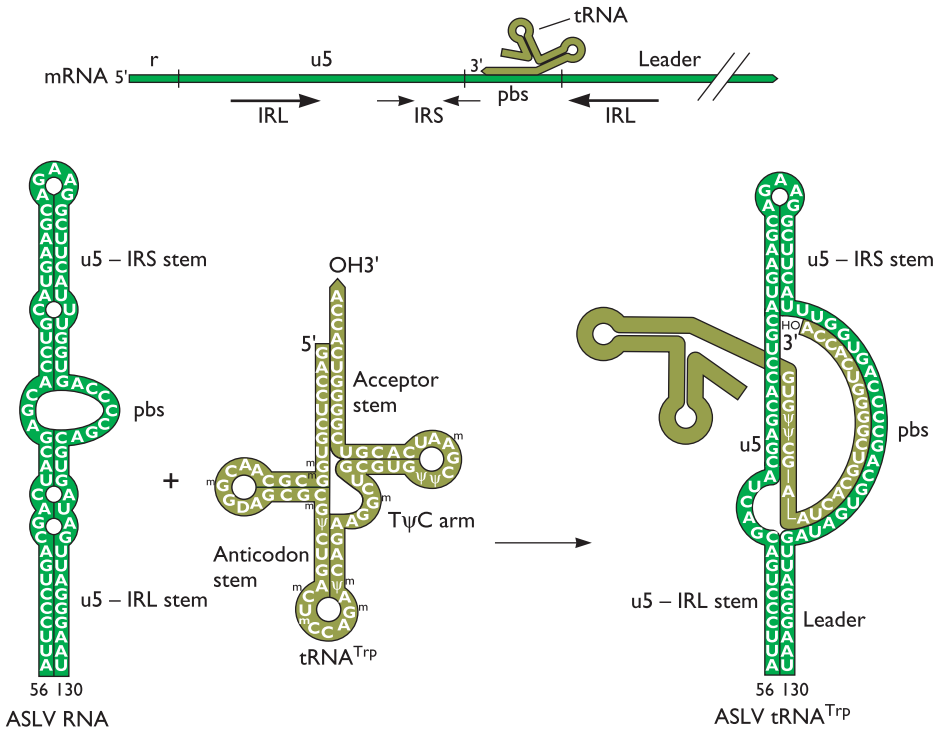


Figure 7.2 Primer tRNA binding to the retroviral RNA genome. (Top) Linear representation of the 5′ terminus of retroviral RNA, indicating locations of the r, u5, and leader regions. A tRNA primer is shown schematically annealed to the pbs. Two inverted-repeat (IR) sequences that flank the pbs are represented by arrows. **(Bottom)** Avian sarcoma/leukosis virus RNA can form an extended hairpin structure around the pbs in the absence of primer tRNA (left). Primer $tRNA^{Trp}$ is shown in the cloverleaf structure (middle). Modified bases are indicated. Viral RNA annealed to $tRNA^{Trp}$, with flanking u5-leader and u5-IR stem structures (right). The TψC arm of the primer and u5 RNA also form hydrogen bonds. Bottom diagram is from J. Leis et al., p. 33–47, *in* A. M. Skalka and S. P. Goff (ed.), *Reverse Transcriptase* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993), with permission.

of RT degrade the genomic RNA after it has been copied into cDNA, form the primer for (+) strand DNA synthesis from the genomic RNA, and, finally, remove this primer and the tRNA primer from the 5′ ends of the nascent viral DNA strands.

Critical Reactions in Reverse Transcription

Initiation of (–) strand DNA synthesis. Based on our understanding of DNA synthesis, the simplest way of copying an RNA template to produce DNA would be to start at its 3′ end and finish at its 5′ end. It was therefore somewhat of a shock for early researchers to discover that retroviral reverse transcription in fact starts near the 5′ end of the viral genome—only to run out of template after little more than about 100 nucleotides (Fig. 7.3). As we will see later, this counterintuitive strategy for initiation of DNA synthesis allows the duplication and translocation of critical transcription and integration signals encoded in both the 5′ and 3′ ends of the genomic RNA, called u5 and u3, respectively.

The 5′ end of the genome RNA is digested by the RNase H domain of RT, after (or as) it is copied to form (–) strand DNA. The short (ca. 100-nucleotide) DNA product of this first reaction, which includes the tRNA primer, accumulates in large quantities in the endogenous and reconstituted systems and is called **(–) strong-stop DNA.** For simplicity, and because genetic analyses indicate that this can occur, the reactions illustrated in Fig. 7.3 to 7.6 are shown as taking place on a single RNA genome. Although a template exchange involving the 5′ end of one RNA genome and 3′ end of the second RNA genome in the virion might also occur, the principles illustrated and the final end products would be the same.

The first template exchange. In the next step (Fig. 7.4), the 3′ end of the RNA genome is engaged as a template via hydrogen bonding between the R sequence in the (–) strong-stop DNA and the complementary r sequence upstream of the poly(A) tail. This reaction, which we call

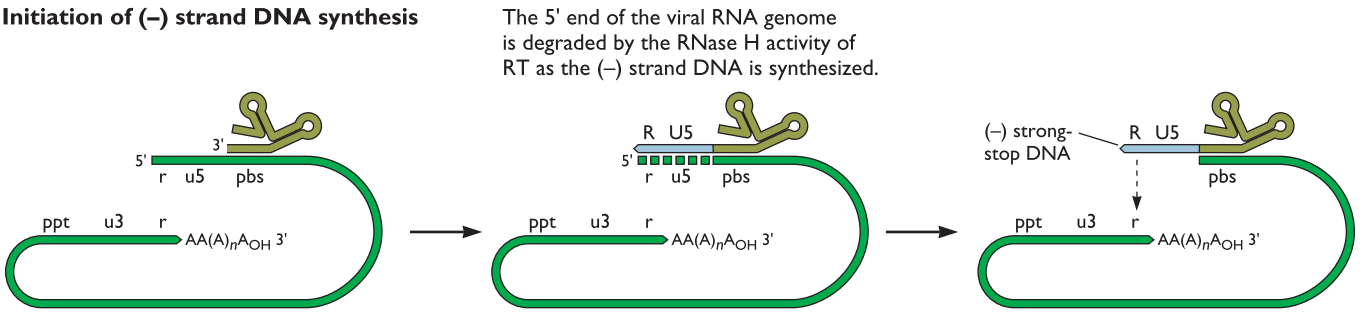


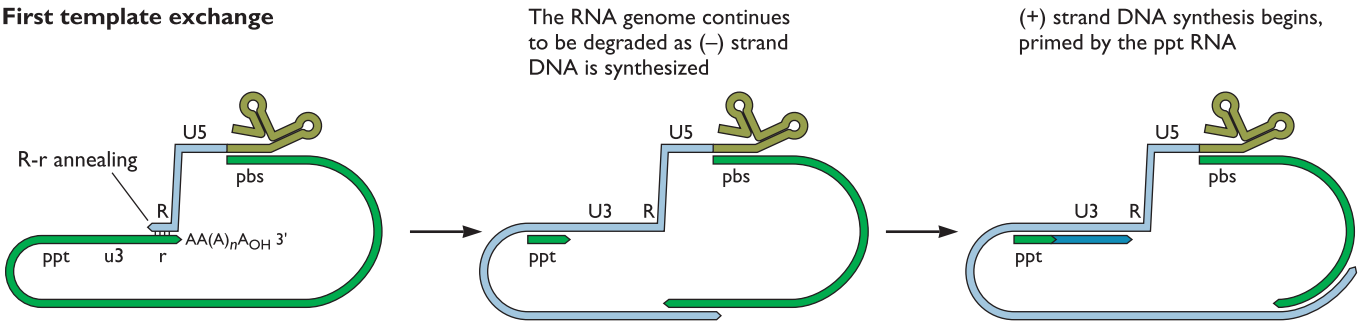
Figure 7.3 Retroviral reverse transcription: initiation of (−) strand DNA synthesis from the tRNA primer.

the **first template exchange,** corresponds to the substitution of one end of the RNA for another to be copied by the RT "machine" (Box 7.3). It has been suggested that base pairing internal to the R and r sequences, followed by branch migration, may allow this exchange to take place without complete digestion of the copied 5′ end of the viral RNA. As (–) strong-stop DNA is barely detectable in infected cells, this first template exchange must be efficient *in vivo*. Once the 3′ end of the genome RNA is engaged, the RNA-dependent DNA polymerase activity of RT can continue copying all the way to the 5′ end of the template, with the RNase H activity digesting the RNA template in its wake.

Initiation of (+) strand DNA synthesis. Among the early products of digestion of genomic RNA by RNase H is a fragment comprising a **polypurine tract (ppt)** of approximately 13 to 15 nucleotides. This RNA fragment is especially important as it serves as the primer for (+) strand DNA synthesis, which begins even before (–) strand DNA synthesis is completed (Fig. 7.4). As illustrated in Fig. 7.5, synthesis of (+) strand DNA proceeds to the nearby end of the (–) strand DNA template and terminates after copying the first 18 nucleotides of the primer tRNA, when it encounters a modified base that cannot be copied by RT. This product is called **(+) strong-stop DNA**. The (–) strand DNA synthesis continues to the end of the viral DNA template, which includes the pbs sequence that had been annealed to the tRNA primer. The production of (+) strong-stop DNA and the converging (–) strand DNA synthesis disengage the template ends. The product is a (–) strand of viral DNA comprising the equivalent of an entire genome (but in permuted order) annealed to the (+) strong stop DNA (Fig 7.5). After removal of the tRNA primer by the RNase H, the single-stranded 3′ end of the (+) strong stop DNA becomes available for annealing to complementary sequences in the single-stranded pbs at the 3′ end of the (–) strand DNA.

The second template exchange. The next steps in the pathway of reverse transcription are facilitated by a **second template exchange** in which annealing of the complementary pbs sequences provides a circular DNA template for continued polymerization by RT (Fig. 7.6, top). Synthesis of the (+) strand DNA can now continue, using the (–) strand DNA as a template. The (–) strand DNA synthesis also continues using the strand-displacement activity of RT, a reaction that opens the DNA circle. Synthesis stops when RT reaches the terminus of each template strand (Fig. 7.6,

Figure 7.4 Retroviral reverse transcription: first template exchange, mediated by annealing of short terminal repeat sequences.



BOX 7.3 **TERMINOLOGY**

Enzymes can't jump!

The exchange of one template for another to be copied by either DNA or RNA polymerases is sometimes referred to as enzyme "jumping." This inappropriate term comes from a too literal reading of cartoon illustrations of the process, in which the templates to be exchanged may be opposite ends of the nucleic acid or different nucleic acid molecules. In actuality, such enzyme movement is quite improbable, and use of this terminology can cloud thinking about these processes. In almost all cases, these polymerases are components of large complexes with architecture designed to bring different parts of the template, or different templates, close to each other. Although some dynamic changes must occur for the proteins to accommodate template exchanges, it seems likely that most of the "movement" is done by the flexible nucleic acid molecules (see, e.g., Fig. 7.3).

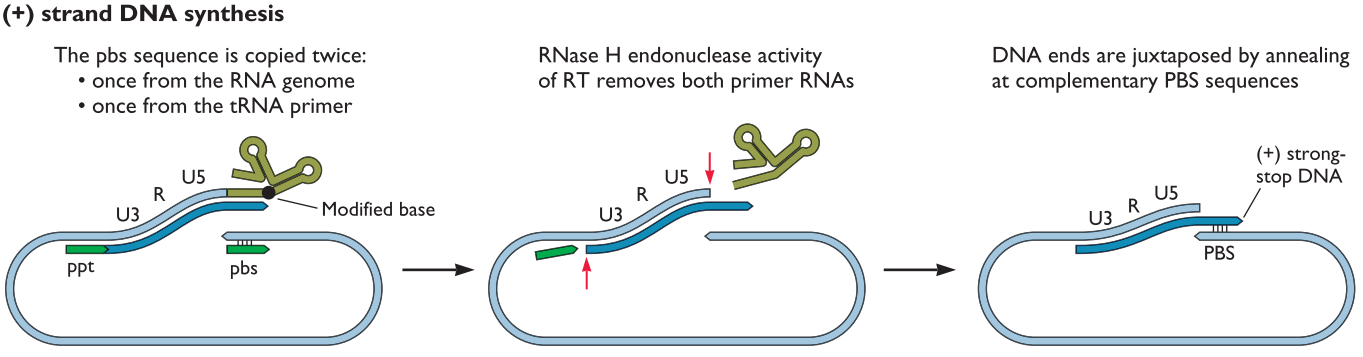
left). The final product is a linear, DNA duplex copy of the viral genome with a **long terminal repeat (LTR)**, of critical *cis*-acting signals at either end. This linear form of viral DNA is the major product of reverse transcription found in the nucleus of infected cells.

Small quantities of two circular DNA products are also invariably present in the nucleus. These are nonfunctional, dead-end products; their presumed origin is illustrated in Fig. 7.6 (right). The smaller of the two, a circle with only one LTR, can arise either from a failure of strand displacement synthesis of RT, or by recombination between the terminal LTR sequences in the linear molecule. The circle with two LTRs is presumed to arise by ligation of the ends of the linear viral DNA. Because formation of this product requires a nuclear enzyme, DNA ligase, and it is easy to detect by polymerase chain reaction (PCR) techniques, the two-LTR circles have been used as convenient markers for the transport of viral DNA into the nucleus.

Retroviral reverse transcription has been called "destructive replication," as there is no net gain of genomes, but rather a substitution of one double-stranded DNA for two molecules of single, (+) strand RNA. However, by this rather complex but elegant pathway, RT not only makes a linear DNA copy of the retroviral genome to be integrated, but also produces the LTRs that contain signals necessary for transcription of the integrated DNA, which is called the **provirus**. The promoter in the upstream LTR is now in the appropriate location for synthesis of progeny RNA genomes and viral mRNAs by host cell RNA polymerase II, and the downstream LTR contains signals for polyadenylation of the mRNA. Integration also ensures subsequent replication of the provirus via the host's DNA synthesis machinery as the cell divides.

Reverse transcription promotes recombination. The above description of reverse transcription has been idealized, for clarity. Analyses of reaction intermediates show that RT pauses periodically during synthesis, presumably at some sequences or breaks that impede copying. If a break is encountered in one RNA template, synthesis can be completed by utilization of the second RNA genome. Such internal template exchanges, known to occur even in the absence of breaks, probably proceed via the same steps outlined for the first template exchange. Indeed, internal exchanges that take place during RNA-directed DNA synthesis are estimated to be the source of at least half of the genetic recombination that occurs in retroviruses, a mechanism known as **copy choice**.

Figure 7.5 Retroviral reverse transcription: (+) strand DNA synthesis primed from ppt RNA.



Second template exchange is facilitated by annealing of PBS sequences

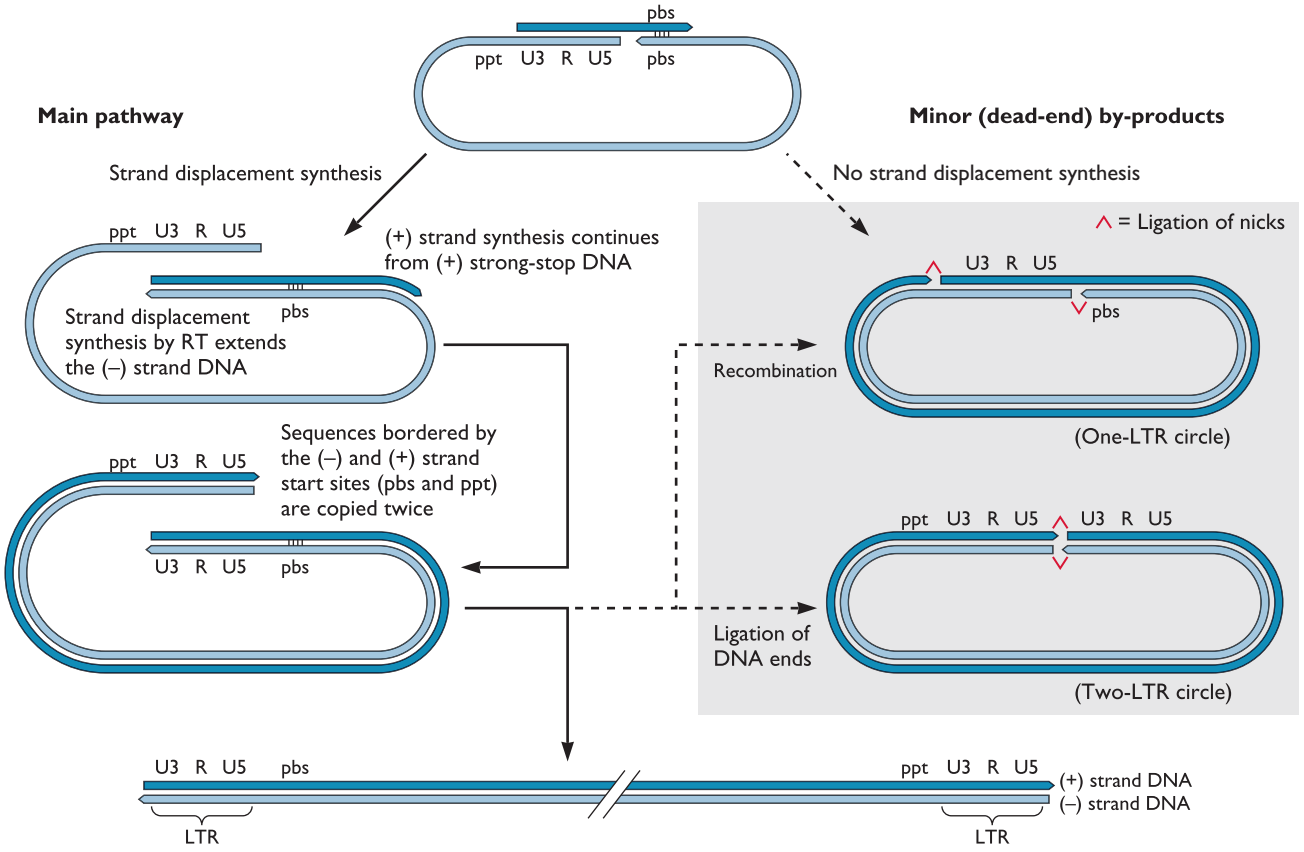


Figure 7.6 Retroviral reverse transcription: the second template exchange and formation of the final linear DNA product. The second exchange is facilitated by annealing of pbs sequences in (+) and (–) strands of retroviral DNA.

In addition to the ppt, other oligoribonucleotides products of RNase H digestion of the RNA template can also serve as primers for DNA-directed DNA polymerization by RT. Human immunodeficiency virus contains a second, specific internal polypurine tract for priming (+) strand synthesis, which is located near the center of the genome. With both the human virus and avian sarcoma/leukosis viruses, (+) strand DNA synthesis can also be initiated at several additional locations. On the other hand, there is little or no evidence of (+) strand initiation at sites other than the 3′ ppt during murine leukemia virus reverse transcription. As illustrated in Fig. 7.7, RT intermediates of murine retroviruses (mouse mammary tumor virus and murine leukemia virus) contain large gaps in the DNA (+) strand. In contrast, avian sarcoma/leukosis virus (+) strands contain single-stranded tails formed by strand displacement synthesis through internal sites of initiation on the DNA template. Human immunodeficiency virus DNA also has a uniquely positioned tail generated by strand displacement synthesis through the internal polypurine tract. This apparent abundance of single-stranded DNA, opposite gaps and in tails, could provide additional opportunities for recombination. It is estimated that approximately half of the genetic recombination that occurs during retroviral reverse transcription takes place during (+) strand synthesis. One proposed mechanism, the **strand displacement-assimilation model**, is illustrated in Fig. 7.8, together with the (–) strand synthesis-dependent mechanism of copy choice.

It is not known how or exactly when linear DNA products with (+) strand discontinuities or branches are processed. From *in vitro* studies it appears unlikely that strand displacement synthesis by RT can displace all downstream (+) strand segments completely. Indeed, a role in transport into the nucleus has been proposed for the displaced (+) strand segment ("flap") that includes the central ppt sequence in human immunodeficiency type 1 DNA (Fig. 7.7). It may

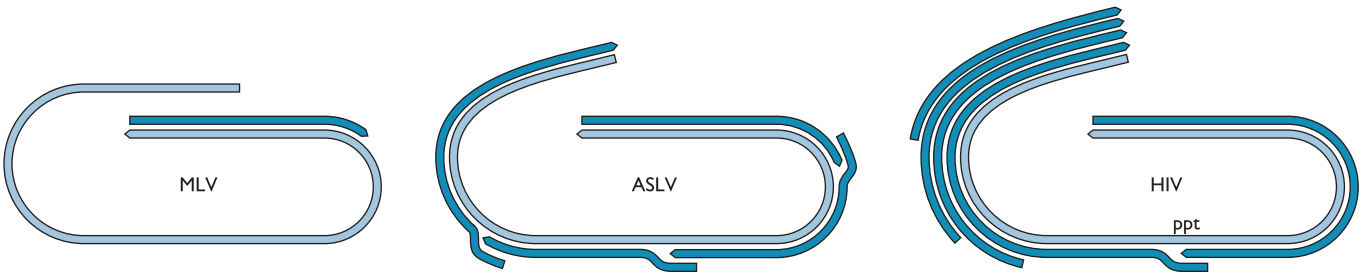


Figure 7.7 Schematic representation of the differences in the structure of retroviral DNAs extracted from infected cells. Most unintegrated murine leukemia virus (MLV) DNA molecules extracted from infected cells have a full-length, continuous DNA (−) strand but only short fragments of the (+) strand. The (+) strands of avian sarcoma/leukosis virus (ASLV) DNA can be initiated at several (apparently random) locations. These are elongated past adjacent initiation sites to generate long, single-stranded tails. Human immunodeficiency virus type 1 (HIV) DNA has two classes of (+) strands. The (+) strands are continuous to a point just beyond the internal polypurine tract (ppt), where they appear to encounter a block to further elongation. Downstream of the internal ppt, there appear to be many possible initiation sites that give rise to a collection of (+) strands of different lengths.

be that cellular repair enzymes in the nucleus participate in the final process. Theoretically, such repair could occur even after the viral DNA is joined to the host DNA, as such joining can proceed as soon as the duplex LTR ends are formed. Indeed, studies with murine leukemia virus show that the first step in the integration process, processing of the blunt ends of the LTRs by integrase (IN), takes place in the cytoplasm, before the viral DNA enters the nucleus. Furthermore, nucleoprotein complexes isolated from the cytoplasmic fraction of human immunodeficiency virus-infected cells are competent to join viral DNA to exogenously supplied target DNA, despite the fact that their (+) DNA strands are not yet continuous.

General Properties and Structure of Retroviral Reverse Transcriptases

Domain Structure and Variable Subunit Organization

The RTs of retroviruses are encoded in the *pol* genes. Despite the sequence homologies and similar organization of coding sequences in their *pol* regions, each of the three Pol polyproteins studied most extensively, those of avian sarcoma/leukosis, murine leukemia, and human immunodeficiency viruses, is processed differently (Fig. 7.9). The mature, functional RT of avian sarcoma virus is a heterodimer, the larger subunit of which includes IN. Murine leukemia virus RT functions as a monomer or homodimer from which IN is absent. As with the avian sarcoma/leukosis virus, the human immunodeficiency virus type 1 RT is a heterodimer. However, in this case only one subunit includes the RNase H domain, and neither includes IN sequences. Little is known about the subunit compositions of RTs from most other retroviruses. In the absence of more structural data, it is difficult to gauge the significance of this apparent structural diversity. However, it seems likely that in the confines of the subviral nucleoprotein complex in which reverse transcription takes place in vivo, the Pol-derived proteins of all retroviruses work together and in similar ways.

Distinctive Catalytic Properties

DNA polymerization is slow. The biochemical properties of RT have been studied with enzymes purified from virions or synthesized in bacteria, and using model templates and primers. Kinetic analyses have revealed an ordered reaction pathway for DNA polymerization similar to that of other polymerases. Like cellular polymerases and nucleases, RTs require divalent cations as cofactors (physiologically, most likely Mg^{2+}). The rate of elongation by RT on natural RNA templates in vitro is 1 to 1.5 nucleotides per second, approximately 1/10 the rate of other eukaryotic DNA polymerases. Assuming that RT begins to act immediately upon viral entry, the long time required to produce a genome's equivalent of retroviral DNA after infection, approximately 4 h for ca. 9,000 nucleotides, supports the view that reverse transcription is a relatively slow process *in vivo*.

In reactions *in vitro*, the rate of dissociation of the enzyme from the template-primer decreases considerably after addition of the first nucleotide, suggesting that initiation and elongation are distinct steps in reverse transcription, as is the case during DNA synthesis by DNA-dependent DNA polymerases. Contrary to most other DNA polymerases, retroviral RTs do not remain attached to their template-primers during a large number of successive deoxyribonucleotide additions; this property is described as "poor processivity."

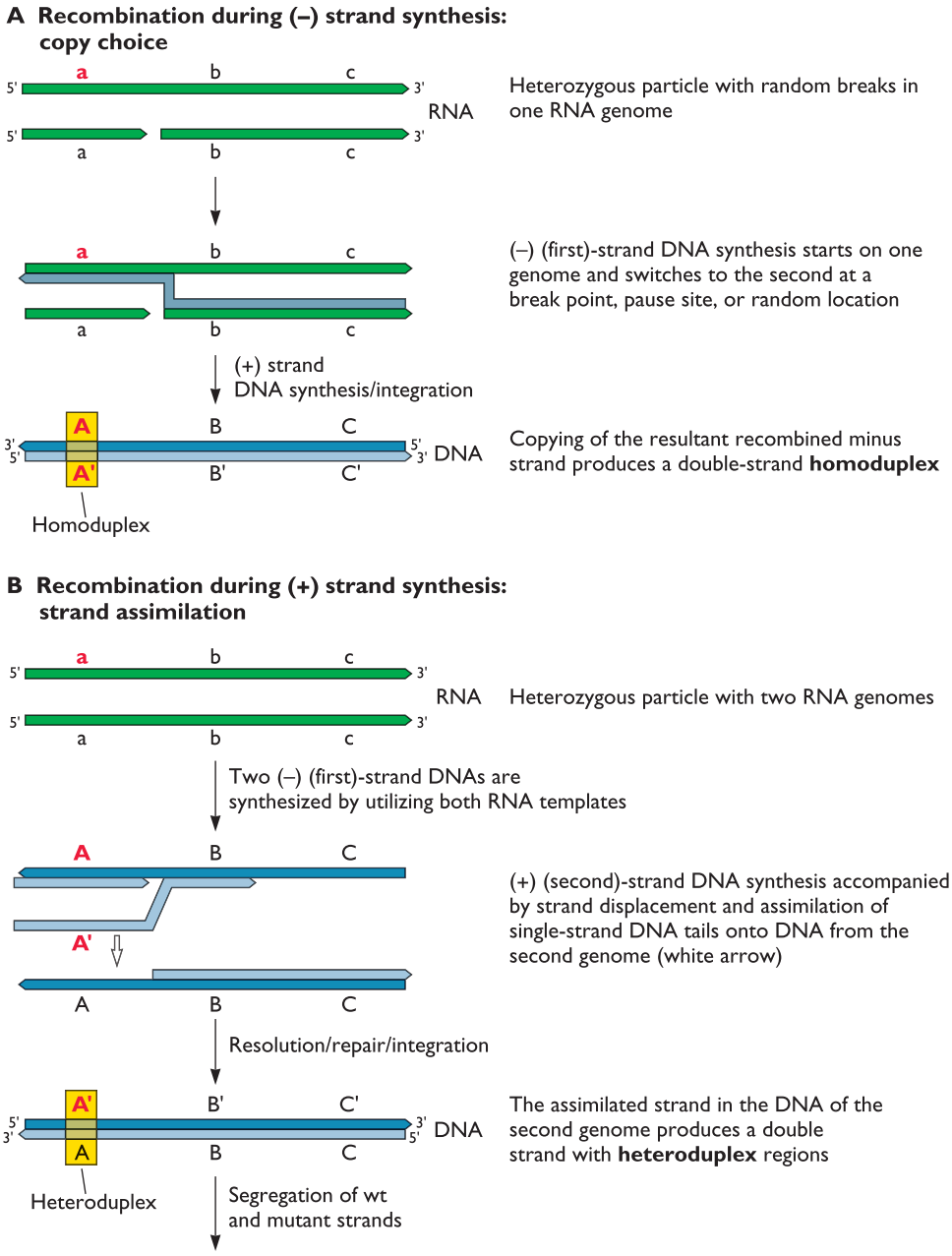


Figure 7.8 Two models for recombination during reverse transcription. Virtually all retroviral recombination occurs between coencapsidated genomes at the time of reverse transcription. The copy choice model **(A)** postulates a mechanism for genetic recombination during (–) strand, DNA synthesis; the strand displacement-assimilation model **(B)** proposes a mechanism for recombination during (+) strand DNA synthesis. These two models are not mutually exclusive, and there is experimental support for both. Viral genetic markers, arbitrarily labeled a, b, and c, are indicated to illustrate recombination. Single recombinations are shown for simplicity, focusing on the a allele, with the mutant form in red. However, multiple crossover events are frequently observed. The boxes highlight the region of recombination. **(A)** Recombination during (–) strand synthesis: copy choice. A break in the RNA is represented as a gap. Exchanges between RNA templates may occur at RNA breaks, as shown, but such breaks may not be required. Introducing intentional breaks, e.g., by gamma irradiation, does not seem to increase the frequency of such recombination. The copy choice mechanism predicts only one DNA homoduplex product from two RNA molecules. **(B)** Recombination during (+) strand synthesis: strand assimilation. (+) strand DNA synthesis initiates at internal sites with RNA primers produced by partial RNase H digestion. The strand assimilation model requires that (+) strand DNA synthesis take place on two templates and predicts formation of a heteroduplex as a consequence of recombination of (+) strands. One DNA molecule is shown for simplicity. Adapted from R. Katz and A. M. Skalka, *Annu. Rev. Genet.* **24:**409–445, 1990, with permission.

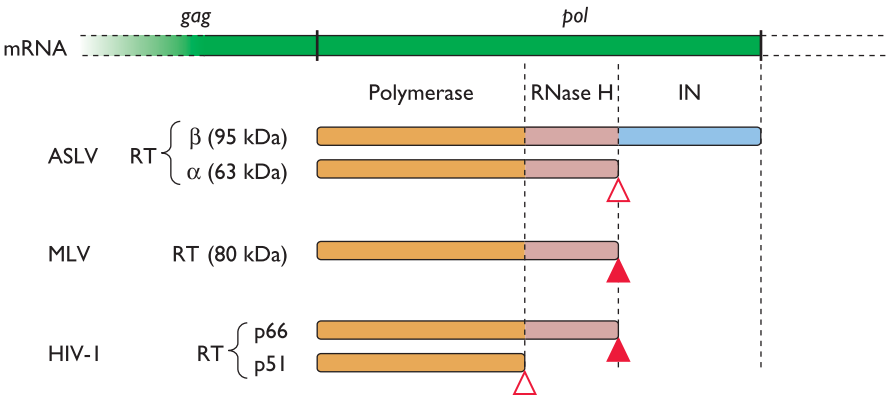
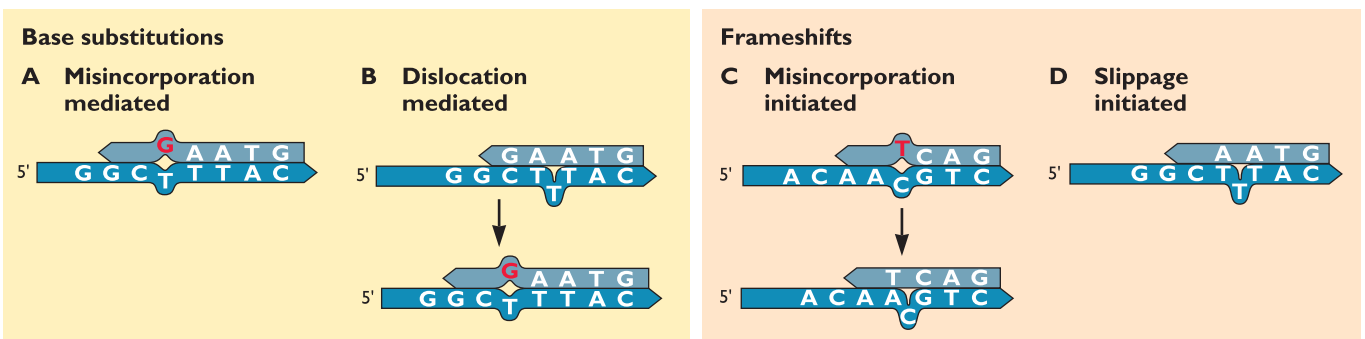


Figure 7.9 Domain and subunit relationships of RTs of different retroviruses. The organization of *pol* domains in retroviral mRNA is shown at the top. The protein products of the three indicated retroviruses are shown below, with arrows pointing to the sites of proteolytic processing that produce the observed diversity of RT subunit composition. Open red arrows indicate partial (asymmetric) processing, and solid red arrows indicate complete processing. ASLV, avian sarcoma/leukosis virus; MLV, murine leukemia virus; HIV-1, human immunodeficiency virus type 1.

Fidelity is low. Retroviral genomes, like those of other RNA viruses, accumulate mutations at much higher rates than do cellular genes. RTs lack an editing activity (resembling the 3′ → 5′ exonuclease of *Escherichia coli* DNA polymerase I, which is capable of excising mispaired nucleotides), and have been shown to be error prone *in vitro*. RTs are presumed therefore to contribute to the high *in vivo* mutation rate of retroviruses.

In vitro, RT errors include not only misincorporations but also rearrangements such as deletions and additions (Fig. 7.10). Misincorporations by human immunodeficiency virus type 1 RT can occur as frequently as 1 per 70 copies at some positions, and as infrequently as 1 per 10^6 copies at others. Many types of genetic experiments have been conducted in attempts to determine the error rates of RTs in a single replication cycle within a cell. The general conclusion is that such rates are also quite high, with reported misincorporations in the range of 1 per 10^4 to 1 per 10^6 nucleotides polymerized, in contrast to 1 per 10^7 to 1 per 10^{11} for cellular DNA replication. As retroviral genomes are approximately 10^4 nucleotides in length, this rate can translate to approximately one lesion per retroviral genome per replication cycle. This high mutation rate explains, in part, the difficulties inherent in treating AIDS patients with inhibitors of RT or other viral proteins; a large population of mutant viruses preexist in every chronically infected individual, some encoding drug-resistant proteins. These mutants can replicate in the presence of a drug and

Figure 7.10 Mutational intermediates for base substitution and frameshift errors. Misincorporated nucleotides are indicated in red. Slippage and dislocations are presumed to be mediated by looping out of nucleotides in the template. Only single-nucleotide dislocations are shown here, but large dislocations leading to deletions are also possible. From K. Bebenek and T. A. Kunkel, p. 85–102, *in* A. M. Skalka and S. P. Goff (ed.), *Reverse Transcriptase* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993), with permission.



quickly comprise the bulk of the population (see Volume II, Chapters 6 and 9).

Several unique activities of RTs are also likely to contribute to their high error rates. The avian sarcoma/leukosis and human immunodeficiency virus enzymes are both proficient at extending mismatched terminal base pairs, such as those that result from nontemplated addition (Fig. 7.10A). This process facilitates incorporation of the mismatched nucleotide into the RT product. RTs also seem to allow a certain type of slippage within homopolymeric runs, in which one or more bases are extruded on the template strand (Fig. 7.10B and C). Mispairing occurs after the next deoxyribonucleotide is added and the product strands attempt to realign with the template. Deletions can also be produced by this mechanism (Fig. 7.10D). Both deletions and insertions are also known to occur during reverse transcription within an infected cell, apparently because template exchanges can take place within short sequence repeats (e.g., 4 or 5 nucleotides) that are not in homologous locations on the two RNA templates. This mechanism is of major importance in the capture of oncogenes (see Volume II, Chapter 7).

RNase H. The RNase H of RT also requires a divalent cation, (most likely Mg^{2+}, which is abundant in cells). Like other RNase H enzymes, present in all prokaryotic and eukaryotic cells, the RNase H of RT digests only RNA that is annealed to cDNA. RNase H cleaves phosphodiester bonds to produce 5′-PO_4 and 3′-OH ends; the latter can be extended directly by the RT. Biochemical studies have uncovered three distinguishable activities for RNase H of human immunodeficiency virus type 1 and murine leukemia virus; recessed 3′ DNA end-directed and recessed 5′ RNA end-directed endonucleases, both of which cleave the RNA strand 12 to 20 nucleotides from the recessed end, and an internal endonuclease that is not end directed. A loose consensus site for all three activities has also been observed.

Structure of RT

Although RTs from avian and murine retroviruses have been studied extensively *in vitro*, the importance of human immunodeficiency virus type 1 RT as a target for drugs to treat AIDS has focused intense interest and resources on this enzyme. As a consequence, we know more about the human viral protein than about any other RT. Three aspartic acid residues in the polymerase region are included in conserved motifs in a large number of polymerases (see Fig. 6.4) and are thought to coordinate the required metal ions and contribute to binding deoxyribonucleoside triphosphates and subsequent catalysis. As illustrated in Figure 7.9, the primary sequence of the p51 subunit is the same as that of p66, minus the RNase H domain. However, as discussed below, the analogous portions of these subunits are arranged quite differently in RT.

The first high-resolution structure of human immunodeficiency virus type 1 RT to be solved was that of the RNase H domain. As illustrated by the ribbon models in Fig. 7.11, the general structure of this domain is almost superimposable on that of *E. coli* RNase H. Included in the regions of structural homology are seven residues that are components of conserved motifs present in all retroviral and bacterial RNases H, clustered at what is thought to be the site at which the metal ion cofactors are bound. The results of site-directed mutagenesis have confirmed that many of the conserved residues are important for the activities of both *E. coli* and human retroviral RNase H.

The high-resolution structure of the intact human immunodeficiency virus type 1 RT heterodimer was obtained for complexes with the nonnucleoside inhibitor nevirapine. Subsequently, several additional structures were determined, including RT with no bound inhibitors, and RT complexed with a short RNA-DNA duplex or a DNA duplex and a deoxynucleotide substrate (Fig. 7.12). The most surprising finding to come from the analysis of the first RT crystals was the **structural asymmetry** in the subunits. Results of biochemical studies showed that the two subunits also perform different functions in the heterodimeric enzyme. The catalytic functions are contributed by the larger subunit, p66, whereas the role of p51 appears to be mainly structural. In the crystal structures, the two subunits are nestled on top of each other, with an extensive subunit interface. The p66 polymerase domain is divided into three subdomains denoted "finger," "palm," and "thumb" by analogy to the convention used for describing the topology of the *E. coli* DNA polymerase I Klenow fragment, described in Chapter 6 (Fig. 6.3 and 6.4). A fourth subdomain lies between the remainder of the polymerase and the RNase H domain, and is therefore called the "connection" domain. It contains the major contacts between the two subunits of RT. The extended thumb of p51 contacts the RNase H domain of p66, an interaction that appears to be required for RNase H activity. Not only are human immunodeficiency virus type 1 RT and *E. coli* DNA polymerase similar topologically, but also this retroviral RT can actually substitute for the bacterial enzyme in *E. coli* cells that carry a temperature-sensitive mutation in their DNA polymerase I gene.

Analysis of the various structural models predicts highly dynamic interactions among the human immunodeficiency virus type 1 RT, its template-primer, and dNTP substrates. The substrates are bound in a defined order: the template-primer first, and then the complementary dNTP to be added. Upon binding the template-primer, the thumb

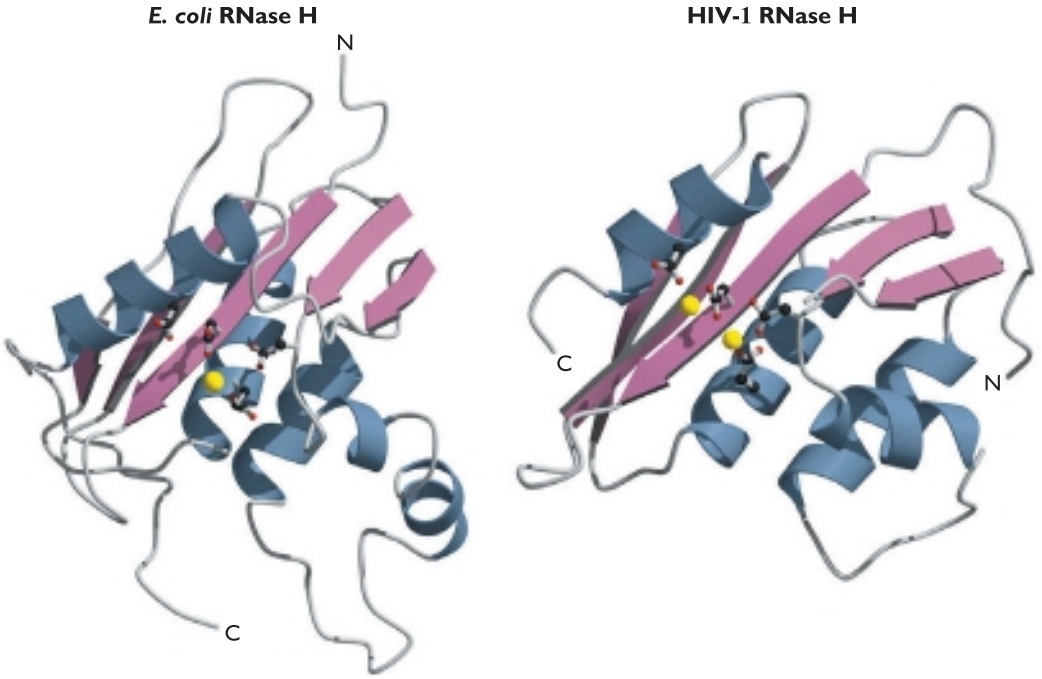


Figure 7.11 Ribbon diagrams based on the crystal structures of *E. coli* and human immunodeficiency virus type 1 (HIV-1) RNase H. The highly conserved acidic residues are shown as ball-and-stick models, with metal ion complexes indicated by the large yellow spheres. Courtesy of Mark Andrake, Fox Chase Cancer Center.

is moved away from the fingers, allowing contacts between the fingers and the 5′ extension of the template. Another conformational change takes place when the dNTP is bound. This substrate interacts directly with two fingertip residues; the interaction may induce closure of the binding pocket. This conformational change facilitates attack of the 3′-OH of the primer on the α-phosphate of the incoming dNTP. After this addition, the fingertips may resume their open position, allowing the diphosphate product to be released and the template-primer to be translocated, so that the next dNTP can be accepted. Such translocation may be driven, in part, by the energy released upon hydrolysis of the previous dNTP substrate.

Figure 7.13 is a schematic drawing of an RNA-DNA heteroduplex bound in the cleft region of the human immunodeficiency virus type 1 RT. It illustrates how the RNA strand is fed into the polymerizing site from the left. As a new DNA chain is synthesized by addition of deoxyribonucleotides to the primer, the template RNA is translocated in stepwise fashion to the RNase H site at the right, where it can be degraded. This schematic is consistent with biochemical evidence for polymerase-coupled RNase H activity that results in cleavage of the RNA template about once for every 50 to 100 dNTPs polymerized. The distance between the polymerizing and RNase H sites can account for the length (ca. 18 nucleotides) of the terminal RNase H oligoribonucleotide product.

Production of the p51 subunit of the human immunodeficiency virus type 1 RT, which possesses identical amino acid sequences to but has both structure and function that are distinct from those of the p66 subunit (Fig. 7.9), appears to be an excellent example of viral genetic economy. Results of cross-linking experiments suggest that the p51 subunit may perform a unique function in the RT heterodimer, that of binding the tRNA primer. The C terminus of the p51 subunit, at the end of the connection domain, is buried within the N-terminal β-sheet of the RNase H domain of p66. This arrangement suggests a model for proteolytic processing in which a p66 homodimer intermediate in the reaction is also arranged asymmetrically and the RNase H domain of the subunit destined to become p51 is unfolded. This mechanism would account for asymmetric cleavage by the viral protease (Fig. 7.9). Without comparable structural data for other retroviral RTs, we can only wonder if an analogous processing strategy applies to these proteins. The asymmetric processing of the avian sarcoma/leukosis virus Pol protein suggests that this may be the case for the alpharetroviruses as well (Fig. 7.9).

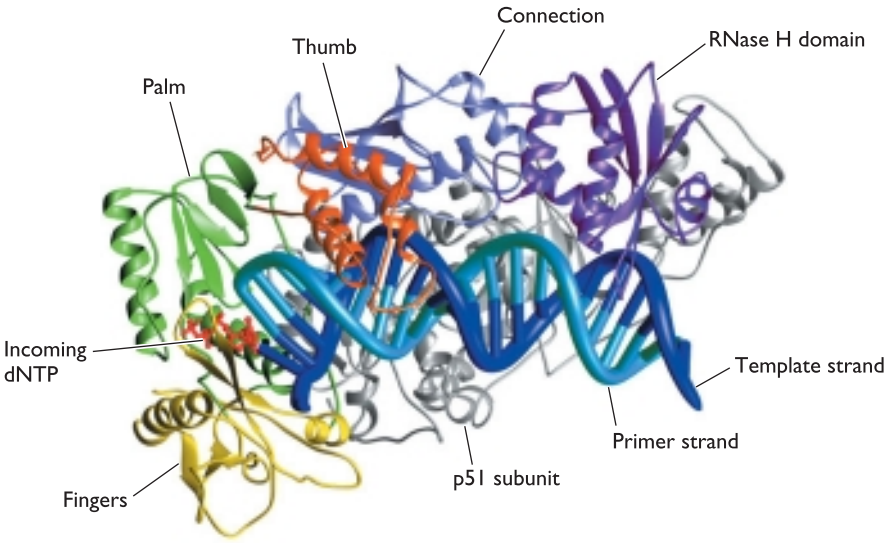
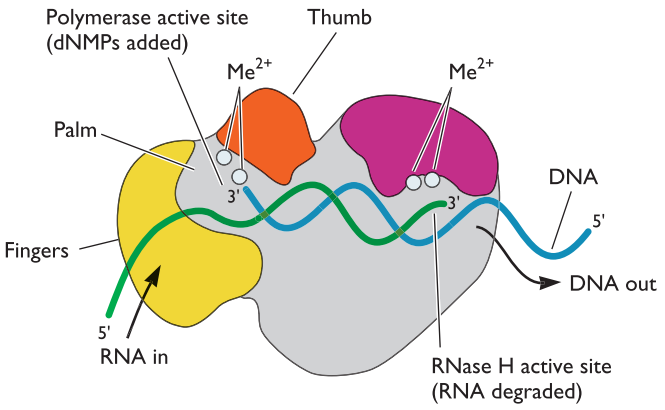


Figure 7.12 A ribbon representation of human immunodeficiency virus type 1 RT in complex with DNA template/primer. dNTP, deoxyribonucleoside triphosphate. Courtesy of Rajiv Chopra and Stephen Harrison, Harvard University.

There Are Many Other Examples of Reverse Transcription

When it was first discovered, RT was thought to be a peculiarity of retroviruses. We now know that other animal viruses, the hepadnaviruses, and some plant viruses, such as the caulimoviruses, also replicate by producing duplex genomic DNA via an mRNA intermediate. All are classified therefore as **retroid viruses**. In fact, the discovery of RT activity in some strains of myxobacteria and *E. coli* places the evolutionary origin of this enzyme before the separation of prokaryotes and eukaryotes. As it is now widely held that the evolving biological world was initially based on RNA molecules, as both catalysts and genomes, the development of the modern (DNA) stage of evolution would have required an RT activity. If so, the retroid viruses may also be viewed as living fossils, shining the first dim light into an ancient evolutionary passageway from the primordial world (Volume II, Chapter 10).

Figure 7.13 Model for a DNA-RNA hybrid bound to human immunodeficiency virus type 1 RT. The RNA template-DNA product duplex is shown lying in a cleft. The polymerase active site and the putative RNase H active site are indicated. Me^{2+} signifies a divalent metal ion. As illustrated, the RNA template enters from the left, and is degraded at the RNase H active site after being copied into DNA at the polymerase active site. The newly synthesized DNA strand exits to the right. Adapted from L. A. Kohlstaedt et al., p. 223–250, *in* A. M. Skalka and S. P. Goff (ed.), *Reverse Transcriptase* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1996), with permission.



Since the discovery of RT in retroviruses, additional RT-related sequences have been found in cellular genomes. Some of these sequences are contained in cellular constituents known generally as **retroelements**, which are derived from retroviruses. During the retroviral life cycle (Appendix, Fig. 20), the double-stranded DNA molecule synthesized by reverse transcription is integrated into the genomes of infected animal cells by a second retroviral enzyme, integrase (discussed in the following section). In some cases, retroviral DNA may be integrated into the DNA of germ line cells in a host organism. These integrated DNAs are then passed on to future generations in Mendelian fashion as **endogenous proviruses**. Such proviruses are often replication defective, a property that may facilitate coexistence with their hosts. Almost 50% of the human genome is now known to comprise mobile genetic elements, including endogenous proviruses and other retroelements such as retrotransposons, retroposons, and processed pseudogenes, all of which have been accumulated during evolution (Box 7.4). Chromosomal

BOX 7.4 DISCUSSION

Retroelements

Retrotransposons are dispersed widely in nature. Their gene content and arrangements are similar to those of retroviruses. Like retroviral proviruses, integrated retrotransposon DNAs have LTRs that include signals for transcription by cellular RNA polymerase II and short direct repeats of host DNA at their borders. RT sequence comparisons (Fig. 7.14) have allowed classification of these elements into two families. Most retrotransposons are distinguished from retroviruses by lack of an extracellular phase; they have no *env* gene, and hence the virus-like particles formed intracellularly are noninfectious. However, in the family *Metaviridae,* members of one genus do include open reading frames corresponding to *env,* and at least one of these elements, the *Drosophila* gypsy, produces infectious extracellular particles. Phylogenetic comparisons of LTR-retrotransposons from invertebrates provide evidence that several of these elements have acquired *env* sequences via genetic recombination with both RNA and DNA viruses. These results support the view that retrotransposons are retroviral progenitors. An alternative possibility, but with less phylogenetic support, is that retrotransposons are degenerate forms of retroviruses.

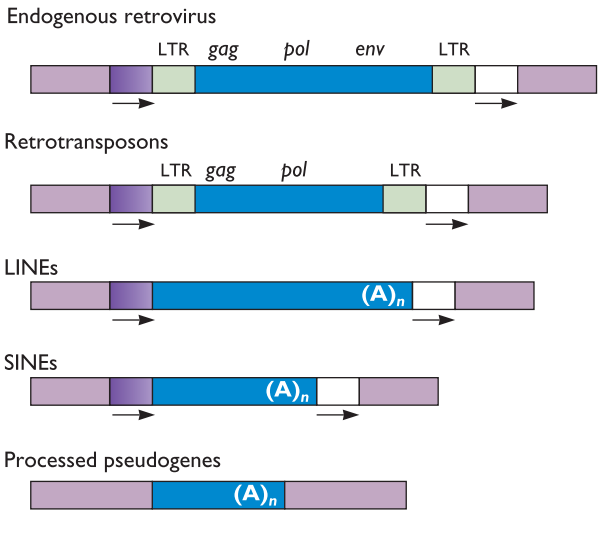
Retroposons, also called non-LTR retrotransposons, are dispersed widely in the DNA of human cells. Like retroviral proviruses and retrotransposons, they are flanked by direct repeats of host DNA at their boundaries. Although they lack LTRs, some contain internal promoters for transcription by cellular RNA polymerase III. All retroposons have A-rich stretches at one terminus, presumed to be derived by reverse transcription of the 3′-poly(A) tails in their RNA intermediates. Retroposons comprise fairly long stretches of related sequences (up to 6 kb). These long interspersed repeat elements are commonly called LINEs. Most LINEs encode RT-related sequences, but they often contain large deletions and translational stop codons and are therefore defective. The sole LINE element of humans, called L-1, comprises approximately 20% of the human genome and, although most L-1 elements are dead, approximately 80 to 100 are transposition competent. Genetic analyses have documented a number of disease-causing genetic lesions resulting from L-1-mediated retrotranspositional events.

Retrosequences, another type of reverse-transcribed sequence, are found as short interspersed repeat elements on the order of 300 bp long. These sequences, called SINEs, comprise approximately 11% of the human genome. SINEs have no known open reading frames and are retrotransposed in *trans* by L-1.

Processed pseudogenes have no introns (hence "processed"), and their sequences are related to exons in functional genes that map elsewhere in the genome. Like retroposons, they include long A-rich stretches at one end and lack LTRs. However, they contain no promoter for transcription, and no RT. Generally, they are thought to arise from rare reverse transcription of cellular mRNAs catalyzed by the RTs of retroviruses or nondefective LINEs. The table summarizes the defining characteristics of the major classes of retroelements.

Malik, H. S., S. Henikoff, and T. H. Eickbush. 2000. Poised for contagions: evolutionary origins of the infectious abilities of invertebrate retroviruses. *Genome Res.* **10:**1037–1318, 2000.

Characteristics of retroelements resident in eukaryotic genomes



Designation	Characteristic	Example	Copy no.
Endogenous retroviruses	RT, LTR (internal Pol II promoter), and *env*	HERVs (human)	$1–10^2$
Retrotransposons	RT, LTR (internal Pol II promoter)	Ty3 (yeast)	$10^2–10^4$
Retroposons (LINEs)	RT, internal Pol III promoter, A-rich sequence at end	LINE 1 (human)	$10^4–10^5$
Retrosequences (SINEs)	A-rich sequence at end, internal Pol III promoter, but no RT	*Alu* (human)	$10^5–10^6$
Processed pseudogenes	A-rich sequence at end, no internal promoter, no RT	β-Tubulin (human)	$1–10^2$

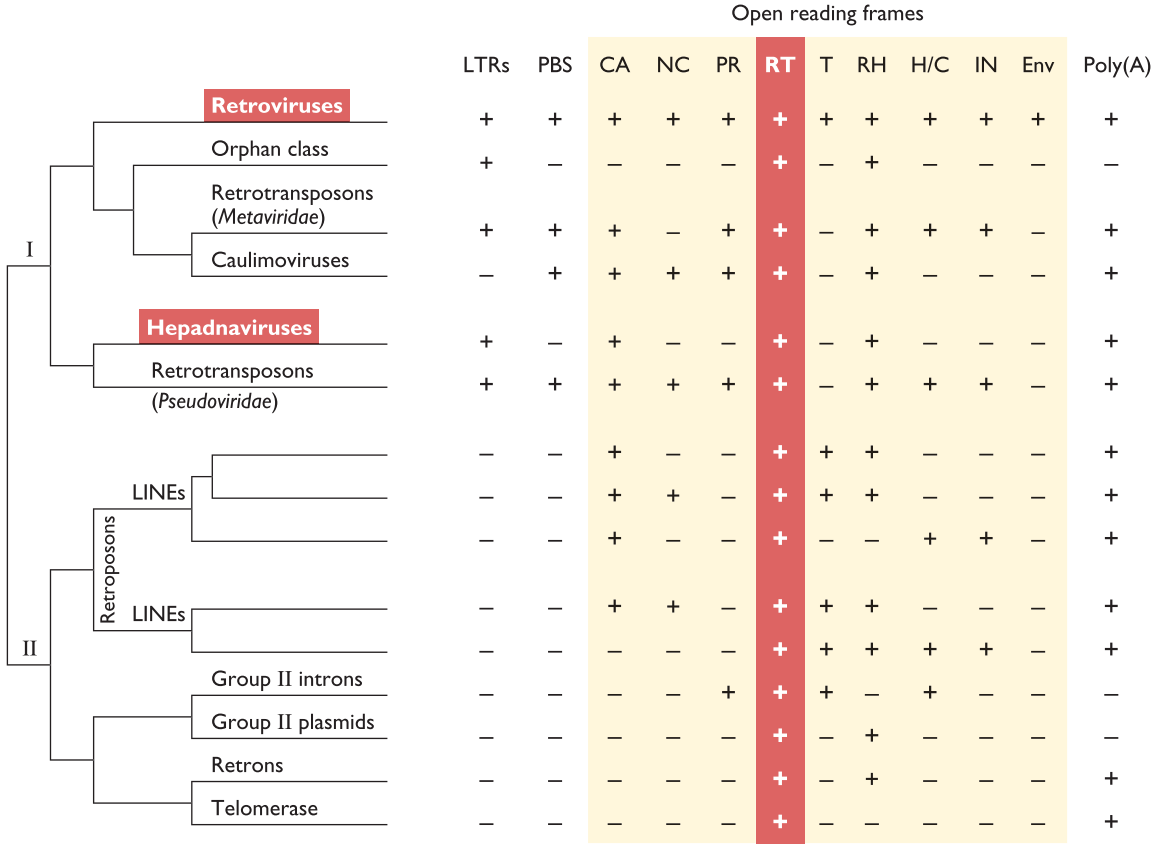
telomeres are also formed via reverse transcription by an enzyme known as telomerase. Comparisons of the predicted RT-related amino acid sequences of representative retroelements provide clues about their relatedness and hints to their possible origin (Fig. 7.14).

Retroviral DNA Integration Is a Unique Process

The **integrase (IN)** of retroviruses and the related retrotransposons catalyzes specific and efficient insertion of the DNA product of RT into the host cell DNA. This activity is unique in the eukaryotic world. Some retroposons also contain IN-related sequences (Fig. 7.14). Establishment of an integrated copy of the genome is a critical step in the life cycle of retroviruses, as this reaction ensures stable association of viral DNA with the host cell's genome. The integrated **proviral DNA** is transcribed by cellular RNA polymerase II to produce the viral RNA genome and the mRNAs required to complete the replication cycle.

IN is encoded in the 3′ region of the retroviral *pol* gene (Fig. 7.9), and the mature protein is produced, in most cases, by viral protease (PR)-mediated processing of the Gag-Pol polyprotein precursor. During progeny virus assembly, all three viral enzymes (PR, RT, and IN) are incorporated into the virion core. Virus particles contain equimolar quantities of RT and IN (ca. 50 to 100 molecules per virion). The viral

Figure 7.14 Schematic representation of the eukaryotic retroid family phylogeny. The unrooted family tree derived from comparison of deduced RT amino acid sequences is displayed at the left. It is separated into two major branches (I and II), based primarily on the presence of long terminal repeats (LTRs) that contain important regulatory sequences in the DNA versions of some of its members. The table compares DNA sequences and deduced open reading frames encoded in all family members with those sequences that are characteristic of retroviruses. The region that defines membership in the retroid family, the RT, is highlighted in red. The *cis*-acting sequences include the following: LTRs, long terminal repeats flanking the retroviral genome; PBS, tRNA primer-binding site; and poly(A), 3′-poly(A) tract in RNA or DNA form. The open reading frames (yellow shaded area) are as follows: CA, capsid protein; NC, nucleocapsid; PR, protease; RT, reverse transcriptase domain; T, "tether" region connecting the RT and RNase H domains; RH, RNase H domain; H/C, histidine and cysteine motif in the N-terminal portion of the integrase; IN, integrase; Env, envelope protein. From M. A. McClure, *in* A. M. Skalka and S. P. Goff (ed.), *Reverse Transcriptase* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1996), with permission.



DNA product of RT is the direct substrate for IN, and genetic and biochemical studies indicate that these enzymes function in concert within infecting particles. As already noted, IN sequences actually are included in one of the subunits of avian sarcoma/leukosis virus RT, and gentle extraction of murine leukemia virus particles yields RT-IN complexes. However, as with RT, virtually nothing is known about the molecular organization of IN within virions.

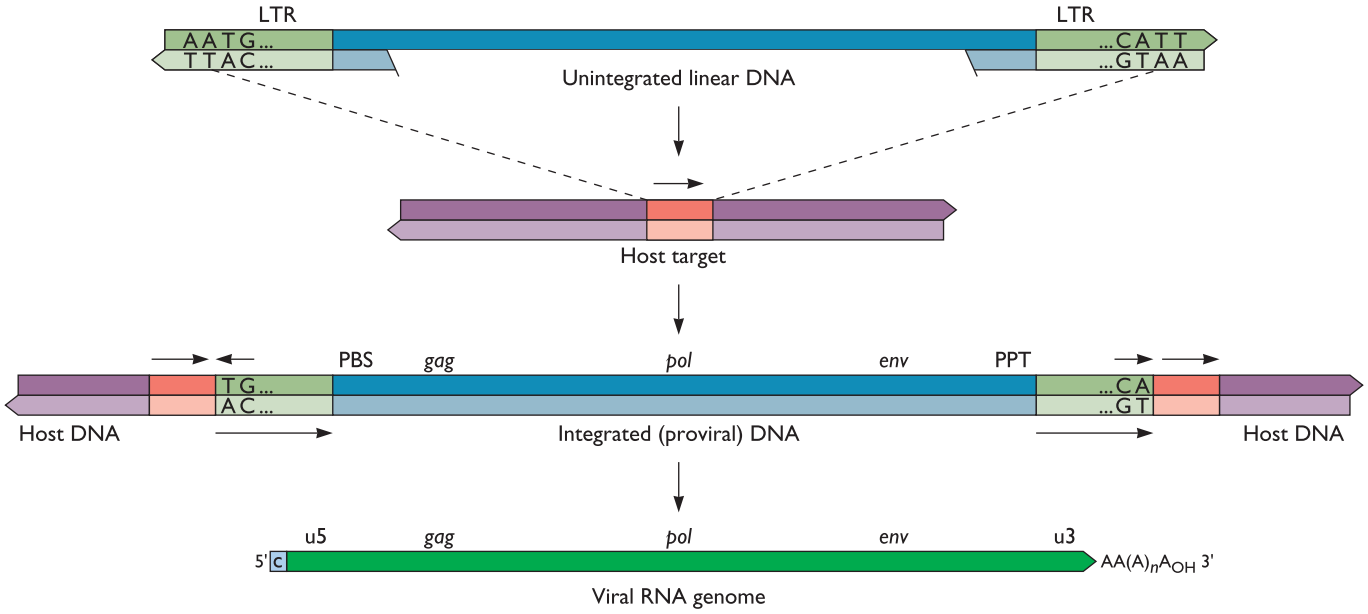
The first insights into the mechanism of the integration process came in the early 1980s, when it was established that proviral DNA is flanked by LTRs and is colinear with the unintegrated viral DNA and the RNA genome (Fig. 7.15). Nucleotide sequencing of cloned retroviral DNAs and host-virus DNA junctions revealed several unique features of the process. Both viral and cellular DNAs undergo characteristic changes; viral DNA is cropped, usually by 2 bp from each end, and a short duplication of host DNA flanks the provirus on either end. Finally, the proviral ends of all retroviruses comprise the same dinucleotide: 5′-TG . . . CA-3′. This dinucleotide is often embedded in an extended, imperfect inverted repeat that can be as long as 20 bp for some viral genomes. The fact that the length of the host cell DNA duplication is characteristic of the virus, provided the first clue that a viral protein must play a critical role in the integration process.

The inverted terminal repeat, conserved terminal dinucleotide sequence, and flanking direct repeats of host DNA were strikingly reminiscent of features observed earlier in a number of bacterial transposons and the *E. coli* bacteriophage Mu (for "mutator"). Homologies to the predicted amino acid sequences in a portion of the retroviral IN were also found in the transposases of certain bacterial insertion sequences and transposons such as Tn*5*. This observation suggested that, like RT, IN probably evolved before the divergence of prokaryotes and eukaryotes. These similarities predicted what is now known to be a common mechanism for retroviral DNA integration and DNA transposition.

Integrase-Catalyzed Steps in the Integration Process

A generally accepted model for the IN-catalyzed reactions has been developed on the basis of results from many different types of experiment, including studies of infected cells and reconstituted systems (Box 7.5). The two ends of viral DNA

Figure 7.15 Characteristic features of retroviral integration. Unintegrated linear DNA of the avian retrovirus avian sarcoma/leukosis virus (top) after reverse transcription has produced blunt-ended LTRs (Fig. 7.3). The break in the bottom (+) strand indicates that this strand may include discontinuities, whereas the top (−) strand must be continuous (Fig. 7.7). Two base pairs (AA·TT) are lost from both termini upon completion of the integration process, and a 6-bp "target site" in host DNA (pink, indicated by an arrow) is duplicated on either side of the proviral DNA. The integrated proviral DNA (middle) includes short, imperfect inverted repeats at its termini, which end with the conserved 5′-TG…CA-3′ sequence; these repeats are embedded in the LTR, which is itself a direct repeat. The gene order is identical in unintegrated and proviral DNA, and is colinear with that in the viral RNA genome (bottom), for which a provirus serves as a template (described more fully in Chapter 8).



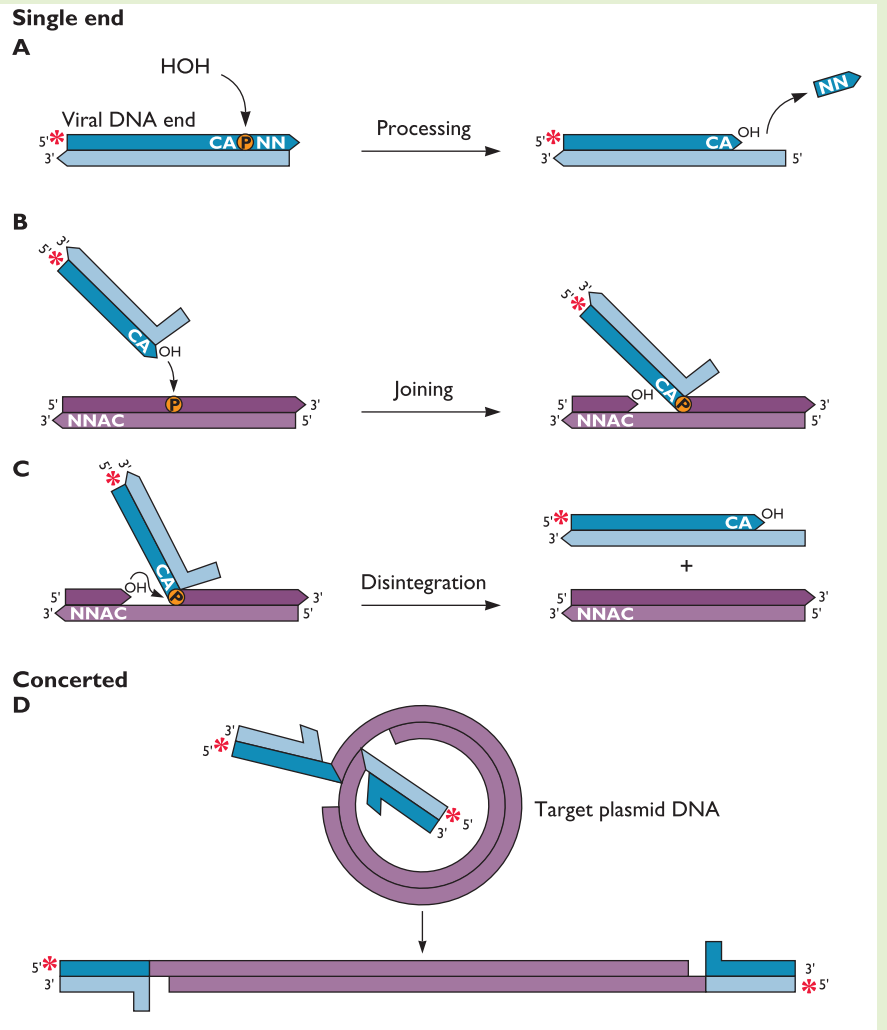
BOX 7.5 BACKGROUND

Model in vitro reactions uncover catalytic mechanisms

The development of simple *in vitro* assays for the processing and joining steps catalyzed by IN marked an important turning point for investigation of the biochemistry of these reactions. With such assays, it was discovered that retroviral IN protein is both necessary and sufficient for catalysis, that no exogenous source of energy (ATP or an ATP-generating system) is needed, and that the only required cofactor is a divalent cation, Mn^{2+} or Mg^{2+}. Use of simple substrates and other biochemical analyses with purified IN protein helped to delineate the sequence and structural requirements for DNA recognition.

The substrates in the simplest assays comprise short duplex DNAs (ca. 25 bp) whose sequences correspond to one retroviral DNA terminus, and are labeled with ^{32}P (red asterisk). The use of such model viral DNA substrates showed that IN can also catalyze an apparent reversal of the joining reaction, which has been called disintegration. Such assays also showed that while the processing, joining, and disintegration reactions produce different products, their underlying chemistry is the same. All comprise a nucleophilic attack on a phosphorus atom by the oxygen in an OH group, and result in cleavage of a phosphodiester bond in the DNA backbone. In **processing (A)**, the OH comes from a water molecule. In **joining (B)**, the OH is derived from the processed 3′ end of the viral DNA, and the result is a direct transesterification. In **disintegration (C)**, also a direct transesterification, a 3′-OH end in the interrupted duplex attacks an adjacent phosphorus atom, forming a new phosphodiester bond and releasing the overlapping DNA. The products of all these reactions can be distinguished by gel electrophoresis.

Although assays with short, single-viral-end model substrates have been invaluable in elucidation of the catalytic mechanisms of IN, they are limited in that the major products represent "half-reactions" in which only one viral end is processed and joined to a target. More recently, conditions for efficient, **concerted processing and joining (D)** of two viral DNA ends to a target DNA have been described, with a variety of specially designed "miniviral" model DNA substrates. The concerted reaction has been shown to be more efficient with longer (150- to 900-bp) DNA "donor" fragments that include viral DNA sequences at a terminus. After preincubation, excess plasmid DNA is added as target, and the concerted joining of two donor fragments produces a linear DNA product that can be detected after electrophoresis in an agarose gel.



are recognized specifically and nicked, and the new 3′ ends are then joined covalently to the host DNA in a relatively sequence-independent manner, at staggered nicks also introduced by IN. The enzyme accomplishes these multiple activities as a multimer in a large nucleoprotein complex, probably assisted by other viral components as well as cellular proteins. As illustrated in Fig. 7.16, IN catalysis occurs in two biochemically and temporally distinct steps.

The first step catalyzed by IN is a processing reaction, which requires duplex ends and therefore can take place

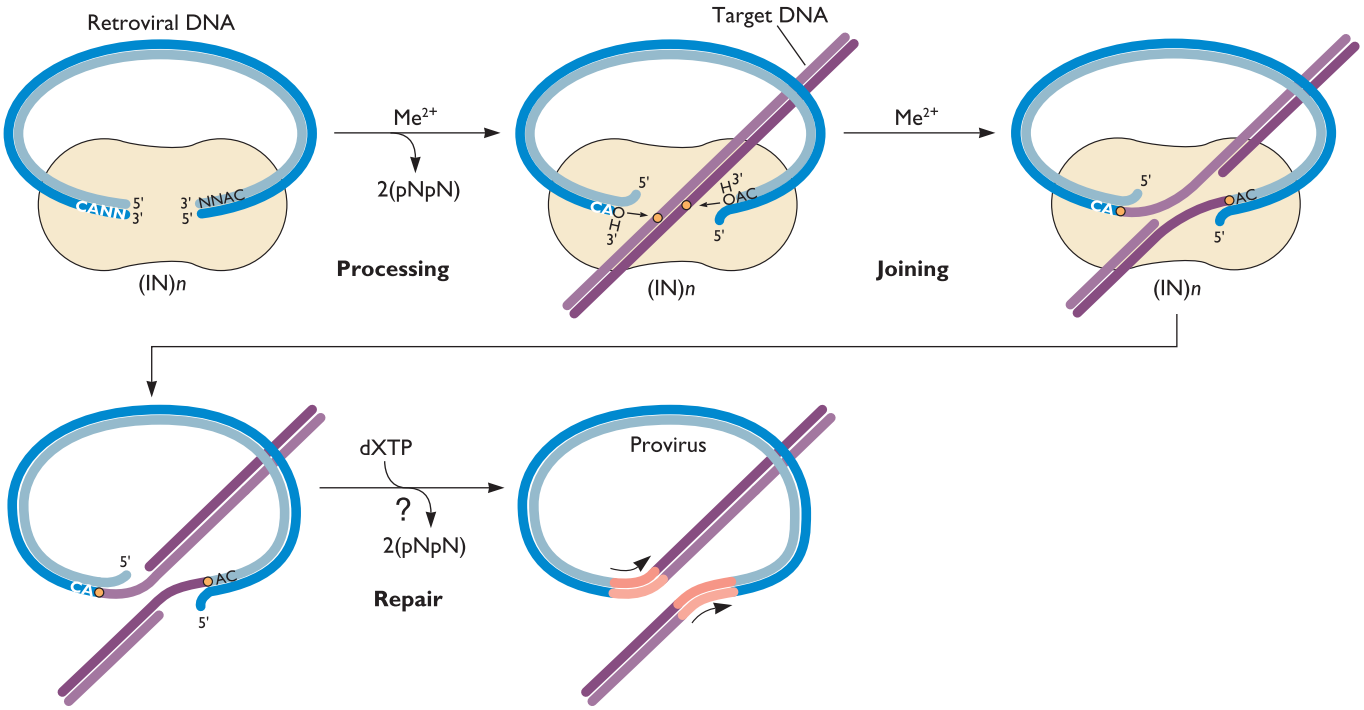


Figure 7.16 Three steps in the retroviral DNA integration process. Endonucleolytic nicking adjacent to the conserved dinucleotide near each DNA end results in the removal of a terminal dinucleotide, with formation of a new, recessed 5′…CA_{OH}-3′ end that will be joined to target DNA in the second step of the IN-catalyzed reaction. Both processing and joining reactions require a divalent metal, Mg^{2+} or Mn^{2+}. The region shaded in beige represents an IN multimer [(IN)*n*]. Results of site-directed mutagenesis of viral DNA ends have shown that the conserved 5′…CA_{OH}-3′ dinucleotide is essential for correct and efficient integration. The small gold circles represent the phosphodiester bonds cleaved and reformed in the joining reaction. The final step in the integration process is a repair reaction that utilizes host enzymes. Adapted from N. D. Grindley and A. E. Leschziner, *Cell* **83:**1063–1066, 1995, with permission.

only when synthesis of the ends of the unintegrated linear viral DNA is complete. This requirement prevents the integration of defective molecules, with imperfect ends. A specific sequence in the duplex viral DNA and close proximity of this sequence to a terminus are critical, but sequences upstream also affect the efficiency of the reaction (Fig. 7.17). It has been shown that the processing reaction can take place in the cytoplasm of an infected cell before viral DNA enters the nucleus, within a subviral structure commonly referred to as the **preintegration complex** (described below). Although there is strong evidence for sequence specificity for processing *in vivo*, only limited sequence-specific binding of purified IN protein to retroviral DNA has been detected in reconstituted systems. It seems likely, therefore, that some structural features or interactions among components within the preintegration complex help to place IN at its site of action near the viral DNA termini. The second step catalyzed by IN is a concerted cleavage and ligation reaction in which the two newly processed 3′ viral DNA ends are joined to staggered (4- to 6-bp) phosphates at the target site in host DNA (Fig. 7.16). The product of the joining step is a **gapped intermediate** in which the 5′-PO_4 ends of the viral DNA are not linked to the 3′-OH ends of host DNA.

Host Proteins Are Recruited for Repair of the Integration Intermediate

Retroviral DNA integration creates a discontinuity in the host cell chromatin, and repair of this damage is required to complete the integration process (Fig. 7.15). As with double-strand breaks produced by ionizing radiation or genotoxic drugs, retroviral DNA integration promotes rapid phosphorylation of the histone variant H2AX in the vicinity of the integration site and recruitment of proteins of the DNA damage-sensing pathways. Various lines of evidence indicate that components of the nonhomologous end-joining DNA repair pathway (DNA-dependent protein kinase, ligase IV, and Xrcc4) are required for postintegration repair. In cells that are defective in any of these components, retroviral DNA integration is essentially a lethal

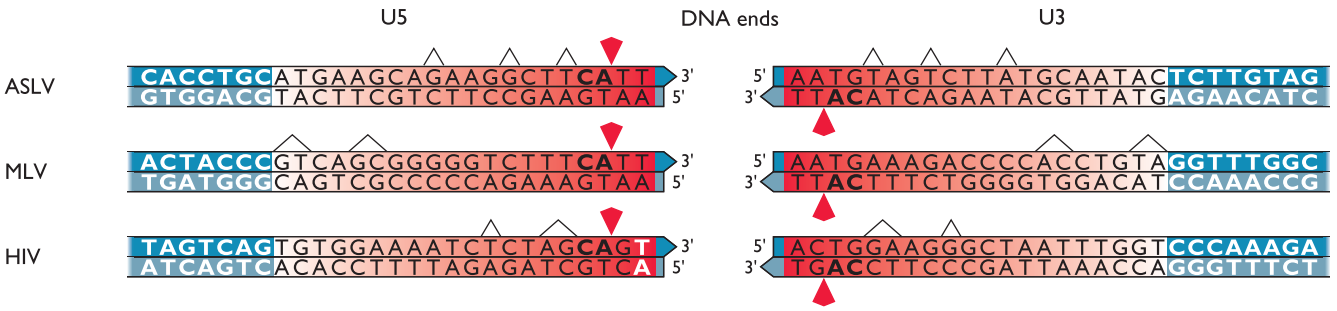


Figure 7.17 Nucleotide sequences at the termini of retroviral DNAs. Sequences are arranged with juxtaposed LTR termini. Red-shaded sections are important for integration. The most intensely shaded regions appear to be most critical, and the conserved 5′…CA…3′ is essential for efficient and accurate integration. Triangles above the sequences mark positions where base pairs are not repeated. Red arrowheads indicate the location of the nick produced during processing by IN.

event, triggering either cell cycle arrest or programmed cell death. It is likely that other host proteins play a role both in postintegration repair and in reconstitution of chromatin structure at the site of integration.

Properties of the Preintegration Complex

Rapidly sedimenting nucleoprotein complexes have been isolated from the cytoplasm of cells infected with avian sarcoma/leukosis virus, murine leukemia virus, and human immunodeficiency virus type 1. These complexes appear to be compact, but the viral DNA associated with them is accessible to nucleases. The nucleoprotein complexes contain IN and viral DNA in a form that can be joined to exogenously provided plasmid or bacteriophage DNA. Such *ex vivo* reactions exhibit all the features expected for products of authentic integration. Other viral proteins, among them RT, capsid, and nucleocapsid proteins, have also been reported to be present in the preintegration complexes of murine leukemia and human immunodeficiency viruses, but no clearly defined role in the integration process has yet been assigned to any of them. Despite intense efforts, the mechanism by which retroviral preintegration complexes enter the nucleus is still unclear, and variable (often contrasting) results have generated a good deal of controversy that has yet to be resolved (see Chapter 5).

Multiple Parameters Affect Selection of Host DNA Target Sites

Many sites in host DNA can be targets for retroviral DNA integration, and *in vitro* studies have revealed little sequence specificity for such sites, although the patterns exhibited by different retroviral INs with the same DNA target are not identical. However, such studies have established a preference for integration into DNA sequences that are intrinsically bent or underwound as a consequence of being wrapped around a nucleosome.

Recent advances in genomics and transcriptional profiling have provided a wealth of both technical and informational resources for studying integration site selection by retroviruses following infection of cultured cells. Results from a number of investigators who, collectively, have mapped thousands of integration sites in human and other cell lines have identified weak consensus sequences for host target sites. As illustrated for the two cases shown in Figure 7.18, the sequence patterns preferred for integration of proviruses of different genera are distinct. Although all of the consensus sequences studied to date form weak palindromes, the significance of this observation is still unclear. The symmetry in the patterns is consistent with the idea that IN complexes function as symmetrical multimers in the preintegration complex (discussed in "Higher-Order Structure in the Preintegration Complex" below). However, this statistically derived feature is also consistent with a mechanism whereby a preferred, nonpalindromic sequence can be used for integration of viral DNA in either direction. The pattern observed after insertion of viral DNA into purified human DNA by isolated preintegration complexes of human immunodeficiency virus type 1 is similar to that seen after infection of cultured cells by this virus (Fig. 7.18). It seems likely, therefore, that recognition by IN proteins depends on structural features that are shaped by primary DNA sequence.

These same large-scale, global analyses have also shown that all human chromosomes are targets for integration, but different retroviruses display distinguishable preferences for defined chromosomal features (Table 7.1). For example, human immunodeficiency virus type 1 shows the highest preference for integration anywhere inside of genes, especially in highly transcribed genes, whereas murine leukemia virus integrates preferentially in and near transcription start sites. The avian sarcoma/leukosis virus also shows a slight preference for integrating within

Random

Base	–5	–4	–3	–2	–1	1	2	3	4	5	6	7	8	9	10
A	0.31	0.29	0.35	0.30	0.28	0.28	0.30	0.28	0.30	0.32	0.30	0.30	0.27	0.31	0.30
C	0.18	0.20	0.20	0.21	0.21	0.22	0.22	0.21	0.23	0.19	0.19	0.22	0.23	0.18	0.25
G	0.21	0.22	0.19	0.22	0.21	0.22	0.22	0.22	0.19	0.21	0.20	0.18	0.19	0.22	0.20
T	0.30	0.29	0.26	0.28	0.29	0.27	0.26	0.30	0.28	0.28	0.31	0.31	0.31	0.29	0.25

ASLV

Base	–4	–3	–2	–1	1	2	3	4	5	6	7	8	9	10
A	0.21	0.22	0.27	0.34	0.18	0.28	0.29	0.32	0.28	0.13	0.26	0.34	0.43	0.23
C	0.28	0.13	0.21	0.14	0.32	0.20	0.23	0.24	0.21	0.32	0.24	0.23	0.17	0.29
G	0.24	0.18	0.24	0.20	0.39	0.19	0.21	0.22	0.23	0.30	0.13	0.21	0.15	0.26
T	0.36	0.47	0.28	0.32	0.11	0.33	0.27	0.23	0.28	0.25	0.31	0.22	0.25	0.22
Preferred base		T			G/C					G/C			A	

HIV-1

Base	–5	–4	–3	–2	–1	1	2	3	4	5	6	7	8	9	10
A	0.34	0.25	0.22	0.29	0.34	0.22	0.24	0.32	0.46	0.10	0.27	0.35	0.46	0.34	0.31
C	0.19	0.25	0.11	0.08	0.13	0.29	0.10	0.16	0.14	0.41	0.34	0.23	0.22	0.16	0.19
G	0.19	0.19	0.24	0.26	0.31	0.40	0.12	0.16	0.10	0.25	0.13	0.10	0.13	0.19	0.21
T	0.28	0.32	0.43	0.37	0.22	0.09	0.54	0.36	0.30	0.24	0.26	0.32	0.19	0.31	0.29
Preferred base			T		G	G	T		A	C	C		A		

Figure 7.18 Palindromic consensus sequences at retroviral integration sites. The frequency of each base at each position around the integration sites was calculated, where 1 equals 100%. Integration occurs between positions –1 and 1 on the top strand. Colored positions have statistically different frequencies of bases from that of randomly generated sequences shown at the top. Bases with a greater than 10% increase of frequency at a position are blue, and bases with a greater than 10% decrease of frequency at a position are yellow. The preferred bases are listed below. Inferred duplicated target sites are in the blue box, and joining to the 3′ ends of viral DNA occurs at positions labeled by arrows. The symmetry of the palindromic patterns is centered on the duplicated target sites. Adapted from X. Wu et al., *J. Virol.* **79:**5211–5214, 2005.

genes, but not necessarily those that are highly transcribed, and it displays no preference for transcription start sites. These observations suggest that the interaction of preintegration complexes with different chromatin-bound proteins promotes integration into specific chromosomal locations. Indeed, recent studies have shown that interaction of lentiviral IN proteins with a 75-kDa transcriptional coactivator, (mis)named lens epithelium-derived growth factor **(Ledgf)**, is a critical component of integration site selection for these viruses. The efficiency of integration of human immunodeficiency virus type 1 is greatly reduced in cells in which Ledgf has been depleted by treatment with small interfering RNA (siRNA), or in which the gene has been deleted. Furthermore, the pattern of preference for various chromosomal features is altered in the small percentage of Ledgf-deficient cells in which integration does occur (Table 7.1). Exactly how this protein promotes such preferential integration is still unclear. Interactions that may be responsible for the preferences observed with other retroviral genera have also yet to be defined. Because integration of the yeast transposon Ty3 into sites upstream of polymerase III promoters has been linked to interaction of the Ty3 IN protein with RNA polymerase III transcription initiation factors, it is possible that a promoter-specific protein could play a role in the preferential integration of murine leukemia virus DNA near transcription start sites.

Integrase Structure and Mechanism

IN Proteins Are Composed of Three Structural Domains

Retroviral IN proteins are approximately 300 amino acids in length, comprising three domains (Fig. 7.19). Attempts to determine the three-dimensional structure of a full-length IN by X-ray crystallography have so far proved unsuccessful. However, considerable insight has been obtained from analyses of single- and two-domain polypeptides.

The **N-terminal domain**, comprising approximately the first 50 amino acids, is characterized by two pairs of invariant, Zn^{2+}-chelating histidine and cysteine residues (HHCC motif). The bound Zn^{2+} ion stabilizes a helix-turn-helix structural motif that is almost identical in topology to the DNA-binding domain of the bacterial *trp* repressor protein. The **catalytic core domain**, included in a central region of approximately 150 amino acids, is characterized by a constellation of three invariant acidic amino acids, the last two separated by 35 residues, the D,D(35)E motif. Results of site-directed mutagenesis experiments have

Table 7.1 Comparison of retroviral integration site preferences

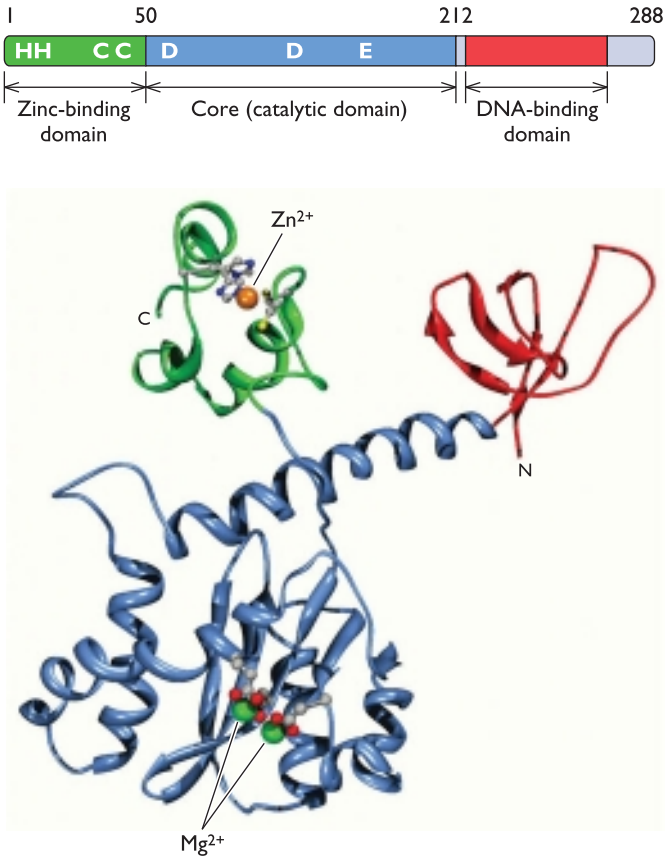
Site or region	% Integration[a]					
	Human cells[b]				Mouse cells[c]	
	Random	ASLV	MLV	HIV	$HIV^{LEDGF+/-}$	$HIV^{LEDGF-/-}$
Within genes	26	42	40	60–70	62	44
Transcription start sites	5	8	20	10	6	17

[a]HIV, human immunodeficiency virus; ASLV, avian sarcoma/leukosis virus; MLV, murine leukemia virus.

[b]Percentages are approximates for integration into human cells and are from A. Narezkina et al., *J. Virol* **78:**11656–11663, 2004.

[c]Percentages for mouse embryo fibroblasts are from M. C. Shun et al., *Genes Dev.*, **21:**1767–1768, 2007. Calculations performed in this study indicated HIV gene usage in mouse $LEDGF^{-/-}$ cells at ~8% above random, which was ~3% less than ASV/MLV gene usage in human cells.

Figure 7.19 Linear map of human immunodeficiency virus type 1 IN and a model showing the three independently folding domains. Numbers at the top indicate amino acid residues starting with 1 at the N terminus. Evolutionarily conserved amino acids are indicated in the single-letter code. In the ribbon model below, conserved amino acids are in ball-and-stick representation with metal ions represented by orange (Zn^{2+}) or green (Mn^{2+} or Mg^{2+}) balls. The conserved Glu residue in the core domain is on an unstructured region in the X-ray structure used for the model. It is presumed to chelate the second metal ion together with one of the conserved Asp residues, as illustrated in Fig. 7.20. Coordinates are from Protein Data Bank codes 1K6Y and 1EX4; figure prepared by Mark Andrake, Fox Chase Cancer Center.



shown that these acidic amino acids are required for all catalytic functions of IN. This finding indicates that there is one catalytic center for both processing and joining. Solution of the crystal structures of the catalytic core domains of the human and avian viral IN proteins was an important milestone in the study of integration. These core domain structures established that IN proteins are members of a large superfamily of nucleases and recombinases that includes the RNase H domain of the human virus RT (Fig. 7.20). The sequence of the 80- to 100-amino-acid **C-terminal domain** is the least conserved among IN proteins from different retroviral genera. However, despite their sequence differences, the three-dimensional structures of this domain are quite similar in the two examples analyzed to date: the human immunodeficiency virus type 1 and the avian leucosis/sarcoma virus IN proteins. This domain contains critical DNA-binding activity and multimerization determinants. A molecular model of a full-length IN protein, which illustrates the structural features of each domain, is shown in Figure 7.19.

Structures of two-domain proteins comprising the catalytic core and either the N- or C-terminal domains of several retroviral IN proteins have been solved by X-ray crystallography. A model of a full-length IN dimer, based on a consolidation of the two-domain structures, is shown in Fig. 7.21. Although no C-terminal domain interactions are depicted in this model, previous biochemical studies indicate that this domain contributes to formation of multimers. C-terminal interactions are included in several computer-derived molecular models of IN tetramers, but to date none of these models has been fully validated.

Higher-Order Structure in the Preintegration Complex

The joining of viral to host DNA comprises a dynamic, multistep reaction in which two separate DNA duplexes are bound to an IN complex, cut, and recombined with a DNA target. We have yet to understand completely how the substrates and protein are organized and acted upon

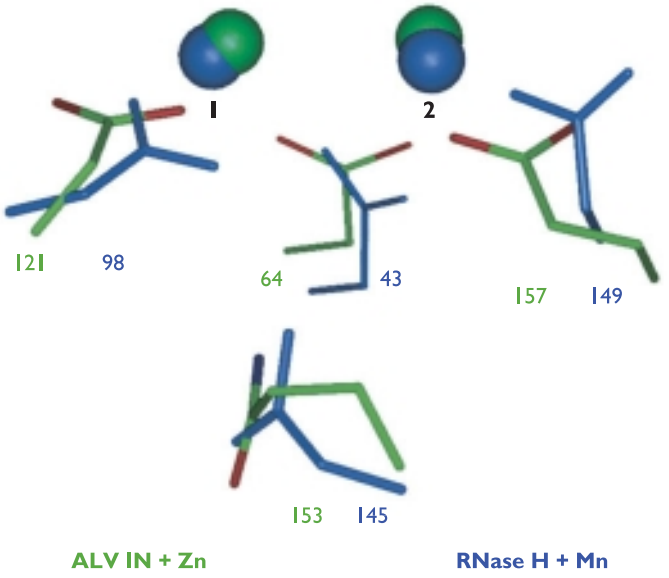


Figure 7.20 Metal complexes in the human immunodeficiency virus type 1 RNase H and the avian sarcoma/leukosis virus (ALV) IN catalytic core domain. The orientation of the side chains and the positions of the metals in the catalytic core domain of human immunodeficiency virus type 1 and avian sarcoma/leukosis virus IN proteins are superimposable on those of metal ion complexes obtained with the RNase H domain of the human immunodeficiency virus type 1 RT (Fig. 7.11). In the comparison shown here, the side chains of critical acidic amino acids form a tripod that interacts with two divalent cations in almost superimposable positions. The IN catalytic core also binds a single Mn^{2+} or Mg^{2+} ion in position 1 with no change in the position of the side chains of D64 and D121. The binding of two metal ions at the active sites of the viral RNase H and IN proteins is consistent with a two-metal mechanism of catalysis by this superfamily, as proposed for the human retroviral RT polymerase activity.

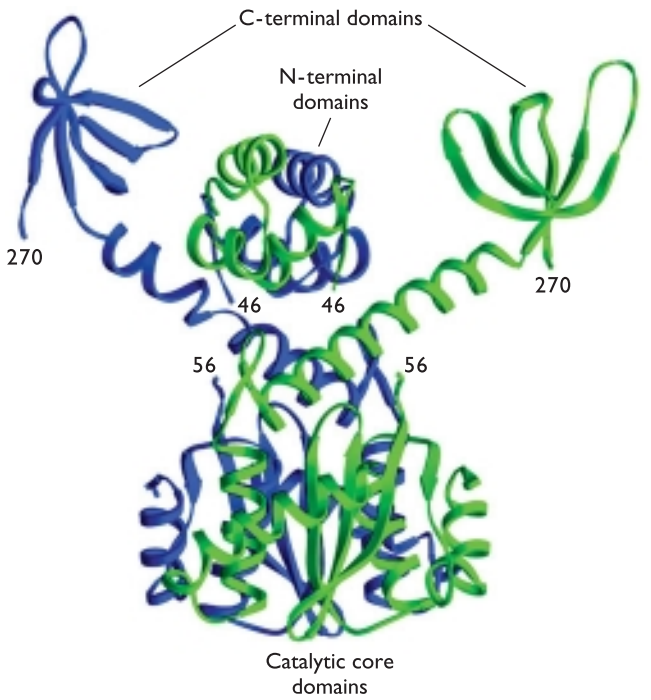


Figure 7.21 Model of a dimer of human immunodeficiency virus type 1 integrase. This ribbon model is derived from the superposition of the nearly identical core domain dimers in the crystal structures of the core with the N-terminal domain (Protein Data Bank code 1K6Y), and the core with the C-terminal domain (Protein Data Bank code 1EX4). The domains comprising the individual monomers are shown in green and blue. Courtesy of Robert Craigie, National Institutes of Health.

in any recombination reaction. However, because details of the structure of IN and substrate DNAs in the preintegration complex are important for the development of antiviral drugs, many investigators have focused on this question.

Organization of DNA ends. In most models of the IN-catalyzed reactions, viral DNA ends are placed near one another, as presumed to be necessary for their concerted processing and joining to a target site. The presence of inverted repeats at these ends (Fig. 7.17) suggests that they are recognized by a multimeric protein with a twofold axis of symmetry. *In vitro* experiments indicate that the invariant dinucleotides at each viral DNA end are bound to IN at a fixed distance from each other during the concerted processing reaction. The optimal distance between them is related to the distance between the staggered cuts in target DNA made during joining. These and other experiments also indicate that the viral DNA ends do not remain double stranded when bound at the active site of the enzyme, but that the two strands are partially unwound and distorted.

A multimeric form of IN is necessary for activity. The results of early biochemical analyses and *in vitro* complementation studies indicated that IN functions as a multimer. A reversible equilibrium among monomeric, dimeric, and tetrameric forms of IN is observed in the absence of DNA substrate. A conservative estimate of the intracapsid concentration of IN gives a value (~150 μM) high enough for most of the IN protein in virions to be in the form of dimers and tetramers. Recent *in vitro* studies indicate that an IN tetramer is stabilized by interaction with a pair of viral DNA ends, and various lines of evidence suggest that each end is held mainly through contacts with C-terminal domain residues in one IN monomer, and then positioned and acted upon by the catalytic core domain of another. In this model, the catalytic core domains of only two of the four subunits in the IN tetramer would provide catalytic function (as illustrated in Fig. 7.22). DNA footprinting experiments with preintegration complexes isolated from

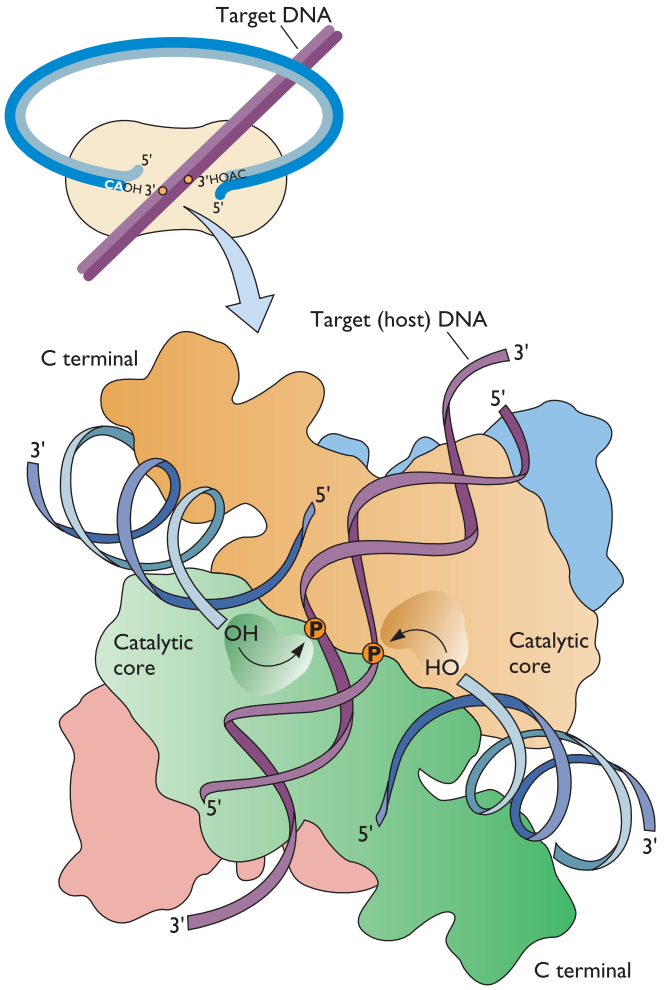


Figure 7.22 Tetramer model for functional integrase. The active sites of only two IN monomers (represented in green and yellow) participate in catalysis during the concerted joining reaction illustrated. As suggested by various lines of evidence, the distal end of viral DNA is held by the C-terminal domain of one monomer and the terminus is acted upon by the catalytic core domain of a second monomer. The other two subunits likely contribute to the stability of the complex. Binding of the target DNA to basic residues at the dimer-dimer interface may help to stabilize the tetramer.

Moloney murine leukemia virus-infected cells suggest that several hundred base pairs of the viral DNA ends are protected by association with IN and therefore that many multimers may be bound. The significance of this association is not yet known.

Host Proteins That May Regulate the Integration Reaction

Two general approaches have been used to identify host proteins that are important for retroviral DNA integration: analysis of the cellular components in purified preintegration complexes, and identification of proteins that can bind to IN. Most of these studies have focused on the clinically important human immunodeficiency virus, but some investigations have been conducted with murine leukemia virus.

The viral DNA within the preintegration complex is not itself a target for joining; such autointegration reactions would be suicidal. Analysis of preintegration complexes of murine leukemia virus showed that this restriction depends on the presence of an 89-amino-acid cellular protein called **barrier-to-autointegration factor (Baf).** This small protein forms dimers in solution, binds to DNA, and can produce intermolecular bridges that compact the DNA. It has been proposed that such compaction prevents autointegration. As purified virions do not contain this cellular protein, it must be acquired from the cytoplasm of a newly infected host cell (Fig. 7.23). The homologous human protein has been shown to block autointegration in human immunodeficiency virus preintegration complexes. Baf was recently shown to bind to a specific domain (the Lem domain) in **lamina-associated polypeptide 2α (Lap2α)**, another cellular protein that accumulates in the preintegration complex of murine leukemia virus. Baf also binds to the Lem domain of **emerin**, a component of the inner nuclear membrane. Transcribed chromatin tends to be associated with the nuclear lamina, and the interaction of Baf with emerin has been proposed to promote access of the preintegration complex to chromatin after the complex has entered the nucleus. However, as deletion of either Lap2α or emerin from mouse cells is reported to have little or no effect on the replication of murine leukemia virus or human immunodeficiency virus type 1, neither interaction can be essential for integration. Another host protein, the high-mobility-group, nonhistone DNA-bending protein **Hmga1**, is present in human immunodeficiency preintegration complexes and was originally thought to play a role in targeting integration to chromatin. However, because cells that lack Hmga1 have no obvious defect in human immunodeficiency virus type 1 replication, this protein is also either redundant or not essential for integration.

The first host protein reported to bind specifically to human immunodeficiency virus type 1 IN was called IN-interacting protein 1 **(Ini-1)**. It was later found to be a core component of the Swi/Snf chromatin remodeling complex. After infection, Ini-1 translocates from the nucleus to the preintegration complex in the cytoplasm. Although the idea that Ini-1 might play a role in targeting the preintegration complex to chromatin initially seemed attractive, no apparent integration defect has been observed in cells that lack this protein. The only human immunodeficiency virus IN-binding protein that has a verified role in mediating access to host chromatin is Ledgf (Fig. 7.23).

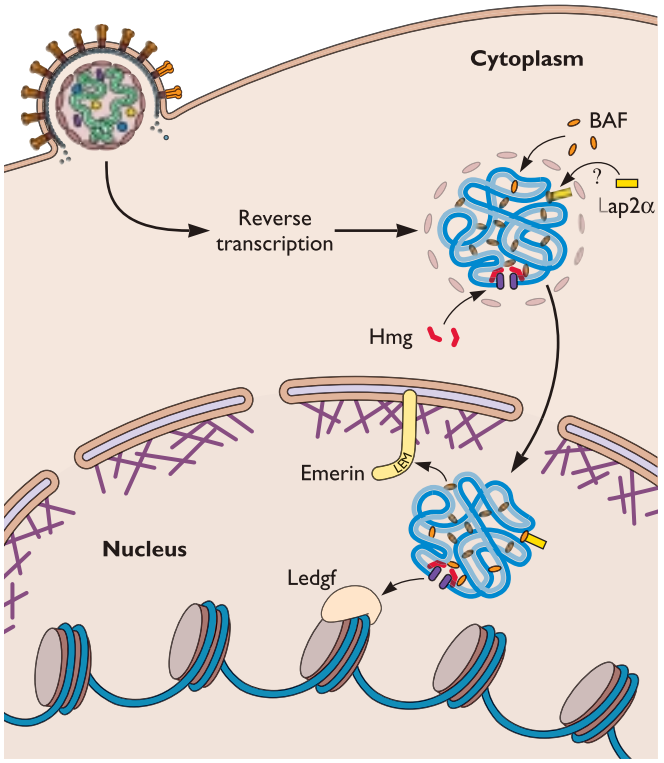


Figure 7.23 Host proteins may regulate the integration process. The barrier-to-autointegration protein (BAF) binds to newly synthesized murine leukemia virus and human immunodeficiency virus type 1 DNAs, causing them to condense. Such compacted DNA may be an unsuitable target for autointegration. The high-mobility-group 1a (Hmga1) and Lap2α proteins also accumulate in the preintegration complexes of the human and murine viruses, respectively, but their functions, if any, are still uncertain. Once inside the nucleus, binding of BAF to emerin is proposed to facilitate access of the IN-DNA complex of human immunodeficiency virus type 1 to chromatin. IN binding to Ledgf tethers this complex to chromatin, thereby increasing integration efficiency.

This normally abundant cellular protein was also found to accumulate in the preintegration complex, and is reported to stabilize IN protein in infected cells and to enhance the catalytic activities of lentiviral IN proteins *in vitro*. Numerous other IN-interacting proteins have been described in the recent literature; the roles of most of these proteins in the integration process have yet to be tested (Box 7.6).

Hepadnaviral Reverse Transcription

A DNA Virus with Reverse Transcriptase?

The revolutionary concept that a virus with an RNA genome can replicate by means of a DNA intermediate was followed, about a decade later, by another big surprise: RNA as an intermediate in the replication of a DNA virus. Early hints that a mechanism other than semiconservative DNA synthesis was responsible for hepadnaviral replication came from the discovery of asymmetries in the genomic DNA and in the endogenous DNA polymerase reaction in isolated virions. The viral DNA comprises one full-length (−) strand and an incomplete, complementary (+) strand (Fig. 7.24), and the endogenous polymerase reaction could extend only the (+) strand. The replication intermediates isolated from infected cells were also unusual, comprising mainly (−) strands of less than unit length, few of which were associated with (+) strands. All this seemed suspiciously like retroviral reverse transcription, and in 1982 landmark studies with duck hepatitis B virus that revealed the unique features of hepadnaviral replication were published. Unlike the endogenous reaction typical of extracellular virions, newly formed intracellular "core" particles were found to incorporate dNTPs into both strands. As with RNA-dependent DNA polymerization in retroviruses, synthesis of the (−) strand was resistant to the DNA-intercalating drug actinomycin D, whereas synthesis of the (+) strand was inhibited by this compound. Furthermore, a portion of the newly synthesized (−) strand DNA sedimented with the density of RNA-DNA hybrids. These and related findings marked an important turning point in our understanding of hepadnaviruses and greatly extended our knowledge of reverse transcription. (See Box 7.7 for yet another, more recent, surprise.)

Role of Reverse Transcription in the Hepadnaviral Life Cycle

A simplified model for the single-cell replication cycle of hepadnaviruses is shown in Fig. 7.25. The gapped DNA of an entering virus particle is imported into the nucleus and repaired to produce a covalently closed circular molecule, by mechanisms that are still undefined. The hepadnavirus genome encodes no integrase, and, unlike retroviral DNA, hepadnaviral DNA is not normally integrated into the host's genome. However, the covalently closed circular DNA persists in the nucleus as an autonomous episome from which viral RNAs are transcribed by the host cell RNA polymerase II. The 3.5-kb **pregenomic mRNA** is exported to the cytoplasm, where it serves as the template for reverse transcription. This process takes place in a newly formed subviral particle that includes the core and polymerase proteins (products of the C and P genes). P protein provides all the activities required for reverse transcription. Following this process, the DNA-containing, nascent core particles can follow one of two pathways. Late in infection, when the cisternae of the endoplasmic reticulum contain an abundance of viral envelope glycoprotein, they can bud into the endoplasmic reticulum and eventually be secreted as progeny virions (Chapter 13). Alternatively, if they

BOX 7.6 WARNING

Determining a role for cellular proteins in viral replication can be quite difficult

Understanding the roles of both viral and cellular proteins at various stages of viral replication is essential for elucidating molecular mechanisms and for developing strategies for blocking pathogenic infections. As viral genomes have a limited set of genes, the viral proteins or genetic elements that are essential at each step can be deduced by introducing mutations and observing phenotypes. Identifying critical cellular genes and proteins is much more difficult. Two general approaches have been used to select likely candidates: identifying cellular proteins that are included in virus particles and/or bind to viral proteins (*in vitro* or in cells).

Once candidates are identified, the contribution of the cellular protein to viral replication may be evaluated by observing the effects of:

- specific small molecule inhibitors of the protein function (inhibitory drugs)
- synthesis of an altered protein, known to have a dominant-negative effect on its normal function
- treatment with siRNAs that reduce the concentration of the cellular protein
- replication in cells in which the candidate gene has been mutated or deleted.

Even after applying the multiple approaches and methods described above, identifying relevant cellular proteins and evaluating their roles in viral replication is seldom easy. Following are some of the problems encountered:

- More than one protein may provide the required function (redundancy).
- The function of the protein might be essential to the cell, and mutation of the gene that encodes it (or inhibition of protein production by siRNA) could be lethal.
- Only small amounts of the protein might be required for viral replication, and reducing its activity with an inhibitor, or its amount with siRNA, may be insufficient to observe a defect.
- The cellular protein might provide a slight enhancement to viral replication that could be difficult to detect, but may be physiologically significant.
- Synthesis of an altered cellular gene, or overexpression of a normal cellular gene, may produce changes that affect virus replication for reasons that are irrelevant to the natural infection (artifacts).

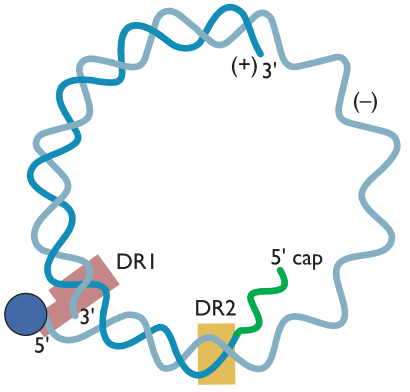
Given these difficulties, it is not surprising that the literature in this area is sometimes contradictory and the results are sometimes controversial. High standards of validation must be met before claims can be accepted!

do not become enveloped, the core particles can be directed to the nucleus, where their DNA is converted to additional copies of covalently closed circular DNA. This pathway predominates at early times after infection, when little envelope protein is available. Eventually, at least for duck hepatitis B virus, as many as 10 to 20 covalently closed circles can accumulate in the nucleus, and a steady-state balance of DNA and cytoplasmic virion protein components is maintained. It seems reasonable to assume that similar regulation occurs during the replication of mammalian hepadnaviruses, but this has not yet been demonstrated.

Covalently closed circular hepadnaviral DNA is not replicated by the host's DNA synthesis machinery; **all** hepadnaviral DNA is produced by reverse transcription. This situation contrasts with that of retroviruses, in which the integrated nuclear form, the provirus, is replicated with the host DNA. Both retroviral and hepadnaviral DNAs are maintained in infected cells for the life of their host, but in quite different ways: retroviral DNA as a single integrated copy that is replicated by host cell machinery, and hepadnaviral DNA as multiple, episomal copies that are amplified by continuous rounds of reverse transcription.

The analysis of hepadnaviral reverse transcription has been difficult for a number of technical reasons. Suitable tissue culture systems were not available until 1986, when hepatoma cell lines that were permissive for viral replication following transfection with cloned viral DNA were identified. Furthermore, mutational studies are confounded by the compact coding organization of the DNA. The tiny genome (~3 kb) is organized very efficiently, with more than half of its nucleotides translated in more than

Figure 7.24 Hepadnaviral DNA. The DNA in extracellular hepadnavirions is a relaxed circular molecule of approximately 3 kb with circularity that is maintained by overlapping 5′ ends. The (−) strand is slightly longer than unit length, and the polymerase, shown as a blue ball, is attached to its 5′ end. The (+) strand has a capped RNA of 18 nucleotides at its 5′ end, and is less than unit length. The 5′ ends are near or in (10- to 12-bp) direct repeats called DR1 and DR2 (colored purple and yellow, respectively). As in retroviruses, these repeat sequences play an important role in facilitating critical template transfers during reverse transcription. Details of the genetic content are provided in the appendix, Fig. 4.



BOX 7.7 BACKGROUND

A retrovirus with a DNA genome?

The *Spumavirinae* comprise a subfamily of retroviruses isolated from primate, feline, and bovine species, among others. Spumaviruses are commonly called **foamy viruses**, because they cause vacuolization and formation of syncytia in cultured cells. These viruses exhibit no known pathogenesis and received little attention from virologists until recently. However, these recent studies have revealed that the foamy viruses are most **unconventional** retroviruses, with many properties that seem more similar to those of hepadnaviruses than of other retroviral family members.

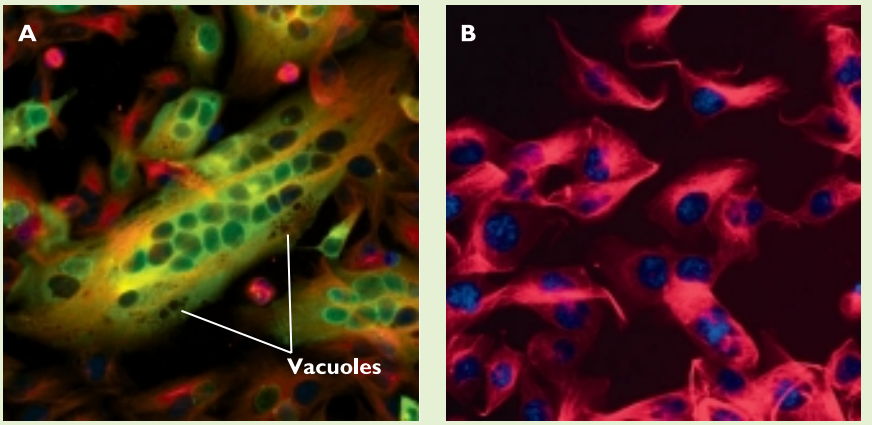
For example:

- Reverse transcription appears to be a late event in viral morphogenesis, and is largely complete before extracellular virus infects new host cells. Furthermore, although they contain both RNA and DNA, the genome-length DNA extracted from such virions can account entirely for virus infectivity. Like other retroviral family members, foamy virus genome replication requires an RNA intermediate, but the functional nucleic acid in extracellular virions appears to be DNA.
- Although the arrangement of genes and the mechanism of reverse transcription are the same as that in other retroviruses, the human foamy virus reverse transcriptase is not synthesized as part of a Gag-Pol precursor, but rather from translation of a separate *pol* mRNA, as is also the case for the hepatitis B virus RT.
- Mature foamy virus particles do not include the usual processed retroviral structural proteins (MA, CA, and NC), but instead contain two large Gag proteins that differ only by a 3-kDa extension at the C terminus. These Gag proteins contain glycine-arginine-rich domains that bind with equal affinity to RNA and DNA, much like the hepadnaviral core protein.
- As with the hepadnaviruses, foamy virus budding requires both Gag and Env proteins, and most budding occurs into the endoplasmic reticulum.
- Most foamy virus particles remain within the infected cell. This property probably accounts for the large quantities of intracellular viral DNA, and might explain why persistently infected cells contain numerous integrated proviruses. It is possible that foamy virus DNA integration occurs through an intracellular recycling pathway of progeny genomes, similar to that which occurs with hepadnaviruses.

The foamy viruses therefore may represent an evolutionary link between the retroviruses and the hepadnaviruses, with genomes that are replicated by reverse transcription.

Linial, M. A. 1999. Foamy viruses are unconventional retroviruses. *J. Virol.* **73:**1747–1755.

Cells infected with primate foamy virus **(A)** show large syncytia and numerous vacuoles. Uninfected cells **(B)** lack such vacuoles and have only single nuclei. Nuclei are stained blue; a-tubulin is red, and viral Gag protein is green. Micrographs were obtained by Alison Yu and generously provided by Maxine Linial, Fred Hutchinson Cancer Center.



one reading frame. This arrangement makes it difficult to produce mutations that change only one protein. Finally, although reverse transcription takes place in newly assembled core particles, until recently it had not been possible to prepare enzymatically active P protein to study the reaction. Despite these difficulties, currently available details reveal fascinating analogies, but also striking differences, in the reverse transcription of hepadnaviruses and retroviruses.

Pathway of Reverse Transcription

Essential Components

Pregenomic RNA. The pregenomic RNA that provides the template for production of hepadnaviral genomic DNA is capped and polyadenylated, and also serves as the mRNA for both C and P proteins. The transcription of pregenomic RNA from covalently closed circular DNA in the nucleus is initiated at a position approximately 6 bp upstream of one copy of a short, direct repeat of 11 to 12 nucleotides called DR1 (Fig. 7.24). Transcription then proceeds along the entire DNA molecule, past the initiation site, to terminate after a polyadenylation signal just downstream of DR1. Consequently, pregenomic RNA is longer than its template DNA. Because the region from the transcription initiation site to the polyadenylation site is copied twice, there is a long direct repeat of ca. 200 nucleotides (r) at either end of the RNA. This long repeat includes dr1 and a structural element of about 100 nucleotides called **epsilon (ε)**

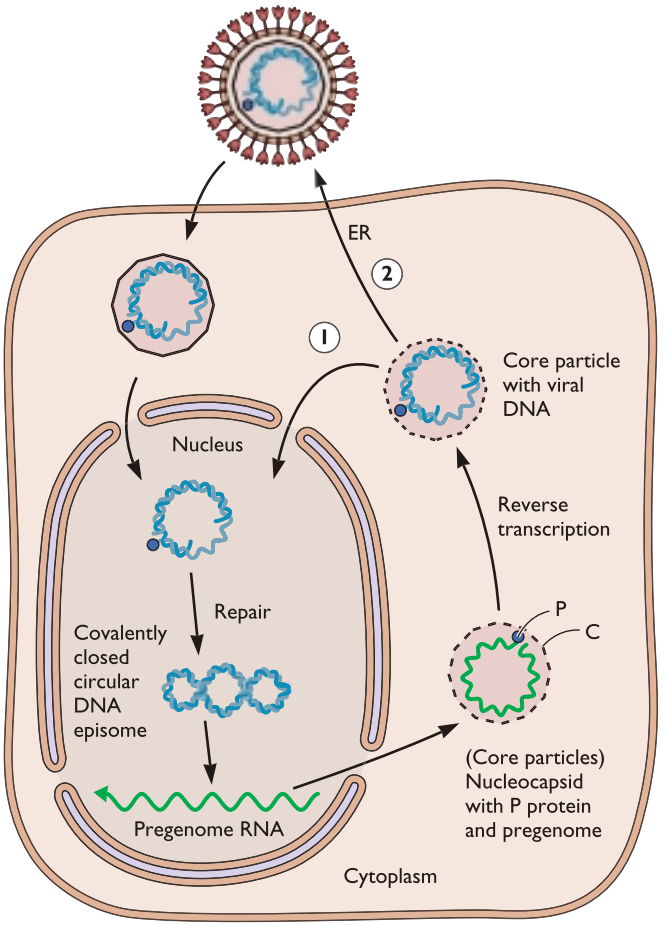


Figure 7.25 Single-cell replication cycle for hepadnaviruses. Pathway 1 provides additional copies of covalently closed circular episomal DNA. Pathway 2 represents exit of enveloped particles through the endoplasmic reticulum (ER).

(Fig. 7.26). Deletion of ε within the 3′ copy of r has no impact on viral replication. In contrast, ε at the 5′ end provides both the site for initiation of (−) strand synthesis and the signal for encapsidation of DNA into core particles. Although all viral transcripts have ε at their 3′ ends, only the pregenomic RNA has this critical copy of ε at its 5′ end.

There is a marked preference for reverse transcription of the pregenomic RNA molecules from which P protein is translated. The basis of such *cis*-selectivity is unknown; C protein, which is also translated from this RNA and has nucleic acid-binding properties, appears to function perfectly well in *trans*. It is possible that the nascent P polypeptide binds to its own mRNA cotranslationally. An attendant benefit from such a mechanism would be the selection for genomes that express functional P protein. Analysis of cytoplasmic core particles suggests that there is one molecule of P protein per molecule of DNA, implying that hepadnaviruses contain one copy of the viral genome per virion (Table 7.2). This selectivity would be determined, in part, by the presence of the encapsidation signal(s) at the 5′ end of the pregenomic RNA.

Primers. The primers for hepadnaviral RT remain attached to the 5′ ends of the viral DNA strands. They are, for (−) strand synthesis, the P protein itself, and, for (+) strand synthesis, a capped RNA fragment derived from the 5′ end of pregenomic RNA. A protein-priming mechanism (Chapter 9) was described first for adenovirus DNA replication and later for the bacteriophage 29. Priming by a viral protein, VPg, is also a feature of poliovirus RNA synthesis. Hepadnaviral reverse transcription is distinguished by the fact that the protein primer and the polymerase are components of the same polypeptide.

Figure 7.26 *cis*-acting signals in pregenomic RNA. The viral pregenomic RNA bears terminal repetitions of ca. 200 nucleotides (r) that contain copies of the packaging signal (ε), but only the 5′ copy has functional activity in vivo. Indicated are positions of initiation for the 5′ ends of (−) and (+) strand DNAs, and the 5′-UUAC-3′ motifs in duck hepatitis B virus within ε and at dr1, that are important for (−) strand DNA synthesis. Both the structural features of ε and the specific sequence in the loop are critical for its function. Adapted from C. Seeger and W. S. Mason, p. 815–832, *in* M. L. DePamphilis (ed.), *DNA Replication in Eukaryotic Cells* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1996), with permission.

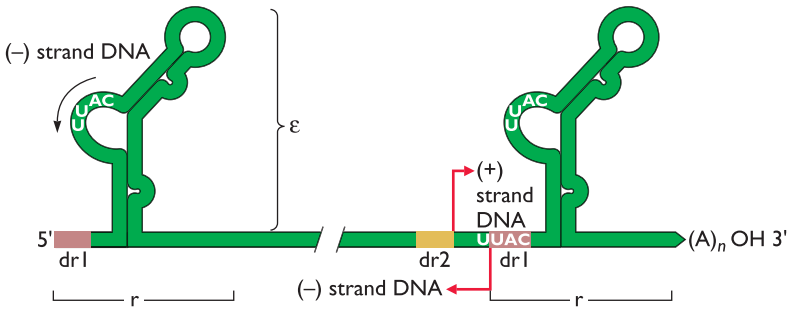


Table 7.2 Comparison of retroviral and hepadnaviral reverse transcription

Parameter	Retroviruses[a]	Hepadnaviruses[a]
Viral genome	RNA (pseudodiploid)	DNA (incomplete duplex)
Template RNA also serves as:	Genomic *RNA mRNA* (*gag*, *pol*)	Pregenomic *RNA mRNA* (C and P proteins)
DNA intermediate	*Circular DNA with 5′ overlaps*	*Circular DNA with 5′ overlaps*
Virus-encoded enzyme	RT	P protein
No. of molecules/core	50–100	1
Functions	*DNA polymerase*, *RNase H*, helicase (strand-displacement)	DNA *polymerase*, *RNase H*, protein priming, template RNA encapsidation
Primer, first (–) DNA strand	tRNA (host)	Viral P protein
Site of initiation	*Near 5′ end of genome*	*Near 5′ end of pregenome*
First DNA product	(–) strong stop DNA, ca. 100 nucleotides	4 nucleotides copied from bulge in 5′ ε
First template exchange	*To complementary sequence in repeated sequence, r, at 3′ end of template RNA*	*To complementary sequence in repeated sequence, R, at 3′ end of template RNA*
Primer, second (+) DNA strand	*Derived from template RNA*, internal RNase H product (ppt)	*Derived from template RNA*, 5′ cap, terminal RNase H product
Site of initiation	*Near 5′ end of (–) DNA*	*Near 5′ end of (–) DNA*
Time of initiation	Before completion of (–) strand	After completion of (–) strand
Type of priming	Priming *in situ*	Primer translocated
Second template exchange	*To the 3′ end of (–) strand DNA via complementary* pbs *sequence*	*To the 3′ end of (–) strand DNA via complementary sequence*
Reverse transcribing nucleoprotein complex	*Subviral "core"* particles, deposited in the *cytoplasm* upon viral entry	Nascent *subviral "cores"*; *cytoplasmic* intermediates in viral assembly
Final product(s)	Double-stranded linear DNA	Circular viral DNA or covalently closed episomal DNA
DNA maintained in the nucleus	Integrated into host genome, proviral DNA	Nonintegrated episome in host nucleus

[a] Italics indicate similarities.

P protein is a self-priming reverse transcriptase. P protein is translated from an internal AUG in the pregenomic RNA (Appendix, Fig. 3). The translation product is a 90-kDa protein with C-terminal enzymatic domains that were first identified by amino acid sequence alignment with the retroviral RTs (Fig. 7.27). The highly conserved residues in the homologous domains are essential for hepadnaviral reverse transcription. Hepadnaviral P protein also contains an N-terminal domain separated from the RT region by a spacer, so called because amino acid substitutions in this region have no functional consequences. The N-terminal domain, referred to as the **terminal protein region**, includes a tyrosine residue utilized for priming (–) strand DNA synthesis. In addition to its other functions, P protein is required for encapsidation of viral RNA, a process that depends on the interaction of both the polymerase and terminal protein domains with the ε region. This is a departure from the retroviral scheme, in which the NC protein sequences in the Gag polyprotein are used for this purpose (Table 7.2). Indeed, the requirement for a DNA polymerase in hepadnaviral RNA encapsidation is unique among retroelements.

Host proteins may facilitate P-protein folding. An important breakthrough in the study of hepadnaviral reverse transcription was achieved with the demonstration that enzymatically active P protein can be produced upon translation of P mRNA from duck hepatitis B virus in a cell-free rabbit reticulocyte lysate. P protein is the only viral protein required for initiation of hepadnaviral DNA synthesis. If the ε region is not present during synthesis of P protein in yeast, the enzyme is inactive, even if the ε region is supplied later. Furthermore, P protein synthesized in the presence of the ε region is more resistant to proteolysis. Therefore, binding to ε may be required for the P protein to fold into an active conformation. Several studies suggest that host cell proteins may also affect P-protein folding. Synthesis of active P protein in the cell-free system requires the presence of cellular chaperone proteins and a source of energy (adenosine triphosphate [ATP]). Furthermore, incorporation of these host cell proteins into viral cores appears to require the P-protein polymerase activity. It has been proposed that chaperones are needed to maintain this viral protein in a conformation that is competent to bind to ε and prime DNA synthesis, and to interact with assembling core subunits (Fig. 7.28).

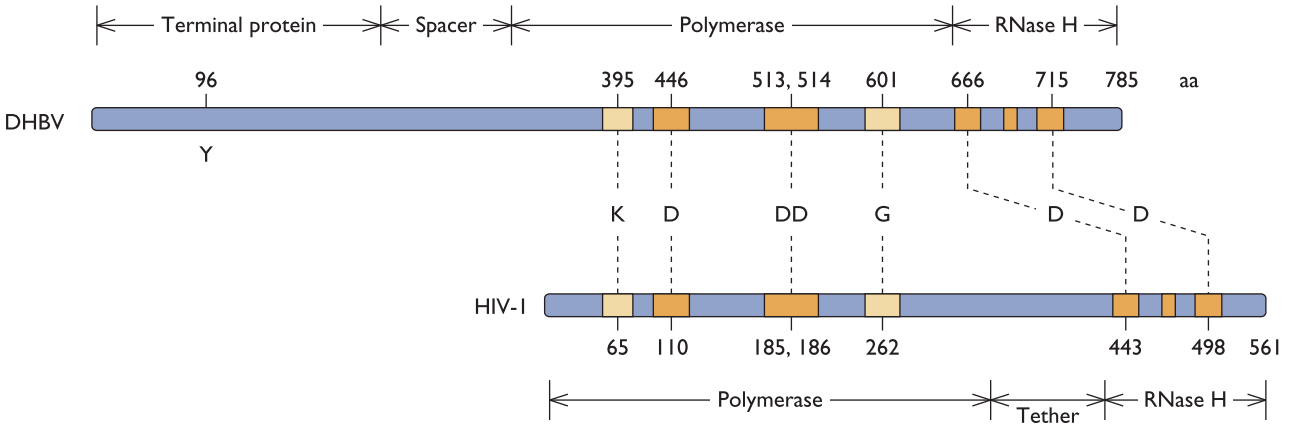
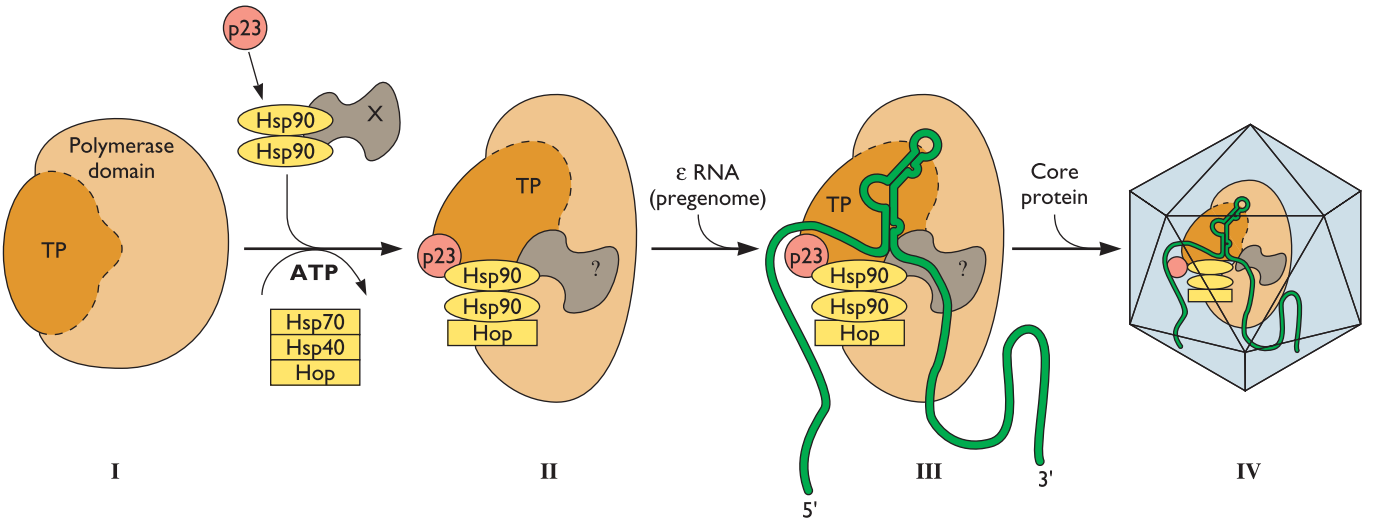


Figure 7.27 Comparison of hepadnaviral and retroviral RT proteins. Linear maps of the duck hepatitis B virus (DHBV) and human immunodeficiency virus type 1 (HIV-1) *pol* gene products. The maps were aligned relative to amino acids that are generally conserved among all RTs. Adapted from C. Seeger and W. S. Mason, p. 815–832, *in* M. L. DePamphilis (ed.) *DNA Replication in Eukaryotic Cells* (Cold Spring Harbor Lboratory Press, Cold Spring Harbor, NY, 1996), with permission.

Critical Steps in Reverse Transcription

Initiation and the first template exchange. Synthesis of the (–) strand of duck hepatitis B virus DNA is initiated by the polymerization of three or four nucleotides primed by the OH group of a tyrosine residue in the terminal protein domain of the single P-protein molecule present in the core. This single protein molecule acts both as primer and catalyst for all subsequent steps in reverse transcription. The template for this reaction is a specific sequence in the bulge of ε at the 5′ end of the pregenomic RNA. Why this copy and not the ε sequence at the 3′ end is normally chosen for initiation by P protein remains a mystery. This initial synthesis is followed by a template exchange in which the enzyme-bound, 4-nucleotide product anneals to another CAUU at the edge of dr1 at the 3′ end of the pregenomic RNA (Fig. 7.26 and 7.29, step 2). Although

Figure 7.28 Model for the assembly of hepadnavirus nucleocapsids. P protein is synthesized in an inactive conformation (I). Interaction of RT with the chaperone complex (heat shock protein 90 [Hsp90], together with four cochaparones, Hsp70, Hop, Hsp40, and p23) induces a conformational change (II) that allows binding of P protein to ε RNA (III), which in turn provides the signal for nucleocapsid assembly and initiation of viral DNA synthesis (IV). Additional protein(s) (marked ?) may also be bound in the P protein-RNA complex. TP denotes the N-terminal domain of the P protein (called terminal protein), which is unique to hepadnaviral RTs and harbors the primer tyrosine residue for the initiation of reverse transcription. Adapted from J. Hu et al., *EMBO J.* **16:**59–68, 1997, and J. Hu et al., *J.Virol.* **78:**13122–13131, 2004, with permission.



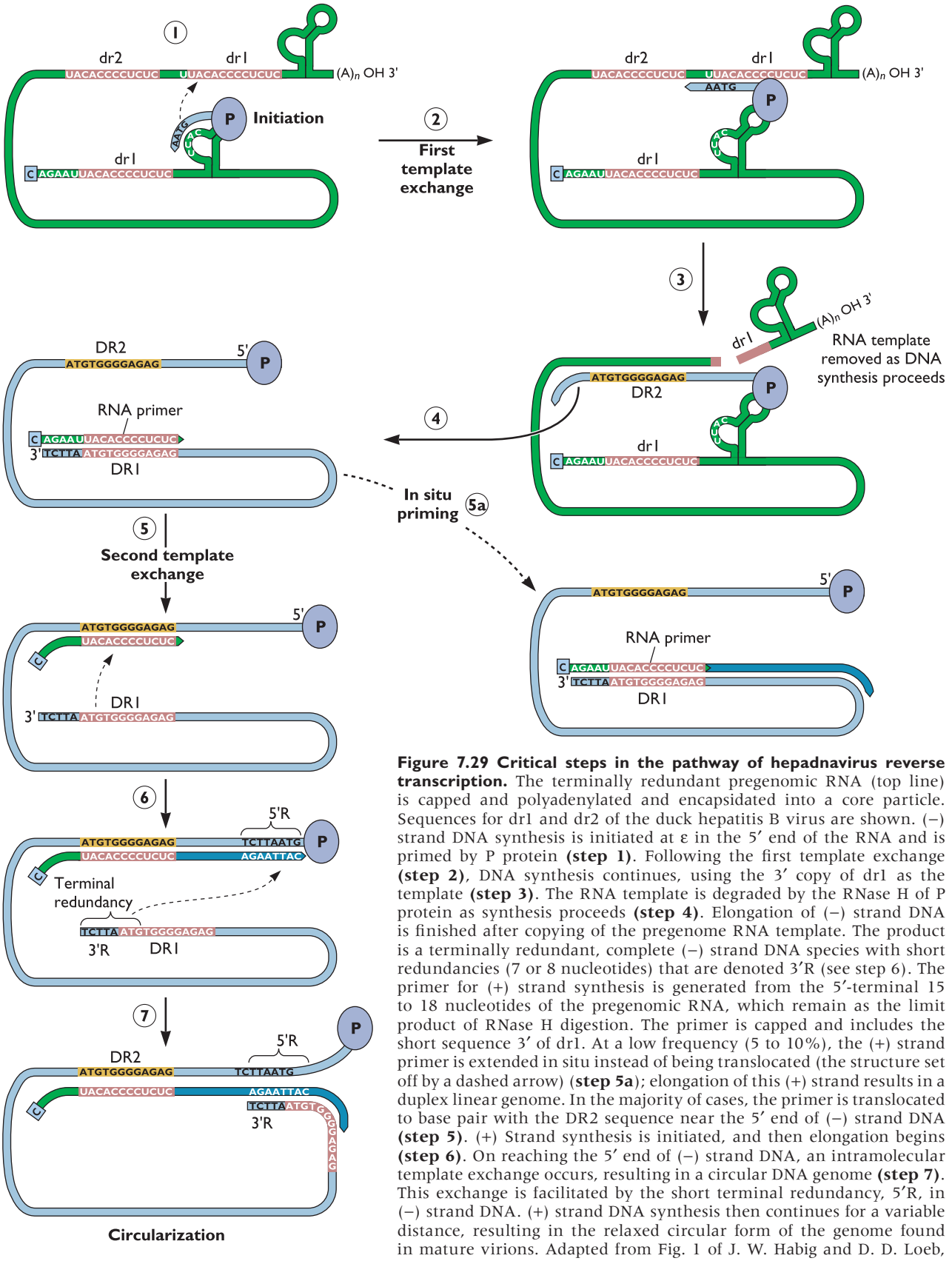


Figure 7.29 Critical steps in the pathway of hepadnavirus reverse transcription. The terminally redundant pregenomic RNA (top line) is capped and polyadenylated and encapsidated into a core particle. Sequences for dr1 and dr2 of the duck hepatitis B virus are shown. (−) strand DNA synthesis is initiated at ε in the 5′ end of the RNA and is primed by P protein **(step 1)**. Following the first template exchange **(step 2)**, DNA synthesis continues, using the 3′ copy of dr1 as the template **(step 3)**. The RNA template is degraded by the RNase H of P protein as synthesis proceeds **(step 4)**. Elongation of (−) strand DNA is finished after copying of the pregenome RNA template. The product is a terminally redundant, complete (−) strand DNA species with short redundancies (7 or 8 nucleotides) that are denoted 3′R (see step 6). The primer for (+) strand synthesis is generated from the 5′-terminal 15 to 18 nucleotides of the pregenomic RNA, which remain as the limit product of RNase H digestion. The primer is capped and includes the short sequence 3′ of dr1. At a low frequency (5 to 10%), the (+) strand primer is extended in situ instead of being translocated (the structure set off by a dashed arrow) **(step 5a)**; elongation of this (+) strand results in a duplex linear genome. In the majority of cases, the primer is translocated to base pair with the DR2 sequence near the 5′ end of (−) strand DNA **(step 5)**. (+) Strand synthesis is initiated, and then elongation begins **(step 6)**. On reaching the 5′ end of (−) strand DNA, an intramolecular template exchange occurs, resulting in a circular DNA genome **(step 7)**. This exchange is facilitated by the short terminal redundancy, 5′R, in (−) strand DNA. (+) strand DNA synthesis then continues for a variable distance, resulting in the relaxed circular form of the genome found in mature virions. Adapted from Fig. 1 of J. W. Habig and D. D. Loeb, *J. Virol.* **76:**980–989, 2002, with permission.

the sequence at this end is complementary to the short, initial product, it is not unique in the pregenomic RNA (Fig. 7.26). Furthermore, mutation of the normal acceptor sequence leads to the synthesis of (–) strands with 5′ ends that map to other sites in the vicinity of the 3′ dr1 that apparently can serve as alternative acceptors. A deletion analysis with the woodchuck virus has suggested that a region 1 kb upstream of the 3′ dr1 includes a signal that specifies the acceptor site. It also seems likely that selection of the normal site is guided by the specific organization of pregenomic RNA in core particles. P protein remains covalently attached to the 5′ end of the (–) strand during the first template exchange and, as noted previously, through **all** subsequent steps.

Elongation and RNase H digestion of the RNA template. Following the first template exchange, (–) strand DNA synthesis continues all the way to the 5′ end of the pregenomic RNA template (Fig. 7.29, steps 3 and 4). Because synthesis is initiated in the 3′ dr1, a short repeat of 7 to 8 nucleotides (3′R) is produced at the end of this elongation step when the 5′ dr1 sequence is copied (Fig. 7.29, step 4). The reader should note this fact, as it will become important in a later step of viral DNA synthesis. The RNA template is degraded by the RNase H activity of P protein as (–) strand synthesis proceeds. Unlike the retroviral RNase H products, none of these hepadnaviral RNAs are used as primers for (+) strand DNA synthesis (Table 7.2). The final product of RNase H digestion is a short RNA molecule, corresponding to the capped end of the pregenomic RNA, which includes the 5′ dr1, and will serve as the primer for (+) strand DNA synthesis. It is noteworthy that (+) strand DNA synthesis can begin only after completion of (–) strand DNA synthesis, because such completion is required for formation of this primer. This 15- to 18-nucleotide product is similar in length to the limit product of polymerase-associated RNase H digestion by human immunodeficiency virus type 1 RT. In the human retroviral protein, such a product provides a measure for the distance between the polymerase and RNase H active sites on the enzyme (Fig. 7.13); a similar relationship may apply to hepadnaviral P protein (Table 7.2).

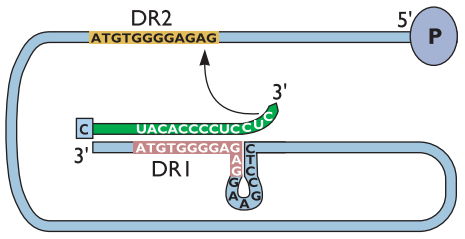
The short, capped RNA primer could also be used *in situ* for synthesis of the (+) strand of DNA (Fig. 7.29, step 5a). Indeed, in 5 to 10% of reverse transcription reactions, such priming does take place, and double-stranded linear genomes are produced. These linear DNAs can be converted to covalently closed circular DNA. However, most of these circular molecules are formed by base-pairing-independent recombination of their linear ends, a process that creates deletions and is therefore mutagenic. What happens most of the time is a translocation of the capped RNA primer to another location on the (–) strand DNA template, DR2 (Fig. 7.29, step 5).

Translocation of the primer for (+) strand DNA synthesis. Translocation of the primer for (+) strand DNA synthesis is likely to be facilitated by the homology between DR1 and DR2 (Fig. 7.29, step 5). The capped RNA primer, which includes dr1 sequences, can anneal to both. How the primer is induced to dissociate from DR1 and associate with DR2 is another mystery. A small hairpin structure that includes the 5′ end of DR1 in the (–) strand of duck hepatitis B virus DNA appears to contribute to the translocation by inhibiting in situ priming and, perhaps, facilitating annealing of the capped RNA fragment with the complementary sequence in DR2 (Fig. 7.30). As in the first template exchange, specific organization of the template in the core particles is thought to be required for this translocation to occur. Furthermore, if the potential for the primer to hybridize with DR2 is disrupted by mutation, the pathway leading to formation of linear duplex DNA molecules predominates.

The (+) strand synthesis primed by the translocated capped hepadnaviral RNA primer is similar to that which produces the strong-stop DNAs in retroviral reverse transcription. The (+) strand DNA synthesis begins near the 5′ end at DR2 and soon runs out of (–) strand template. As in the retroviral case, this problem is solved by a second template exchange, in this instance facilitated by the short repeat, 5′R, produced during synthesis of the (–) strand (Fig. 7.29, step 6).

The second template exchange creates a circle. The structural requirements for the next step in hepadnaviral reverse transcription must be complicated, because P protein is still attached to the 5′ end of the (–) strand, which needs to be displaced to allow a template transfer. Studies in which genomes of heron and duck hepatitis viruses

Figure 7.30 Model for (+) strand priming. Formation of a putative hairpin in the (–) strand DNA template displaces the 3′ end of the capped RNA fragment, preventing *in situ* priming and facilitating annealing with the homologous sequence in DR2. The ensuing translocation of the RNA primer allows initiation of (+) strand DNA synthesis. Adapted from Fig. 2 of J. W. Habig and D. D. Loeb, *J. Virol.* **76:**980–989, 2002, with permission.



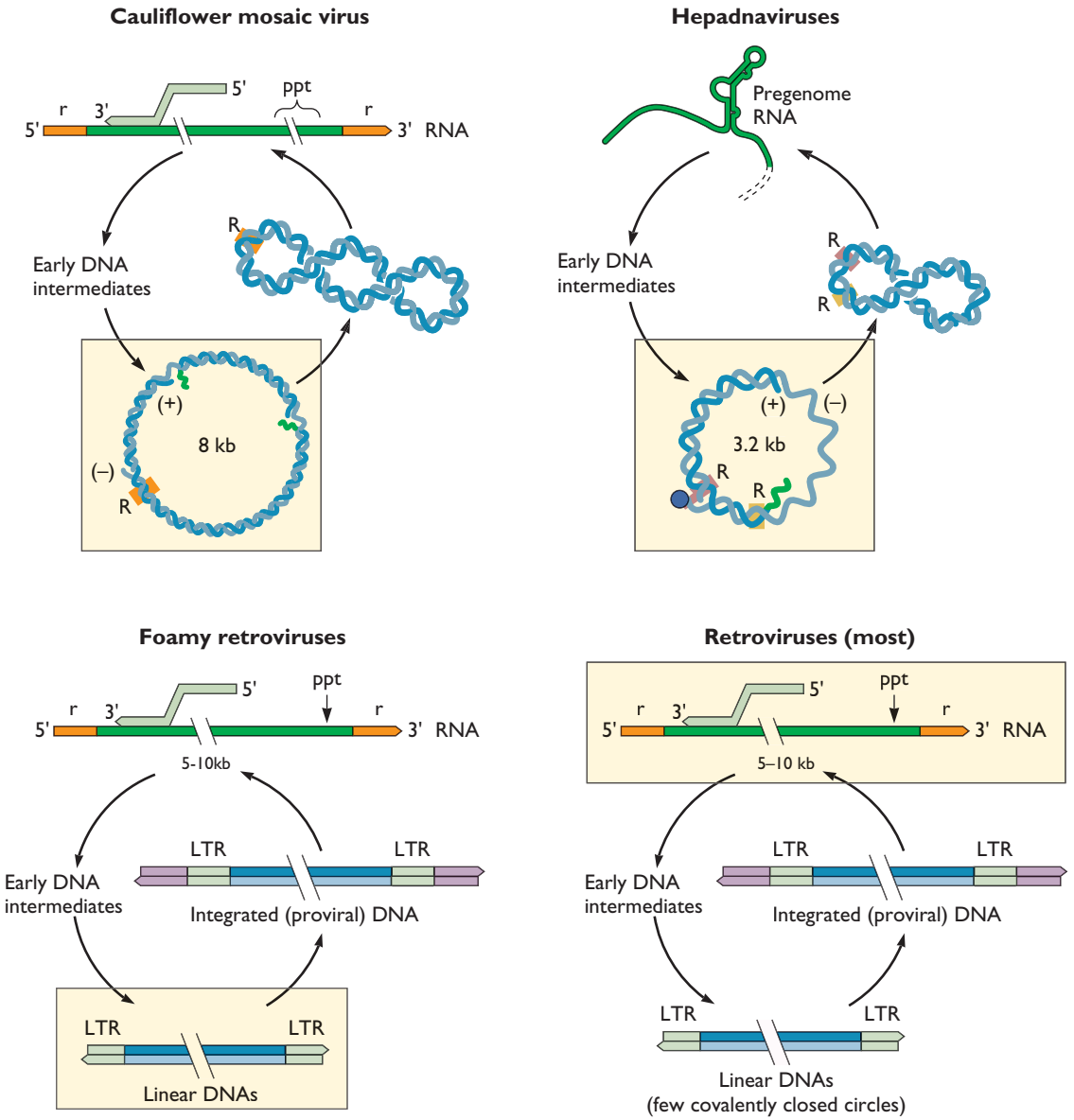


Figure 7.31 Replication cycles of cauliflower mosaic virus, hepadnaviruses, and retroviruses. The double-stranded DNA circle found in cauliflower mosaic virus particles contains three interruptions. At each interruption there is a short 5′ overlap of DNA as if formed by strand displacement synthesis. Ribonucleotides are often found attached to the 5′ ends. The (–) strand starts with either a ribo- or a deoxyriboadenosine. The 5′ ends of the (+) strand each contain 8 to 10 purine-rich matches to the viral DNA at the same location, suggesting a primer function. r, short sequence at both ends of viral RNA; R, same sequence in DNA. Shaded boxes show the nucleic acid (genomes) encapsidated in the virions of each virus, which represent different components in analogous pathways. Updated from H. E. Varmus, *Nature* **304:**116–117, 1983, with permission.

were exchanged indicate that, in addition to DR1 and DR2, *cis* interactions among other sequences, at the ends and in a central region of (–) strand DNA, are important in this final step. It has been suggested that the simultaneous interaction of the central region with both ends may hold the termini in a position that facilitates both (+) strand primer translocation and the second template exchange. However, even with such "help," it is difficult to envision how a single protein accommodates all three DNA ends at once, and catalyzes polymerization while still attached to

one of them. Nevertheless, this exchange does occur with high efficiency in vivo, and subsequent incomplete elongation of the (+) strand produces the partially duplex circle that comprises virion DNA (Fig. 7.29, step 7).

It is not clear what causes premature termination during synthesis of the (+) strand of hepadnaviral DNA. Synthesis of (+) strand DNA is affected by mutations in C protein, consistent with the idea that this protein is somehow involved in this reaction. It has been proposed that DNA synthesis induces a change in the outer surface of the core, and that envelopment is regulated by interaction of the envelope proteins with the altered core. Once the cores are enveloped, DNA synthesis stops, presumably because dNTP substrates can no longer enter the particle.

In mammalian hepadnaviruses, the (+) strand is only about half as long as the (−) strand, but in avian hepadnaviruses, it is nearly full length. However, in both cases the primer is **not** removed by the P protein RNase H, as occurs during retroviral replication. Furthermore, strand displacement synthesis, an essential activity of retroviral RT, is also not observed with P protein (Table 7.2).

Perspectives

The description of critical steps in hepadnaviral reverse transcription reveals interesting points of similarity to and contrast with retroviral systems, as summarized in Table 7.2. Amino acid sequences and functions are conserved among retroviral RT and hepadnaviral P proteins, and both enzymes use terminal nucleic acid repeats to mediate template exchanges. However, the mechanisms by which their templates are reverse transcribed are quite distinct. Differences in the form and function of the final products of the two pathways are especially striking. A relaxed DNA circle with overlapping 5′ ends is an intermediate in the formation of the final product of retroviral reverse transcription, a linear duplex DNA. Repair of this relaxed-circle intermediate is an aberrant reaction, and the covalently closed circle formed is a dead-end product. In contrast, linear DNA is an aberrant product of hepadnaviral reverse transcription, and the covalently closed circle is the functional form for transcription.

The single-cell replication cycles of retroviruses and hepadnaviruses are, in a sense, permutations of one another. In comparing them and the unconventional foamy viruses (Box 7.7), it is also instructive to consider cauliflower mosaic virus, a plant retroid virus that seems to combine some features of both animal viruses during reverse transcription (Fig. 7.31). This plant virus has a relaxed circular DNA genome and directs synthesis of a covalently closed episomal form, but its reverse transcription and priming mechanisms are quite analogous to those of retroviruses and retrotransposons. On the other hand, as with hepadnaviruses, all, or remnants of, the RNA primers remain attached to the 5′ ends of cauliflower mosaic virus DNA. As suggested by the family tree of retroelements (Fig. 7.14), retroid viruses represent a continuum in evolution and remind us of the varied combinations of strategies that exist in nature for replicating viral genomes and related genetic elements.

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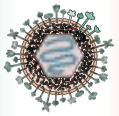
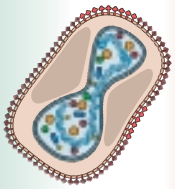




8

Transcription Strategies: DNA Templates

It is possible that nature invented DNA for the purpose of achieving regulation at the transcriptional rather than at the translational level. The level of control in small DNA viruses and large RNA viruses should be interesting to study.

A. CAMPBELL, 1967

Introduction

During replication of viruses with DNA genomes, viral messenger RNA (mRNA) synthesis must precede production of proteins. In most cases, this step is accomplished by the host cell enzymes that produce cellular mRNA (Table 8.1). The proviral DNA of retroviruses is also transcribed by this cellular enzyme. The signals that control expression of the genes of these viruses are therefore similar to those of cellular genes. In fact, much of our current understanding of the mechanisms of cellular transcription stems from study of transcription of viral DNA templates. In contrast, the genomes of poxviruses are transcribed by a viral DNA-dependent RNA polymerase and accessory viral proteins, which control the recognition of viral promoters.

The expression of viral genes in a strictly defined, reproducible sequence is a hallmark of cells infected by DNA viruses. In general, enzymes and regulatory proteins are made during the initial period of infection, whereas structural proteins of virions are made only after viral DNA synthesis begins. Such orderly gene expression is primarily the result of transcriptional regulation by viral proteins. This pattern is quite different from the continual expression of all viral genes characteristic of the infectious cycles of many RNA viruses (Chapter 6). As discussed in this chapter, the elucidation of the molecular strategies that ensure sequential transcription of the genes of DNA viruses, one of the primary goals of studies of DNA viruses, has identified a number of common mechanisms executed in virus-specific fashion. As a collateral dividend, we have gained insights into the cellular mechanisms that control progression through the cell cycle.

Properties of Cellular RNA Polymerases That Transcribe Viral DNA

Eukaryotes Have Three Transcriptional Systems

A general feature of eukaryotic cells is the division of transcriptional labor among three DNA-dependent RNA polymerases. These enzymes, designated

Table 8.1 Strategies of transcription of viral DNA templates

Origin of transcriptional components	Virus
Host only	Simple retroviruses
Host plus one viral protein	
The viral protein transcribes late genes	Bacteriophages T3 and T7
The viral protein regulates transcription	Complex retroviruses, parvoviruses, papillomaviruses, polyomaviruses
Host plus several viral proteins that act sequentially to stimulate transcription of particular sets of viral genes.	Adenoviruses, bacteriophage T4, herpesviruses
Viral	Poxviruses

RNA polymerases I, II, and III, synthesize different kinds of cellular RNA (Table 8.2). RNA polymerase II makes mRNA species or their precursors, as well as the precursors of small, regulatory RNA molecules (see Chapter 10). RNA polymerases I and III produce stable RNAs, such as ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs), respectively. Synthesis of the latter "housekeeping" RNAs must be adjusted to match the rates of cell growth and division. But regulation of mRNA synthesis is crucial for orderly development and differentiation in eukaryotes, as well as for the responses of cells to their environment. The evolution of RNA polymerases with distinct transcriptional responsibilities appears to be a device for maximizing opportunities for regulation of mRNA synthesis, while maintaining a constant and abundant supply of the RNA species essential for the metabolism of all cells.

The three eukaryotic RNA polymerases transcribe different classes of genes and must meet different regulatory requirements. Nevertheless, several of the 12 to 16 subunits of these large enzymes are identical, and others are related in sequence to one another, or to subunits of bacterial RNA polymerases. Such conservation of sequence can be attributed to the common biochemical capabilities of the enzymes. These activities include binding of ribonucleoside triphosphate substrates, binding to template DNA and to product RNA, and catalysis of phosphodiester bond formation. The structure of a 10-subunit form of yeast RNA polymerase II revealed that the organization of its active centers is similar to that of smaller DNA-dependent RNA polymerases, as well as of enzymes that make DNA from DNA or RNA templates (Fig. 6.4).

Table 8.2 Eukaryotic RNA polymerases synthesize different classes of cellular and viral RNA

Enzyme	RNAs synthesized[a]	
	Cellular	Viral
RNA polymerase I	Pre-rRNA	None known
RNA polymerase II	Pre-mRNA, pre-miRNA, snRNAs	Pre-mRNA, mRNA, pre-miRNA, HDV genome RNA
RNA polymerase III	Pre-tRNAs, 5S rRNA, U6 snRNA	Ad2 VA-RNAs, EBV EBER RNAs

[a]snRNA, small nuclear RNA; miRNA, micro-RNA; Ad2, adenovirus type 2; EBV, Epstein-Barr virus; HDV, hepatitis delta virus.

Transcription of cellular and viral genes requires not only template-directed synthesis of RNA, but also correct interpretation of DNA punctuation signals that mark the sites at which transcription must start and stop. **Initiation of transcription** comprises recognition of the point at which copying of the DNA should begin, the **initiation site**, and synthesis of the first few phosphodiester bonds in the RNA. During the **elongation** phase, nucleotides are added rapidly to the 3′ end of the nascent RNA, as the transcriptional machinery reads the sequence of a gene. When termination sites are encountered in the DNA, both the RNA product and the RNA polymerase are released from the template. Purified RNA polymerases I, II, and III perform the elongation reactions *in vitro* but, despite their complexity, are incapable of specific initiation of transcription without the assistance of additional, polymerase-specific proteins.

Cellular RNA Polymerases II and III Transcribe Viral Templates

The transcription of viral DNA genomes to produce viral mRNAs (or pre-mRNA) is analogous to the expression of many bacteriophage genes by bacterial RNA polymerases (Table 8.1). However, some animal viral DNA genomes also encode small, noncoding RNAs that are made by RNA polymerase III. This phenomenon was initially observed in human cells infected by adenovirus, but RNA polymerase III transcription units are also present in the genomes of other viruses. The cellular transcriptional machinery can also contribute to viral reproduction in unique ways. The acquisition of 5′ caps and primers for viral mRNA synthesis from cellular pre-mRNAs or mRNAs in cells infected by certain (−) strand RNA viruses (Chapter 6) is a dramatic example of such virus-specific mechanisms. Perhaps even more remarkable is transcription of an RNA template by RNA polymerase II to produce hepatitis delta satellite virus genomes and mRNA.

Some Viral Genomes Must Be Converted to Templates for Transcription

All viral DNA molecules that are transcribed by cellular RNA polymerases must enter the infected cell nucleus.

Table 8.3 Prerequisites for transcription of viral genes by RNA polymerase II

Requirement	Virus	Form of the intracellular template
Entry of genome into nucleus	Adenoviruses	Linear double-stranded DNA
	Papillomavirus, polyomaviruses	Closed, circular, double-stranded DNA organized by cellular nucleosomes
Entry of viral genome plus a virion protein (e.g., VP16) into the nucleus	Herpesviruses	Circular double-stranded DNA formed by ligation of genomic DNA; associated with cellular histones
Production of double-stranded DNA from genomic DNA and entry into nucleus	Hepadnaviruses	Closed-circular DNA synthesized from genomic molecules with a gap in the (+) strand and a nick in the (−) strand; organized into nucleosomes
	Parvoviruses	Linear double-strand DNA synthesized from single-stranded DNA genome during replication
Reverse transcription of RNA genome, entry of DNA product into nucleus, and integration into cellular genome	Retroviruses	Linear double-stranded DNA integrated into the cellular genome; associated with cellular nucleosomes

However, there is considerable variation in the reactions needed to produce templates that can be recognized by the cellular transcriptional machinery (Table 8.3). Some of the viral genomes, like that of the host cell, are double-stranded DNA molecules that can be transcribed as soon as they reach the nucleus. Transcription of specific genes is therefore the first biosynthetic reaction in cells infected by adenoviruses, herpesviruses, papillomaviruses, and polyomaviruses. Although viral DNA synthesis is not required prior to transcription, this process dramatically increases the concentration of templates and, as we shall see, is crucial for transcription of specific viral genes.

Other viral DNA genomes must be converted from the form in which they enter the cell to double-stranded molecules that serve as transcriptional templates (Table 8.3). The hepadnaviral genome is an incomplete circular DNA molecule with a large gap in one strand (Appendix, Fig. 3B) that is repaired by cellular enzymes to form a fully double-stranded DNA molecule. During the initial period of the hepadnaviral infectious cycle, the supply of templates is supplemented when pregenomic RNA transcribed by RNA polymerase II is copied into double-stranded DNA by the viral DNA polymerase. The prerequisites for expression of retroviral genetic information are even more demanding, for the (+) strand RNA genome must be both converted into viral DNA and integrated into the cellular genome, following import into the nucleus. Reverse transcription creates both an appropriate double-stranded DNA template and the signals needed for its recognition by components of the cellular transcriptional machinery (Chapter 7).

The cellular templates for transcription by RNA polymerase II are DNA sequences in chromatin, which contains the conserved histones and many other proteins. The fundamental structural unit of chromatin is the nucleosome, which comprises some 140 bp of DNA wrapped around an octamer containing two copies each of histones H2A, H2B, H3, and H4. As the organization of DNA into nucleosomes both blocks recognition of specific sequences and imposes barriers to transcriptional elongation, many of the proteins that regulate transcription function by overcoming such obstacles. Because they are integrated into the cellular genome, the proviral DNA templates for retroviral transcription are organized into chromatin indistinguishable from that of the host cell. The DNA genomes of papillomaviruses and polyomaviruses enter cells as "minichromosomes" in which the viral DNA is bound to nucleosomes. Furthermore, the results of recent studies indicate that histones are associated with herpesviral genomes soon after viral DNA molecules enter infected cell nuclei (Box 8.1). Transcription of viral DNA templates associated with histones therefore appears to be a common phenomenon.

Transcription by RNA Polymerase II

Accurate initiation of transcription by RNA polymerase II is directed by specific DNA sequences located near the site of initiation and called the **promoter** (Fig. 8.1). The promoter and the set of DNA sequences that control transcription make up the **transcriptional control region.** These control sequences of DNA viruses and retroviruses were among the first to be examined experimentally. For example, the human adenovirus type 2 major late promoter was the first from which accurate initiation of transcription was reconstituted *in vitro* (Box 8.2). Subsequently, the study of viral transcription yielded fundamental information about control signals, and the mechanisms by which RNA polymerase II transcription is initiated and regulated.

Biochemical studies of model transcriptional control regions, such as the adenoviral major late promoter, established that initiation of transcription by RNA polymerase II is a multistep process. The initiation reactions include promoter recognition, formation of an open initiation

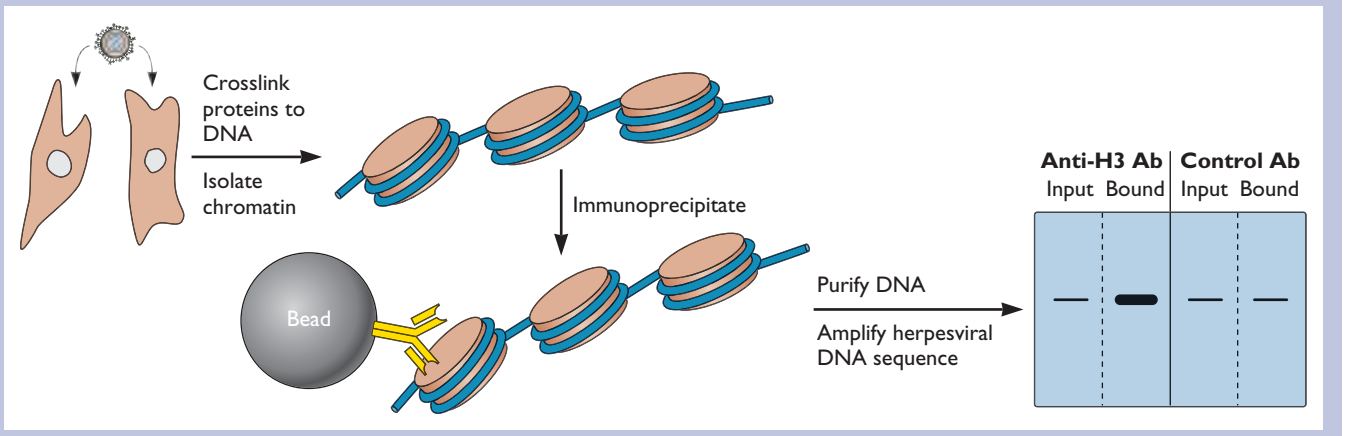
BOX 8.1 METHODS

Association of histones and other proteins with DNA in vivo: *the chromatin immunoprecipitation assay*

The chromatin immunoprecipitation (ChIP) assay is widely used to investigate the association of specific proteins with viral or cellular DNA within cells. Proteins are initially cross-linked to DNA by exposure of intact cells to formaldehyde; in this example, cells infected by herpes simplex virus type 1 are used. Protein-DNA complexes are then isolated after the DNA has been fragmented by sonication or enzymatic digestion. DNA fragments bound to the protein of interest are isolated by immunoprecipitation with antibodies against that protein. Enrichment for herpesviral DNA in the immunoprecipitate is then assessed by PCR amplification of specific sequences.

Application of this assay to herpes simplex virus type 1-infected cells has demonstrated that an immediate-early, an early, and a late gene are associated with histone H3 throughout the infectious cycle. However, nucleosomes do not organize viral DNA into a regular structure like that of cellular chromatin, in which the histone octamers are spaced at regular intervals on DNA.

Kent, J. R., P.-Y. Zeng, D. Atanasice, N. W. Fraser, and S. L. Berger. 2004. During lytic infection herpes simplex virus type 1 is associated with histones bearing modifications that correlate with active transcription. *J. Virol* **78:**10178–10186.



complex in which the two strands of the DNA template in the vicinity of the initiation site are unwound, and promoter clearance, movement of the transcribing complex away from the promoter (Fig. 8.2). At least 40 proteins, which comprise RNA polymerase II itself and auxiliary initiation proteins, are needed to complete the intricate process of initiation. Our understanding of the functions of these proteins and of the DNA sequences that control initiation of transcription is based largely on *in vitro* systems or simple assays for detecting gene expression within cells (Box 8.3). Application of these methods has identified a very large number of transcriptional control sequences. Fortunately, all of them can be assigned to one of the three functionally distinct regions identified in Fig. 8.1.

Core promoters of viral and cellular genes contain all the information necessary for recognition of the site of initiation of assembly of precisely organized **preinitiation complexes** (Box 8.4). These assemblies contain RNA polymerase II and a common set of general initiation proteins that begin transcription from the appropriate sites. A hallmark of many core RNA polymerase II promoters is the presence of a TA-rich TATA sequence 20 to 35 bp upstream of the site of initiation (Fig. 8.1 and 8.3A and B). This sequence, which was initially thought to be a definitive feature of core promoters, is recognized by the TATA-binding protein (Tbp) (Box 8.4). Short sequences, termed **initiators**, which specify accurate (but inefficient) initiation of transcription in the absence of any other promoter sequences, are also commonly found (Fig. 8.1 and 8.3). Either an initiator or an appropriately positioned TATA element can be sufficient to establish specific initiation, but both may be present (Fig. 8.3).

Many of the interactions among components of the transcriptional machinery take place before a promoter is encountered: RNA polymerase II is present in cells in extremely large assemblies that contain the initiation proteins, as well as other proteins that are essential for transcription, or that participate in regulation. One such multisubunit required for regulation is the mediator. Such assemblies, termed **holoenzymes**, appear to be poised to

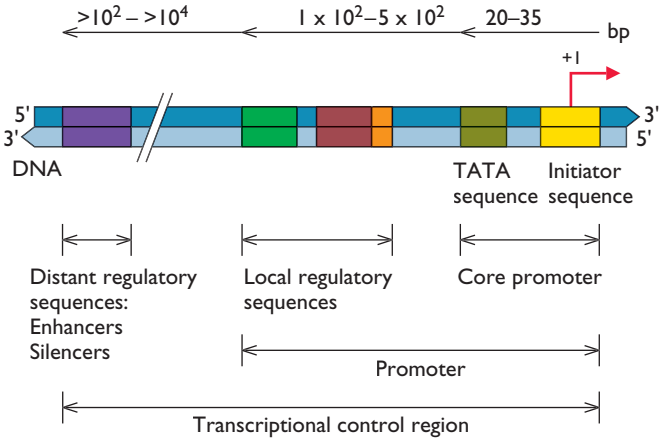


Figure 8.1 RNA polymerase II transcriptional control elements. The site of initiation is represented by the red arrow drawn in the direction of transcription on the nontranscribed DNA strand, a convention used throughout this text. The core promoter comprises the minimal sequence necessary to specify accurate initiation of transcription. The TATA sequence is the binding site for TfIId (Box 8.4), and the initiator is a sequence sufficient to specify initiation at a unique site. The activity of the core promoter is modulated by local regulatory sequences typically found within a few hundred base pairs of the initiation site. The location of these sequences upstream of the TATA sequence as shown is common, but such sequences can also lie downstream of the initiation site (see, e.g., Fig. 8.3). Distant regulatory sequences that stimulate (enhancers) or repress (silencers) transcription are present in a large number of transcriptional control regions.

initiate transcription as soon as they are recruited to a promoter.

Regulation of RNA Polymerase II Transcription

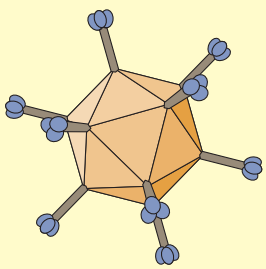
Numerous patterns of gene expression are necessary for eukaryotic life: some RNA polymerase II transcription units must be expressed in all cells, whereas others are transcribed only during specific developmental stages, or in specialized differentiated cells. Many others must be maintained in a silent ground state, from which they can be activated rapidly in response to specific stimuli, and to which they can be returned readily. Transcription of viral genes is also regulated during the single-cell life cycles of most of the viruses considered in this chapter. Large quantities of viral proteins for assembly of progeny virions must be made within a finite (and often short) infectious cycle. Consequently, some viral genes must be transcribed at high rates. As noted previously, in many cases viral genes are transcribed in a specific temporal sequence. Such regulated transcription is achieved in part by means of cellular control mechanisms, for example, signal transduction cascades that transmit specific environmental stimuli to the transcriptional machinery, or cellular proteins that repress transcription. However, viral proteins are generally critical components of the circuits that establish orderly transcription of viral genes.

BOX 8.2

EXPERIMENTS

Mapping of a human adenovirus type 2 initiation site and accurate transcription in vitro

When cellular RNA polymerase II was identified in 1969, investigators had access to only preparations of total cellular DNA. Furthermore, nothing was known about the organization of eukaryotic transcription units. The genomes of smaller DNA viruses, such as simian virus 40 and human adenovirus type 2, were therefore perceived as a valuable resource for investigation of mechanisms of transcription, a view that inspired many investigators to enter virology. Indeed, it was detailed information about a particular adenoviral transcription unit that finally allowed biochemical studies of the mechanism of initiation. In 1978, the site at which major late transcription begins was mapped precisely, by determining the sequence of the 5′ end of the RNA transcript. This knowledge was exploited to develop a simple assay for accurate initiation of transcription, the "runoff" assay (Box 8.3). Purified RNA polymerase II produced no specific transcripts in the runoff assay, but unfractionated nuclear extracts of human cells were shown to contain all the components necessary for accurate initiation of transcription.



Weil, P. A., D. S. Luse, J. Segall, and R. G. Roeder. 1979. Selective and accurate initiation of transcription at the Ad2 major late promotor in a soluble system dependent on purified RNA polymerase II and DNA. *Cell* **18:**469–484.

Ziff, E. B., and R. M. Evans. 1978. Coincidence of the promoter and capped 5′ terminus of RNA from the adenovirus 2 major late transcription unit. *Cell* **15:**1463–1475.

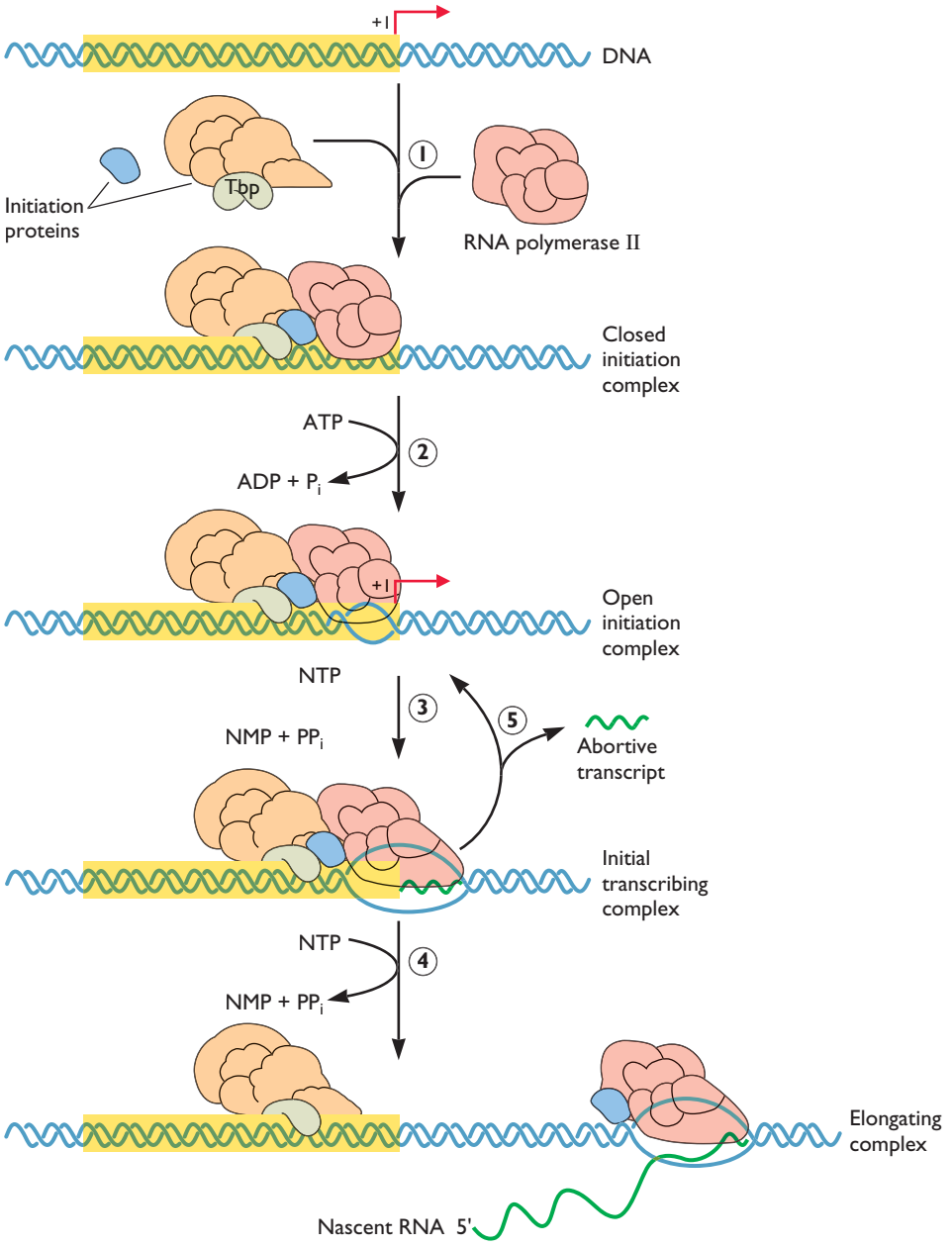
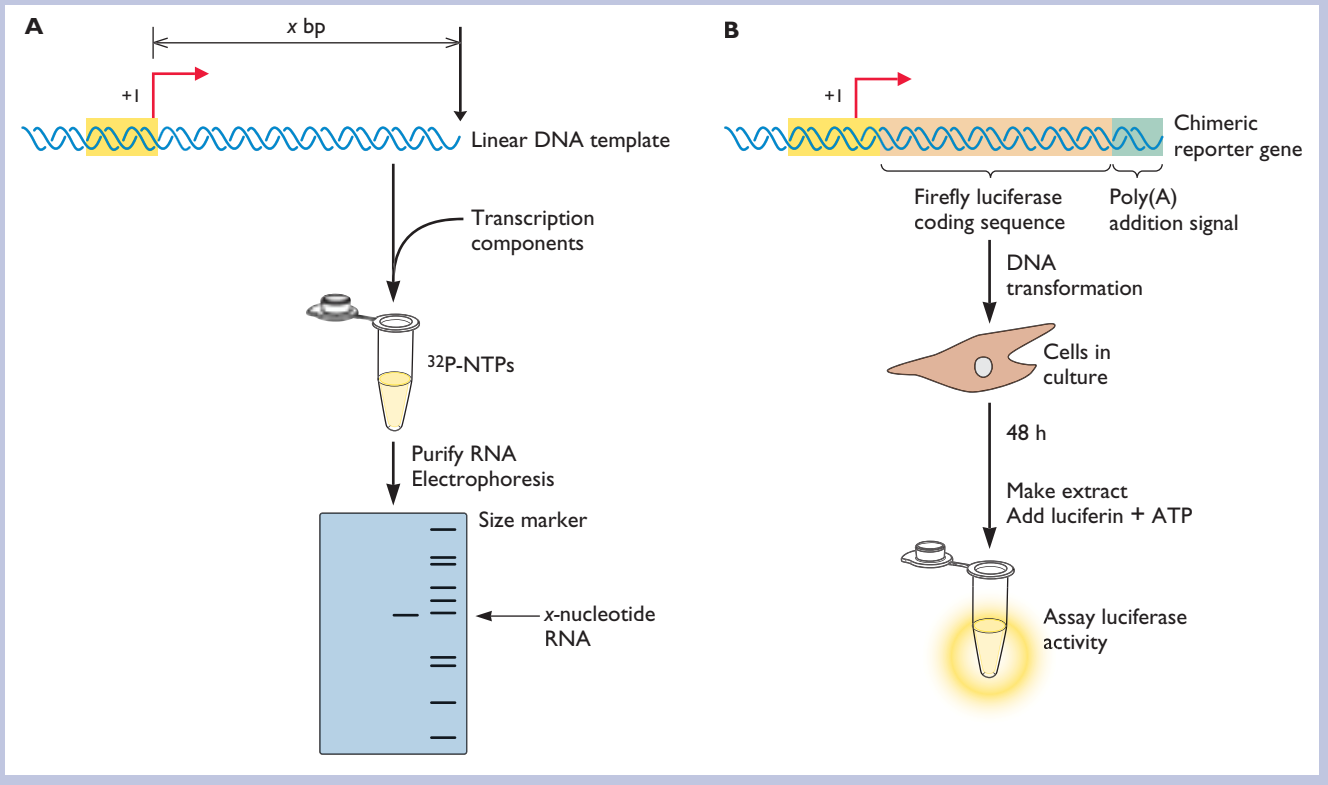


Figure 8.2 Initiation of transcription by RNA polymerase II. Assembly of the closed initiation complex (step 1) is followed by unwinding of the DNA template in the region spanning the site of initiation (step 2). RNA polymerase II then synthesizes an initial short transcript (less than 10 to 15 nucleotides) by template-directed incorporation of nucleotides (step 3). The initial transcribing complex is thought to be conformationally strained, because RNA polymerase II remains in contact with promoter-bound initiation proteins as it synthesizes short RNAs. The severing of these contacts allows the transcribing complex to escape from the promoter and proceed with elongation (step 4). This promoter clearance step is often inefficient, with abortive initiation (step 5) predominating. In the latter process, initial transcripts are cleaved and released, reforming the open initiation complex. The elongating transcriptional complex contains some but not all of the proteins that form the preinitiation complex, as well as proteins that stimulate elongation.

BOX 8.3 METHODS

Assays for the activity of RNA polymerase II promoters

(A) *In vitro* transcription assay. In this simple assay, linear DNA templates are prepared by restriction endonuclease cleavage (black arrow), a known distance, *x* bp, downstream of the initiation site (+1). When the template is incubated with the transcriptional machinery and nucleoside triphosphate (NTP) substrates, transcription initiated at position +1 continues until the transcribing complex "runs off" the linear template. Specific transcription is therefore assayed as the production of ^{32}P-labeled RNA *x* nucleotides in length. This runoff transcription assay is convenient and can be used to assess both specificity and efficiency of transcription. However, it can be applied only to linear DNA templates and often suffers from high background, for example, nonspecific initation of transcription at the ends of the template. These problems can be circumvented by the use of circular templates and an indirect assay for specific transcripts, for example, copying of an unlabeled transcript by reverse transcriptase to form a labeled, complementary DNA. Such *in vitro* transcription assays differ from transcription under normal intracellular conditions in several important ways. Transcription templates are typically provided as naked circular or linear DNA molecules, physical states that may not resemble those of cellular or viral genes within cells. Another important parameter that may be altered is the relative, as well as the absolute, concentrations of the proteins necessary for transcription. **(B)** Transient-expression assay. A segment of DNA containing the transcriptional control region of interest (yellow) is ligated to the coding sequence (orange) of an enzyme not synthesized in the recipient cells to be used (luciferase in this example), and RNA-processing signals, such as those specifying polyadenylation, shown by the green box at the end of the coding sequence. Plasmids containing such chimeric reporter genes are introduced into cells in culture by any one of several methods, including electroporation and incubation with synthetic vesicles containing the plasmid DNA. The proportion of cells that will take up foreign DNA varies with a number of parameters, including cell type. Within a cell that takes up the reporter gene, the DNA enters the nucleus, where the transcriptional control region directs transcription of chimeric RNA. The RNA is exported from the nucleus following processing, and translated on cytoplasmic polyribosomes. The activity of the luciferase enzyme is then assayed, generally 48 h after introduction of the reporter gene. Note that this indirect measure of transcription assumes that it is **only** the activity of the transcriptional control region that determines the concentration level of the enzyme. Alternatively, the concentration of the chimeric reporter RNA can be measured.



BOX 8.4 BACKGROUND

The RNA polymerase II closed initiation complex

The closed initiation complex is shown on a promoter that contains both a TATA and an initiator sequence (e.g., the adenovirus major late promoter). The TfIId protein contains a subunit that recognizes the TATA sequence (TATA-binding protein [Tbp]) and 8 to 10 additional subunits, termed Tbp-associated proteins (Tafs). X-ray crystal structures of DNA-bound Tbp, such as that of *Arabidopsis thaliana*, bound to the adenoviral major late TATA sequence shown in the inset, revealed that this protein induces sharp bending of the DNA. One popular hypothesis is that such bending facilitates interaction among proteins bound to local regulatory sequences located upstream of the TATA sequence and the basal transcriptional machinery. TfIId is required for transcription from all RNA polymerase II promoters. It can recognize those that lack TATA sequences (see, e.g., Fig. 8.3) by binding of a Taf to an initiator or an internal sequence, or it can be recruited to the promoter by interactions with proteins bound to specific sequences near the initiation site. The largest subunit of TfIIh supplies DNA-dependent ATPase and helicase activities essential for transcription. Two others are components of a kinase that can phosphorylate the C-terminal segment of the largest subunit of RNA polymerase II. The preinitiation complex assembles via an ordered pathway *in vitro*. In the cell, however, many of these proteins are associated with RNA polymerase II prior to encounter with a promoter.

Buratowski, S., S. Hahn, L. Guarente, and P. A. Sharp. 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell* **56:**549–561.

Kim, J. L., D. B. Nikolov, and S. K. Burley. 1993. Co-crystal structure of TBP recognizing the minor groove of a TATA element. *Nature* **365:** 520–527.

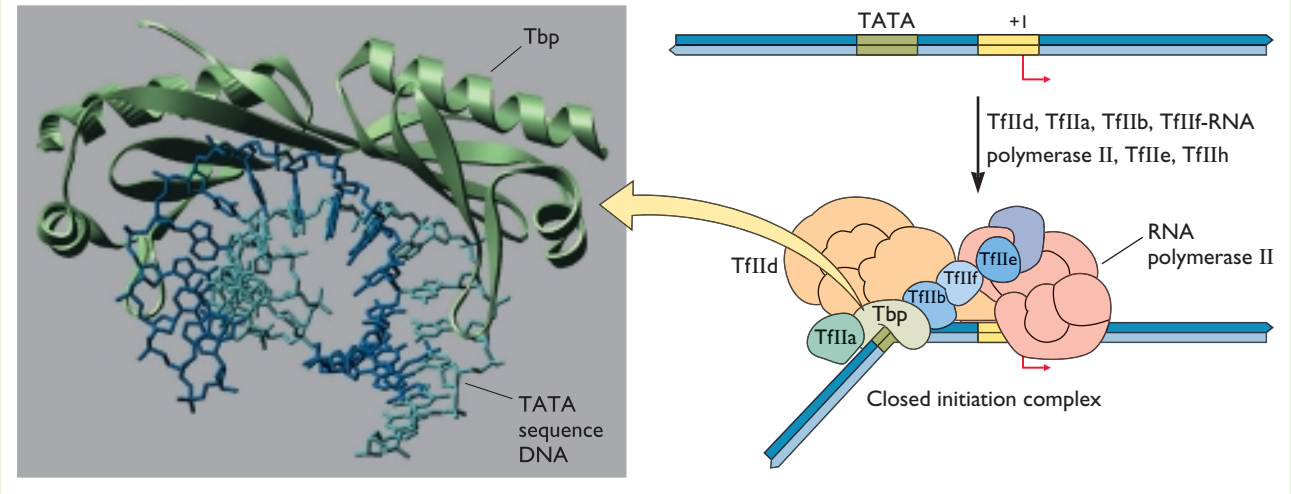
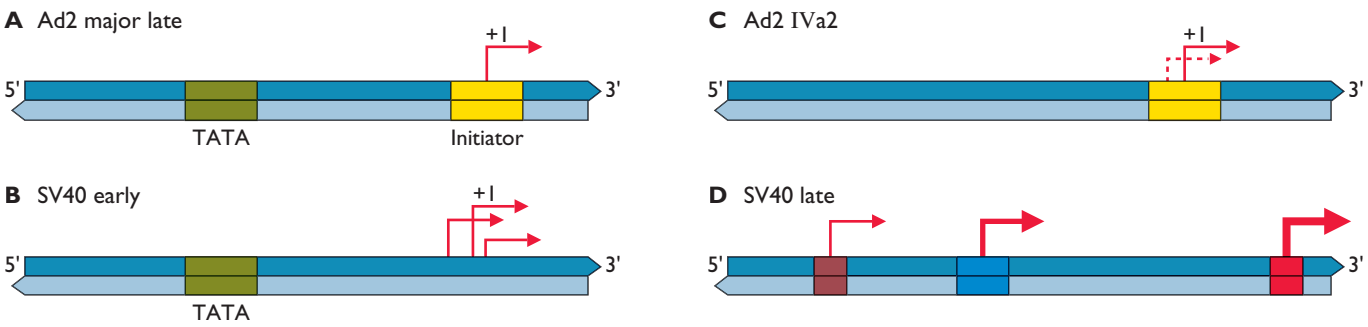


Figure 8.3 Variations in core RNA polymerase II promoter architecture. Variations in promoter architecture are illustrated using four viral promoters represented as in Fig. 8.1. The TATA or initiator sequences of the different promoters are not identical in DNA sequence. In the case of the simian virus 40 (SV40) late transcription unit, each of the sites of initiation is included within a DNA sequence resembling an initiator of another promoter. It has not been shown experimentally that all actually function as autonomous initiator sequences. The relative frequencies with which different initiation sites in a single promoter are used are indicated by the thickness of the red arrows. Ad2, adenovirus type 2.



Recognition of Local and Distant Regulatory Sequences

Both local and distant sequences (Fig. 8.1) can control transcription from core promoters. However, in many cases, local sequences are sufficient for proper transcriptional regulation. These local regulatory sequences arerecognized by sequence-specific DNA-binding proteins (Fig. 8.4), a property first demonstrated with the simian virus 40 early promoter. An enormous number of sequence-specific proteins that regulate transcription are now known, many first identified through analyses of viral promoters (Fig. 8.4). Unfortunately, the nomenclature applied to these regulatory proteins (Table 8.4) presents serious difficulties for both writer and reader, for it is unsystematic and idiosyncratic (Box 8.5).

Proper regulation of transcription of many viral and cellular genes also requires more distant regulatory sequences in the DNA template, which possess properties that were entirely unanticipated. The first example was discovered in the genome of simian virus 40 and was termed an **enhancer**, because it stimulated transcription to a large degree. Enhancers are defined by their position- and orientation-independent stimulation of transcription of homologous and heterologous genes over distances as great as 10,000 bp in the genome. Despite these unusual properties, enhancers are built with binding sites for the proteins that recognize local promoter sequences.

The Simian Virus 40 Enhancer: a Model for Viral and Cellular Enhancers

The majority of viral DNA templates described in this chapter contain enhancers of transcription that are recognized by cellular DNA-binding proteins. The simian virus 40 enhancer has been studied intensively, in part because it was the first such regulatory sequence to be identified. Its properties and mechanism of action are considered characteristic of many enhancers, whether of viral or cellular origin.

The simian virus 40 enhancer displays the hierarchical organization typical of these regulatory regions (Box 8.6). It is built from three units, termed enhancer elements, which are subdivided into smaller sequences recognized by DNA-binding proteins (Fig. 8.5). The DNA-binding proteins that interact with this viral enhancer are differentially produced in different cell types. For example, Nf-κb and certain members of the octamer-binding protein (Obp) family are specific to cells of lymphoid origin, and their binding sites are necessary for enhancer activity in these cells. Other elements of the simian virus 40 enhancer, such as the activator protein 1 (Ap-1)-binding sites, confer responsiveness to cellular signaling pathways. The combination of different DNA sequences ensures the activity of the enhancer, and therefore initiation of the viral infectious cycle, in many different cellular environments. This property is exhibited by several other viral enhancers, including those of avian retroviruses (Table 8.5). In contrast, some viral templates for RNA polymerase II transcription contain

Figure 8.4 Local regulatory sequences of three viral transcriptional control regions. The TATA sequences, initiator sequences, and sites of transcription initiation are depicted as in Fig. 8.3. The local regulatory sequences of each promoter, which are recognized by the cellular DNA-binding proteins listed (see Table 8.4), are drawn to the scale shown at the bottom, where +1 is the major initiation site. The black arrows below the Ad2 E2 early promoter indicate the orientation of the E2f-binding sites.

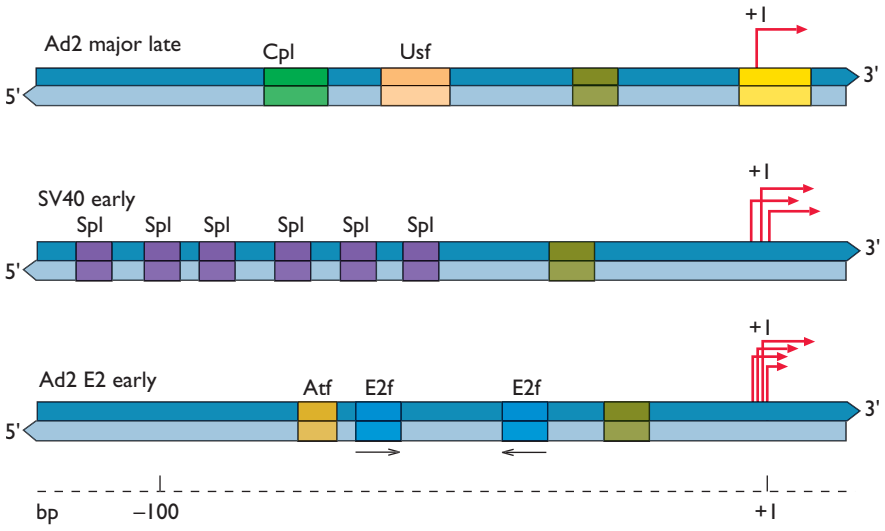


Table 8.4 Some cellular transcriptional regulators that recognize specific DNA sequences

Abbreviation	Full name	Characteristics	Viral or cellular promoters/enhancers recognized
Ap-1	Activator protein 1	Dimers of various basic-leucine zipper proteins, including c-Fos and c-Jun; regulated by dimerization and phosphorylation; mediate transcriptional responses to a variety of extracellular stimuli	Simian virus 40 enhancer
Atf-2	Activating transcription factor 2	Member of large Atf/Creb family; basic-leucine zipper proteins, related to Ap-1 family; mediate transcriptional responses to second-messenger cyclic AMP	Adenovirus type 2 E2E, E3, and E4 promoters; virus-responsive enhancer of human beta interferon gene.
E2f	E2 factor	Member of family of dimeric activators; activity regulated by association with Rb and Rb-related proteins; important in cell cycle regulation	Adenovirus type 2 E2E promoter
Ebps, e.g., A1/Ebp and C/Ebp-α	CCAAT/enhancer-binding proteins	Members of basic leucine-zipper family; C/Ebp α enriched in hepatocytes	Rous sarcoma virus enhancer; hepatitis B virus enhancer
Gata-3	GATA (binding protein) 3	Member of family defined by conserved Zn finger DNA-binding domain; expressed in T cells and specific neurons of the central nervous system	Human immunodeficiency virus type 1 enhancers
Ibp	Initiator-binding protein	Member of nuclear hormone receptor superfamily; binds via Zn fingers as dimers	Simian virus 40 major late promoter
Nf-κb	Nuclear factor κB	Dimer of p50-p65 Rel family proteins; activity regulated by sequestration in cytoplasm (Fig. 8.11)	Human immunodeficiency virus type 1 core enhancer; simian virus 40 enhancer
Nf-Il6	Nuclear factor for Il6 expression	Member of basic-leucine protein family	Rous sarcoma virus and human immunodeficiency virus type 1 enhancers
Oct-1	Octamer-binding protein 1	Ubiquitously expressed member of Obp family	Herpes simplex virus type 1 IE promoters, in complex with VP16 and Hcf (see the text)
Sp1	Stimulatory protein 1	Binds as monomer via Zn finger DNA-binding domain; first sequence-specific transcription factor identified	Simian virus 40 E promoter
Srf	Serum response factor	Member of Mads box DNA-binding domain family; mediates transcriptional response to serum growth factors	Rous sarcoma virus enhancer/ promoter; interacts with HTLV-1 Tax
Tef-1	Transcriptional enhancer factor 1	Complex DNA binding domains; no independent activation domain identified; requires limiting coactivator	Simian virus 40 enhancer; human papillomavirus type 16 E6/E7 promoter
Usf	Upstream stimulatory factor	Contains basic, helix-loop-helix and leucine zipper domains; binds as dimer	Adenovirus type 2 major late promoter

enhancers that are active only in a specific tissue (for example, hepatitis B virus DNA enhancer in liver cells) or only in the presence of inducers or viral proteins (Table 8.5).

The simian virus 40 enhancer is located within 200 bp of the transcription initiation site. More typically, enhancers are found thousands, or tens of thousands of base pairs up- or downstream of the promoters that they regulate. The most popular model of the mechanism by which these sequences exert remote control of transcription, the DNA-looping model, invokes interactions among enhancer-bound proteins and the transcriptional components assembled at the promoter, with the intervening DNA looped out. Compelling evidence in favor of this model has been collected by using the simian virus 40 enhancer (Box 8.7). These regulatory sequences can also facilitate access of the transcriptional machinery to chromatin templates. For example, the simian virus 40 enhancer lies within, and contains DNA sequences necessary for formation of, a nucleosome-free region of the viral genome in infected cells. Enhancers can, therefore, stimulate RNA polymerase II transcription by

BOX 8.5 TERMINOLOGY

The idiosyncratic nomenclature for sequence-specific DNA-binding proteins that regulate transcription

When proteins that bind to specific promoter sequences to regulate transcription by RNA polymerase II were first identified, no rules for naming mammalian proteins (or the genes encoding them) were in place. Consequently, the names given by individual investigators were based on different properties of the protein.

- Some names indicate the function of the regulator, e.g., the glucocortoid receptor, Gr.
- Some names indicate the promoter sequence to which the protein binds, e.g., cyclic AMP response element (CRE)-binding protein, Creb.
- Some names are based on the promoter in which binding sites for the regulator were first identified, e.g., adenovirus E2 transcription factor, E2f.
- Some names report some very general property of the regulator, e.g., stimulatory protein 1, Sp1 (the first sequence-specific activator to be identified), and upstream stimulatory factor, Usf.

Such inconsistency, coupled with the universal use of acronyms, can mystify rather than inform: the historical origins of the names of transcriptional regulators are not known to most readers. The subsequent recognition that many "factors" are members of families of closely related proteins compounds such difficulties.

multiple molecular mechanisms. The primary effect of these mechanisms is to increase the probability that the gene to which an enhancer is linked will be transcribed.

Proteins That Regulate Transcription Share Common Properties

All viral templates transcribed by cellular RNA polymerase II contain transcriptional control regions directly recognized by cellular regulatory proteins (see, e.g., Fig. 8.4). Indeed, the transcriptional programs of retroviruses with simple genomes are executed by the cellular transcriptional machinery alone. Consequently, cellular, sequence-specific, transcriptional regulators play pivotal roles in expression of viral genes and its regulation. However, the genomes of many viruses also encode transcriptional regulatory proteins, some of which resemble cellular proteins that recognize local promoter or more distant enhancer sequences. The cellular and viral DNA-binding proteins necessary for transcription from viral DNA templates share a number of common properties. Their most characteristic feature is modular organization: they are built from discrete structural and functional domains (Fig. 8.6). The basic modules are a DNA-binding domain and an activation domain, which function as independent units. Other common properties are binding to DNA as dimers, and membership in families of related proteins that share the same types of DNA-binding and dimerization domains (Fig. 8.6).

Regulation of transcription by sequence-specific DNA-binding proteins usually requires additional proteins termed **coactivators**. In general, these proteins cannot bind specifically to DNA, nor can they modulate transcription on their own. However, once recruited to a promoter by interaction with a DNA-bound sequence-specific regulator, they dramatically augment (or damp) transcriptional responses. Coactivators can cooperate with multiple, sequence-specific transcriptional activators and stimulate transcription from many promoters, but are not required at all. A common feature of many coregulators is their ability to alter the structure of nucleosomal templates for transcription, either directly or by interaction with appropriate enzymes. For example, several coactivators, including p300/Cbp, are histone acetyltransferases that catalyze the addition of acetyl groups

BOX 8.6 DISCUSSION

Typical properties of the simian virus 40 enhancer

- An enhancer is composed of multiple units, termed **enhancer elements** (e.g., the B1, B2, and A elements of the simian virus 40 enhancer) (Fig. 8.5).
- Enhancer elements operate synergistically: a single element has little activity, but the complete set stimulates transcription more than 100-fold.
- Multimerization of a single inactive enhancer element can create an active enhancer.
- Enhancer activity is relatively insensitive to the orientation or position of individual elements: stimulation of transcription generally depends on the presence of multiple elements, rather than on the exact way in which these are arranged.
- Enhancer elements comprise multiple sequences recognized by sequence-specific DNA-binding proteins that can also bind to promoter sequences (Fig. 8.5, 8.7, and 8.10).

Table 8.5 Some viral enhancers and their recognition

Virus	Enhancer location	Enhancer-binding proteins		Enhancer properties and functions
		Cellular	Viral	
Adenovirus				
Human adenovirus type 2	Enhancer 1, sequences repeated at −300 and −200 of the E1A gene	E2f, Ets family members	None	Broad cell type specificity
Hepadnavirus				
Hepatitis B virus	Enhancer I, adjacent to X gene promoter	Nf-κb, Ap-1, Nf-1, C/Ebp, hepatocyte nuclear factor 3 (Hnf3), Hnf4	None	Strong specificity for hepatocytes, because C/Ebp, Hnf3 and Hnf4 are specific for, or enriched in, these cells; activity may be increased by the viral X protein
Herpesvirus				
Human cytomegalovirus	Immediate-early proximal enhancer, 613 to −70 of major immediate-early transcription unit	Nf-κb, Ap-1, Creb, Srf, Ets family member Elk1, Rxr	None	Can function as a strong basal enhancer and an inducible enhancer activated by signal transduction pathways
Papillomavirus				
Human papillomavirus type 16	"Constitutive" enhancer in the long control region (LCR)	Nf-κb, Ap-1, Tef-1, Tef-2, Oct-1, Nf-1	None	Epithelial cell specific; Ap-1-binding sites confer responsiveness to epidermal growth factor
	E2 protein-dependent enhancers formed by E2-binding sites	None	E2 protein dimers	Active only in cells synthesizing the viral E2 protein; activates transcription from all early genes
Polyomavirus				
Simian virus 40	Between early and late promoters; tandem copies of a 72-bp repeat sequence	Nf-κb, Ap-1, Tef-1, octamer family members	None	Active in many mammalian cell types; activated by signal transduction pathways that converge on Ap-1
Retrovirus				
Human immunodeficiency virus type 1	Core enhancer, −95 to −50 of viral transcription unit	Nf-κb, Ets-1	None	Active only in cells in which Nf-κb is activated (e.g., T cells exposed to various growth factors)
Rous sarcoma virus	LTR enhancer, −250 to −130 of the viral transcription unit	bZip proteins, Srf, Y-box-binding proteins	None	Active in many cell types

to specific lysine residues in histones. Such enzymes, and the histone deacetylases associated with corepressors, help establish the histone codes that distinguish transcriptionally active from inactive chromatin (Box 8.8). A second class of coactivators, exemplified by members of the Swi/Snf family, contain ATP-dependent chromatin remodeling enzymes that alter the way in which DNA is bound to the histone octamer in a nucleosome. It is thought that the coordinated action of these two types of enzyme helps remove the nucleosomal barriers to transcription described previously.

As noted above, the RNA polymerase II system can mediate many patterns of transcription. Such plasticity stems in part from the variety in the nature of core RNA polymerase II promoters, and in the constellations of sequence-specific proteins and coactivators that regulate their activity. Equally important is the power of the transcriptional machinery to integrate signals from multiple, promoter-bound regulators. The transcriptional machinery must also be able to sense and respond to developmental and environmental cues. The proteins that control transcription are therefore frequently regulated by mechanisms that govern their activity, availability, or intracellular concentration. These mechanisms include regulation of the phosphorylation (or other modification) of specific amino acids, which can determine how

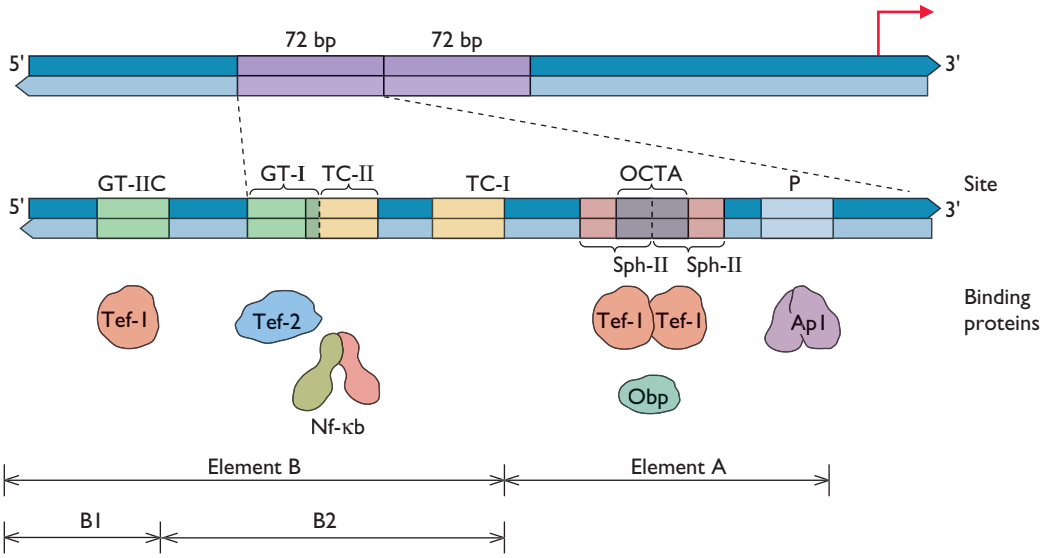


Figure 8.5 Organization of the simian virus 40 enhancer. The positions of the 72-bp repeat region containing the enhancer elements are shown relative to the early promoter at the top. Shown to scale below are functional DNA sequence units of the early promoter-distal 72-bp repeat and its 5′ flanking sequence, which forms part of enhancer element B, and the proteins that bind to them. All the protein-binding sites shown between the expansion lines are repeated in the promoter-proximal 72-bp repeat. The complete enhancer contains one copy of the enhancer element B1 and two directly repeated copies of the enhancer elements B2 and A. Some enhancer elements are built from repeated binding sites for a single sequence-specific protein. For example, cooperative binding of Tef-1 to the two Sph-II sequences forms a functional enhancer element. Such cooperative binding renders enhancer activity sensitive to small changes in the concentration of a single protein. A second class of enhancer elements comprise sequences bound by two different proteins, as illustrated by the enhancer element B GT-IIC and GT-I sequences. Binding of Tef-1 and Tef-2 is not cooperative, but these proteins interact once bound to DNA to form an active enhancer element. A third kind of enhancer element forms an active enhancer element upon oligomerization of a single sequence. The TC-II sequence recognized by Nf-κb functions in this manner.

well a protein binds to DNA, its **oligomerization** state, or the properties of its regulatory domain(s). In some cases, the intracellular location of a sequence-specific DNA-binding protein, or its association with inhibitory proteins within the nucleus, is controlled. Autoregulation of expression of the genes encoding transcriptional regulators is also common. This brief summary illustrates the varied repertoire of mechanisms available for regulation of transcription of viral templates by RNA polymerase II. Not surprisingly, virus-infected cells provide examples of all items on this menu, with the added zest of virus-specific mechanisms.

Transcription of Viral DNA Templates by the Cellular Machinery Alone

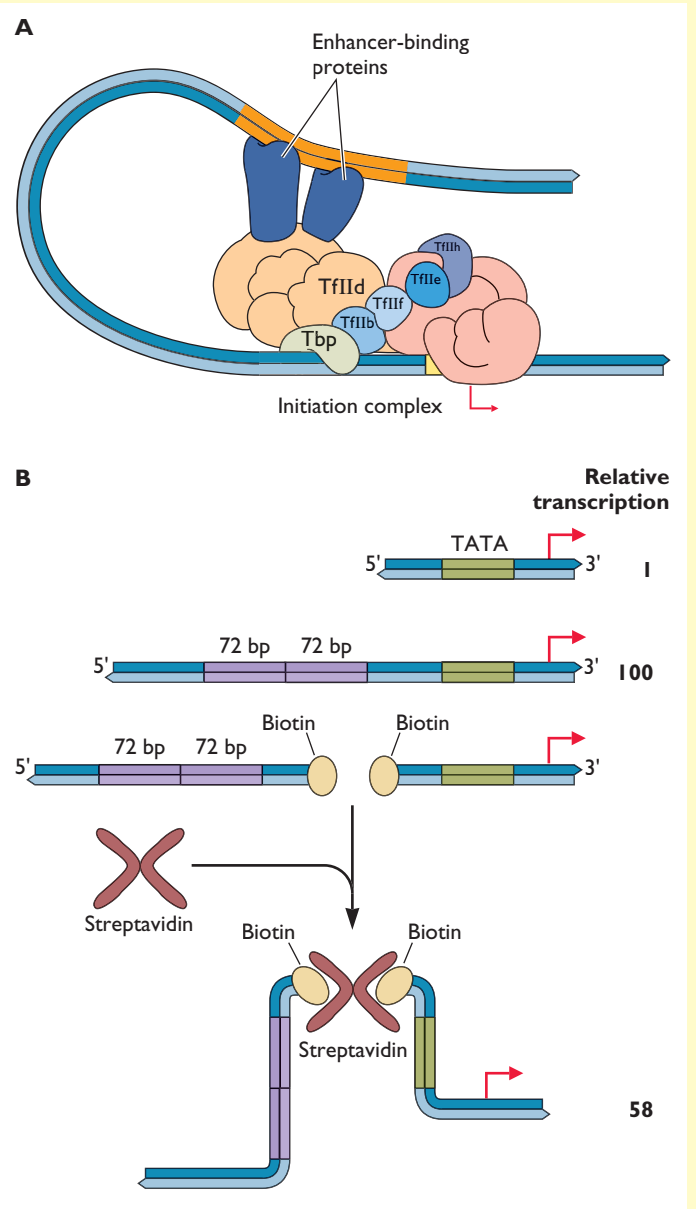
Retroviral transcription is characterized by production of a single viral transcript, which serves as both the genome for assembly of progeny virions and the source of viral mRNA species. In cells infected by many retroviruses, the components of the cellular transcriptional machinery described in the previous section complete the viral transcriptional program without the assistance of **any** viral proteins. The proviral DNA created by reverse transcription and integration (Table 8.3) comprises a single RNA polymerase II transcription unit organized into chromatin, exactly like the cellular templates for transcription. Such integrated proviral DNA is a permanent resident in the cellular genome. Because the genomes of these retroviruses do not encode transcriptional regulators, the rate at which proviral DNA is transcribed is determined by the constellation of cellular transcription proteins present in an infected cell. This rate may be influenced by the nature and growth state of the infected cell, as well as by the organization of cellular chromatin containing the proviral DNA. Transcription of viral genetic information can occur throughout the lifetime of the host cell, indeed even in descendants of the cell initially infected. This strategy for transcription of viral DNA is exemplified by avian sarcoma and leukosis viruses, such as Rous-associated viruses.

The long terminal repeat (LTR) of these proviral DNAs contains a compact enhancer located immediately upstream of the viral promoter (Fig. 8.7). As noted previously, the close proximity of the avian proviral LTR enhancer to promoter

BOX 8.7 EXPERIMENTS

Mechanisms of enhancer action

(A) The DNA-looping model postulates that proteins bound to a distant enhancer, here shown upstream of a gene (orange), interact directly with components of the transcription initiation complex, with the intervening DNA looped out. These interactions of proteins bound to a distal enhancer are analogous to those that can take place when the enhancer is located close to the promoter. Such interactions might stabilize the initiation complex and therefore stimulate transcription. **(B)** An enhancer noncovalently linked to a promoter via a protein bridge is functional. When placed immediately upstream of the rabbit β-globin gene promoter, the simian virus 40 enhancer stimulates specific transcription from circular plasmids *in vitro* by a factor of 100. In the experiment summarized here, the enhancer and promoter were separated by restriction endonuclease cleavage. Under this condition, the enhancer cannot stimulate transcription. Biotin was added to the ends of each DNA fragment by incorporation of biotinylated UTP. Biotin binds the protein streptavidin noncovalently, but with extremely high affinity (K_d, 10^{-15} M). Because streptavidin can bind four molecules of biotin, its addition to the biotinylated DNA fragments allows formation of a noncovalent protein "bridge" linking the enhancer and the promoter. Under these conditions, the viral enhancer stimulates *in vitro* transcription from the rabbit β-globin promoter almost as efficiently as when present in the same DNA molecule, as summarized in the column shown on the right. Because this result indicates that an enhancer can stimulate transcription when present in a separate DNA molecule (i.e., in *trans*), it rules out models in which enhancers are proposed to serve as entry sites for RNA polymerase II (or other components of the transcription machinery): such a mechanism requires that RNA polymerase II slide along the DNA from the enhancer to the promoter, a passage that would be blocked by the protein bridge. The results of this experiment are therefore consistent with the looping model shown in panel A.



Muller, H. P., J. M. Sogo, and W. Schappner. 1989. An enhancer stimulates transcription in trans when attached to the promoter via a protein bridge. *Cell* **58**:767–777.

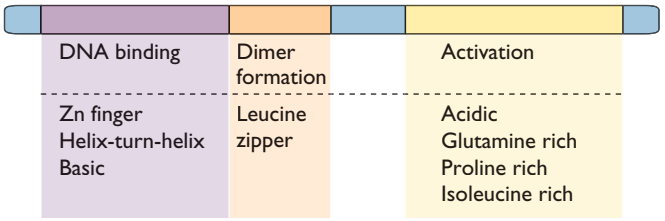


Figure 8.6 Modular organization of sequence-specific transcriptional activators. Common functional domains of eukaryotic transcriptional regulators are shown at the top, with some of the types of each domain listed below. DNA-binding and activation domains are defined by their structure (e.g., Zn finger or helix-turn-helix) and chemical makeup (e.g., acidic, glutamine rich), respectively. Transcriptional activators are often more complex than illustrated here. They can contain two activation domains, as well as regulatory domains, such as ligand-binding domains.

sequences is typical of viral genomes (for examples, see Fig. 8.5 and 8.10). The avian and mammalian serum response proteins that bind to the enhancer also bind to a specific sequence in the promoter (Fig. 8.7). This arrangement emphasizes the fact that enhancer and promoter sequences cannot be distinguished by the kinds of protein that recognize them. The other proteins that bind to this enhancer are all members of the basic-leucine zipper family of proteins (Fig. 8.6). Such proteins share a "leucine zipper" dimerization motif located immediately adjacent to a DNA-binding domain rich in basic residues (Fig. 8.8). As with many other transcriptional regulators, dimerization is essential for DNA binding, in this case to align the adjacent basic regions of the monomers to form the DNA-binding surface (Fig. 8.8).

The most remarkable property of the avian retroviral transcriptional control region is that it is active in many different cell types of both the natural avian hosts and mammals. This unusual feature can be explained by the widespread distribution of the cellular proteins that bind to it. The enhancer- and promoter-binding proteins, presumably assembled on the LTR transcriptional control region in different combinations in different cell types, allow efficient transcription of the provirus in both avian and mammalian cells. This property of the LTR enhancers/promoters of avian retroviruses has been exploited in the development of viral vectors.

Despite these properties, transcription of proviral DNA is not an inevitable consequence of integration, but can be blocked or impaired by specific cellular proteins. The avian retroviral integrase binds to the cellular protein Daxx, which becomes associated with proviral DNA. This association leads to repression of viral transcription. It is thought that once it has bound to chromatin containing the proviral template, Daxx recruits histone deacetylases (corepressors). As discussed in Volume II, Chapter 3, such epigenetic silencing of proviral transcription is but one example of general antiviral defense mechanisms.

Because the LTRs are direct repeats of one another (Fig. 8.7), transcription directed by the 3′ LTR extends into cellular DNA, and cannot contribute to the expression of retroviral genetic information. In fact, the transcriptional control region of the 3′ LTR is normally inactivated by a process called **promoter occlusion**: the passage of transcribing complexes initiating at the 5′ LTR through the 3′ LTR prevents recognition of the latter by enhancer- and promoter-binding proteins. Occasionally, transcription from the 3′ LTR does occur, with important consequences for the host cell (see Volume II, Chapter 7).

Absolute dependence on cellular components for the production of viral transcripts avoids the need to devote limited viral genetic information to transcriptional regulatory proteins. Nevertheless, such a strategy is not the rule.

Viral Proteins That Regulate RNA Polymerase II Transcription

Patterns of Regulation

Transcription of many viral DNA templates by the RNA polymerase II machinery results in the synthesis of large quantities of viral transcripts (in some cases, more than 10^5 copies of individual mRNA species per cell) in relatively short periods. Such bursts of transcription are elicited by transcriptional regulatory proteins. Viral proteins that stimulate RNA polymerase II transcription establish one of two kinds of regulatory circuit. The first is a **positive autoregulatory loop**, epitomized by transcription of human immunodeficiency virus type 1 proviral DNA (Fig. 8.9A). A viral activating protein stimulates the rate of transcription, but does not alter the nature of viral proteins made in infected cells. The second is a **transcriptional cascade**, in which different viral transcription units are activated in a fixed sequence (Fig. 8.9B). This mechanism, which ensures that different classes of viral proteins are made during different periods of the infectious cycle, is characteristic of viruses with DNA genomes. The participation of viral regulatory proteins presumably confers a measure of control lacking when the transcriptional program is executed solely by cellular components. The following sections describe some well-studied examples of regulatory circuits established by viral proteins.

The Human Immunodeficiency Virus Type 1 Tat Protein Autoregulates Transcription

Like those of their simpler cousins, the proteins of retroviruses with complex genomes are encoded in a single proviral transcription unit controlled by an LTR enhancer and promoter. However, in addition to the common structural proteins and enzymes, these genomes encode auxiliary proteins, including transcriptional regulators. Some of these regulatory proteins, such as the Tax protein of

BOX 8.8 DISCUSSION

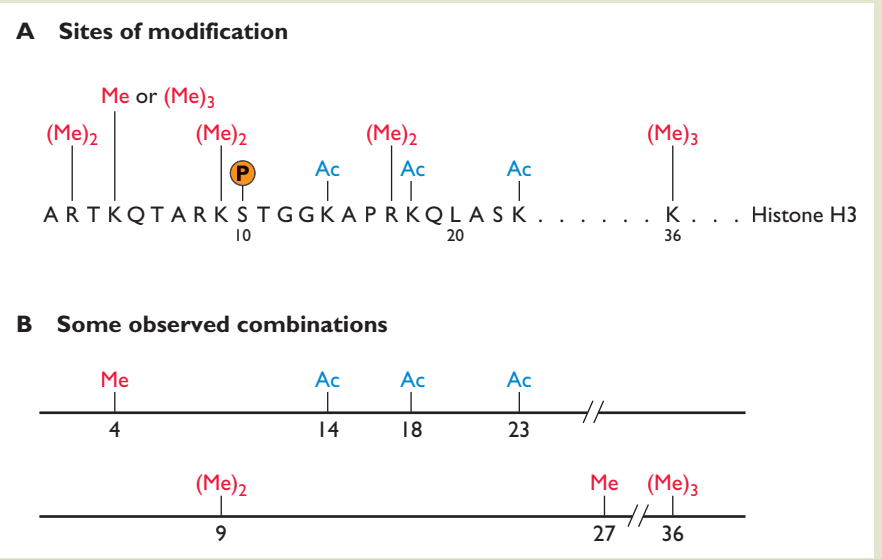
The histone code hypothesis

In eukaryotic cells, genomic DNA is organized and highly compacted by histones and many other chromosomal proteins in chromatin. Transcriptionally active DNA is present as less condensed euchromatin. Although it has been known for decades that the nucleosomal histones present in euchromatin are enriched in acetylated residues, the complexity and regulatory importance of posttranslational modification of nucleosomal histone has become apparent only within the past decade.

The N-terminal tails of the four core histones of the nucleosome (H2A, H2B, H3, and H4) are subject to acetylation, methylation, and phosphorylation of specific residues. Panel A of the figure summarizes modifications of this segment of histone H3. The N-terminal segment of histone H4 can also be methylated or acetylated at several positions, while H2A and H2B can be modified by acetylation, phosphorylation and ubiquitinylation. The large number of modifications results in a much greater number of possible combinations. For example, over 150 combinations present in different molecules of histone H3 in human cells have been identified by mass spectrometry, just 2 of which are shown in panel B.

It was initially proposed that particular combinations of posttranscriptionally modified histones identify transcriptionally active or inactive DNA. Consistent with this "histone code" hypothesis, some combinations, such as S10 phosphorylation plus K9 and/or K14 acetylation of histone H3, are characteristic of transcriptionally active genes. Conversely, methylation of K9 in this histone has been implicated in formation of heterochromatin. These modified amino acids serve as recognition sites for proteins that modify histones, remodel nucleosomes, or facilitate transcription by other mechanisms.

Although the idea of a simple code of histone posttranslational modifications has great appeal, it is now clear that it may be more appropriate to consider this a complex "language": for example, the same modification can recruit either activators or repressors of transcription, probably depending on the context of other posttranscriptional modifications. Furthermore, histone modifications are dynamic, changing, for example, during transcriptional elongation, or from one transcriptional cycle to another.



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human T-lymphotropic virus type 1, resemble activators of other virus families, and stimulate transcription from a wide variety of viral and cellular promoters (Table 8.6). Others, exemplified by the transactivator of transcription (Tat) of human immunodeficiency virus type 1, are unique sequence-specific activators of transcription: they recognize an RNA element in nascent transcripts.

The positive feedback loop that is established once a sufficient concentration of Tat has accumulated in an infected cell is simplicity itself (Fig. 8.9A). Cellular proteins initially direct transcription of the proviral DNA in infected cells at some basal rate. Among the processed products of viral primary transcripts are spliced mRNA species from which the Tat protein is synthesized. This protein is imported into the nucleus, where it stimulates transcription of the proviral template upon binding to its RNA recognition site in nascent viral transcripts. However, the molecular mechanisms that establish this autostimulatory loop are sophisticated and unusual. Their elucidation has been an important area of research, because the Tat protein is essential for virus propagation and represents a good target for antiviral therapy.

Cellular Proteins Recognize the Human Immunodeficiency Virus Type 1 LTR Transcriptional Control Region

The Tat protein is necessary for efficient human immunodeficiency virus type 1 transcription, but it is not sufficient: cellular proteins that bind to the LTR enhancer

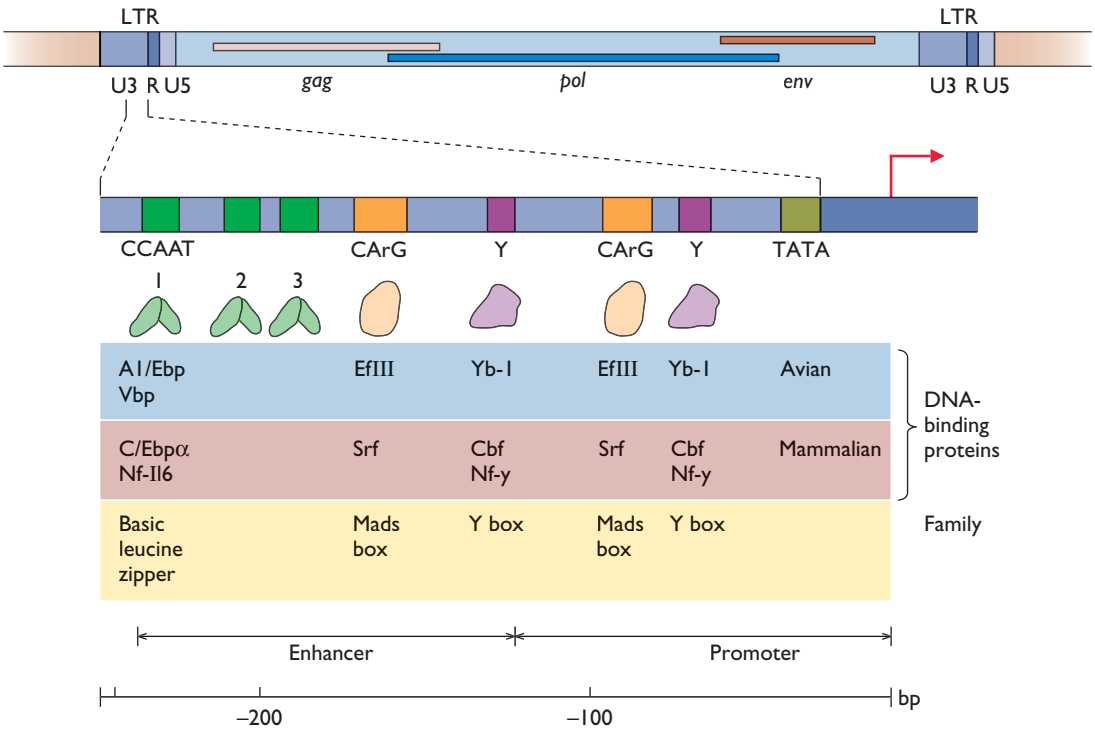


Figure 8.7 The transcriptional control region of an avian retrovirus. The proviral DNA of an avian leukosis virus is shown at the top. The enhancer and promoter present in the U3 regions of the LTRs are drawn to scale below. Each of the multiple CCAAT, CArG, and Y-box sequences, which are required for maximally efficient transcription, is recognized by the avian and mammalian proteins listed below. The chicken protein EfIII is the avian homolog of mammalian serum response factor (Srf), which plays an important role in the activation of transcription in response to serum growth factors. These proteins are members of a large, widespread family defined by a conserved sequence motif within the DNA-binding domain (the Mads box) and named for four of the originally identified members.

and promoter are also required. These proteins support a low level of proviral transcription before Tat is made in infected cells.

In contrast to avian retroviruses, human immunodeficiency virus type 1 propagates efficiently in only a few cell types, notably $CD4^+$ T lymphocytes and cells of the monocytic lineage. Viral reproduction (i.e., transcription) in infected T cells in culture is stimulated by T-cell growth factors, indicating that viral transcription requires cellular components available only in such stimulated T cells. Indeed, the failure of the virus to propagate efficiently in unstimulated T cells correlates with the absence of active forms of particular enhancer-binding proteins. The distribution of cellular enhancer-binding proteins is therefore an important determinant of the host range of retroviruses with both simple and complex genomes. The difference is that the transcription of proviral DNA of avian retroviruses depends on proteins that are widely distributed, whereas human immunodeficiency virus type 1 transcription requires proteins that are found in only a few cell types, or active only under certain conditions.

Within the LTR, the promoter is immediately preceded by two important regulatory regions (Fig. 8.10). The promoter-proximal enhancer core is essential for enhancer function in transient-expression assays (Box 8.3) and virus reproduction in T cells. The upstream enhancer region is also necessary for efficient viral transcription in both peripheral blood lymphocytes and certain T-cell lines. Both the core and the upstream enhancers are densely packed with binding sites for cellular proteins, many of which are enriched in the types of cell in which the virus can reproduce (Fig. 8.10). For example, the human Gata-3 and Ets-1 proteins, which stimulate viral transcription in transient-expression assays, are restricted to cells of the T-lymphocyte and hematopoietic lineages, respectively. However, it is not known whether these proteins are important for transcription of the provirus in infected cells. Transient-expression assays do not reproduce physiological conditions (Box 8.9). Consequently, a positive result in this kind of assay establishes only that a certain protein **can** stimulate transcription, not that it normally does so.

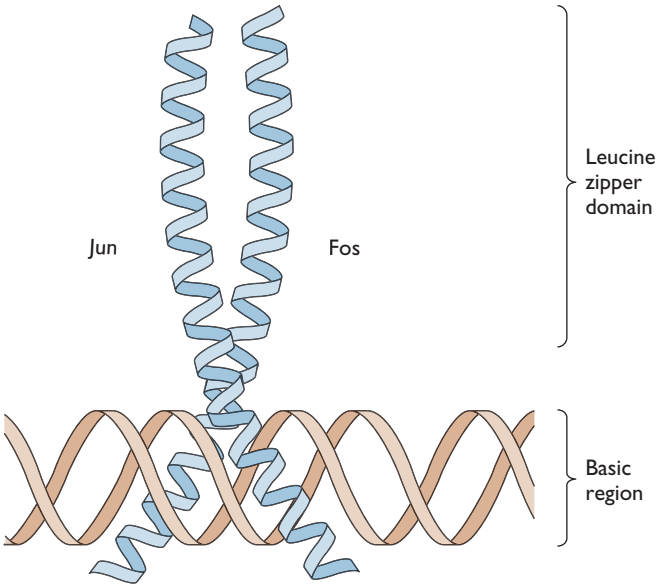


Figure 8.8 The structure of a basic-leucine zipper domain bound specifically to DNA. The model of the leucine zipper and adjacent basic region of the Jun-Fos heterodimer (Ap1) bound to a specific recognition sequence in DNA is based on the structure determined by X-ray crystallography. The leucine zipper forms an α-helical coiled coil, both in crystals and in solution. In the protein-DNA complex, the basic (DNA-binding) region is also α-helical, but in the absence of DNA it is disordered: DNA binding induces a major conformational change in proteins of this class. The α-helical regions containing the basic amino acids that make specific contact with DNA fit snugly into successive major grooves. Consequently, the basic-leucine zipper dimerization and DNA-binding domains have been likened to DNA "forceps." Adapted from J. N. M. Glover and S. C. Harrison, *Nature* **373:**257–261, 1995, with permission.

The mechanisms by which viral transcription is regulated by cellular pathways are illustrated by the cellulartranscriptional activator Nf-κb, which plays a critical role in relication of human immunodeficiency virus type 1 in T cells (as well as in cells infected by several other viruses) (Fig. 8.11). Unstimulated T cells display no Nf-κb activity, because the protein is retained in inactive form in the cytoplasm by binding of inhibitory proteins of the Iκb family. Upon T-cell activation by treatment with any one of several growth factors, Iκb proteins rapidly disappear from the cytoplasm, with the concomitant appearance of active Nf-κb in the nucleus. Phosphorylation of Iκb at specific sites, the result of signaling pathways activated by growth factors at the cell surface, targets the inhibitor for degradation by the cytoplasmic multiprotease complex (the **proteasome**) (Fig. 8.11). Consequently, Nf-κb is free for transit to the nucleus, where it can bind to its recognition sites within the viral LTR core enhancer (Fig. 8.10 and 8.11). This pathway can account for the induction of human immunodeficiency virus type 1 transcription observed when T cells are stimulated. The severe, or complete, inhibition of virus reproduction (transcription) in normal human $CD4^+$ lymphocytes, caused by mutations in the Nf-κb-binding site, emphasizes the importance of activation of Nf-κb in the infectious cycle of this virus. Nf-κb is also indispensable for viral transcription in macrophages, which, when differentiated, contain nuclear pools of constitutively active protein. Nevertheless, Nf-κb and the other cellular proteins that act via LTR enhancer- or promoter-binding sites do not support efficient expression of viral genes. This process depends on synthesis of the viral Tat protein (see the next section).

The efficient production of progeny human immunodeficiency virus type 1 genomes by transcription of the provirus depends ultimately on the constellation or activation state of cellular enhancer-binding proteins. Transcription of even small quantities of full-length viral transcripts in response to these proteins allows the synthesis of Tat mRNA and protein, and consequently activation of the positive, autoregulatory loop (Fig. 8.9A). The provirus can be considered dormant in cells that do not contain the necessary enhancer-binding proteins, or in which these proteins are inactive. However, it is important to keep in mind that such transcriptional inactivity is **not** the cause of the clinical latency characteristic of human immunodeficiency virus type 1 infections. In clinical latency, few, if any, symptoms are manifested. Nevertheless, virus is produced continuously (Volume II, Chapter 6), because the positive, autoregulatory circuit is triggered whenever infected cells can support LTR enhancer-dependent transcription of proviral DNA.

The Unique Mechanisms by Which the Tat Protein Regulates Transcription

Tat recognizes an RNA structure. Stimulation of human immunodeficiency virus type 1 transcription by Tat requires an LTR sequence, termed the **transactivation response (TAR)** element, which lies within the transcription unit (Fig. 8.12A). This sequence is active only in the sense orientation and only when located close to the initiation site of the promoter, properties that distinguish it from enhancer elements (see Box 8.4). The observation that mutations that disrupted the predicted secondary structure of TAR RNA inhibited Tat-dependent transcription suggested that the TAR element is recognized as RNA. Indeed, the Tat protein binds specifically to a trinucleotide bulge and adjacent base pairs in the stem of the TAR RNA stem-loop structure (Fig. 8.12B). Such specific binding requires an arginine-rich basic region and adjacent sequences of Tat. Binding of Tat to this region of TAR induces a local conformational rearrangement in the RNA, resulting in formation of a more stable and compact structure (Fig. 8.12C), and is therefore energetically

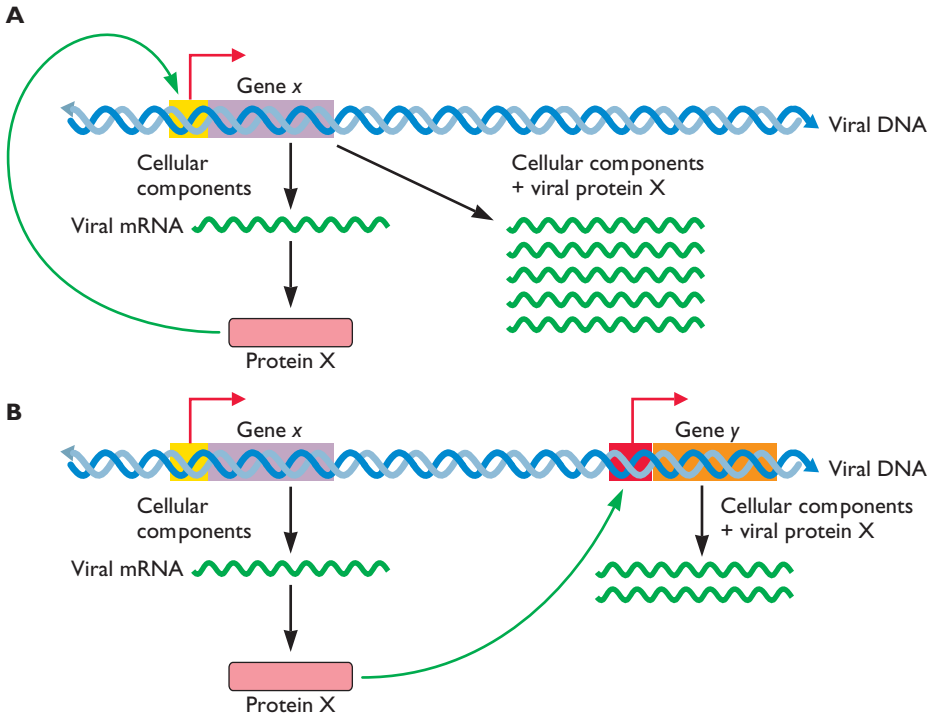


Figure 8.9 Mechanisms of stimulation of transcription by viral proteins. Cellular transcriptional components acting alone transcribe the viral gene encoding protein X. Once synthesized and returned to the nucleus, viral protein X can stimulate transcription either of the same transcription unit **(A)** or of a different one **(B)**. In either case, viral protein X acts in concert with components of the cellular transcriptional machinery.

favorable. Tat may also make contact with residues within the TAR loop (Fig. 8.12B). Recognition of a transcriptional control sequence as RNA by a regulatory protein remains unique to Tat proteins.

Tat stimulates transcriptional elongation. Binding of Tat to TAR RNA stimulates production of viral RNA as much as 100-fold. In contrast to many cellular and viral proteins that stimulate transcription by RNA polymerase II, the Tat protein has little effect on initiation. Rather, it greatly improves elongation. Complexes that initiate transcription in the absence of Tat elongate poorly, and many terminate transcription within 60 bp of the initiation site (Fig. 8.13A). Consequently, in the absence of Tat, full-length transcripts of proviral DNA account for no more than 10% of the total. This property resolves the paradox of why the human immunodeficiency virus type 1 LTR enhancer and promoter do not support efficient viral RNA synthesis: they direct initiation effectively, but the transcription complexes formed cannot carry out sustained transcription of the proviral genome over long distances. The Tat protein overcomes such poor **processivity** of elongating complexes to allow efficient production of full-length viral transcripts.

How Tat stimulates transcriptional elongation. A search for cellular proteins that stimulate viral transcription when bound to the N-terminal region of Tat (Fig. 8.12C) identified the human Ser/Thr kinase p-Tefb (positive-acting transcription factor b), which was known to stimulate elongation of cellular transcripts. This cellular protein is essential for Tat-dependent stimulation of processive viral transcription both *in vitro* and in infected cells. One subunit of the p-Tefb heterodimer is a **cyclin**, cyclin T. Cyclins are so named because members of the family accumulate during specific periods of the cell cycle. Cyclin T regulates the activity of the second subunit of p-Tefb, cyclin-dependent kinase 9 (Cdk9). The active kinase phosphorylates an unusual domain at the C terminus of the largest subunit of RNA polymerase II, which is essential for Tat-dependent stimulation of viral transcription. This domain comprises multiple, tandem copies of a heptapeptide sequence rich in Ser and Thr residues. The C-terminal domain of RNA polymerase II present in preinitiation complexes is hypophosphorylated, and its hyperphosphorylation has been implicated in successful promoter clearance and transcriptional elongation.

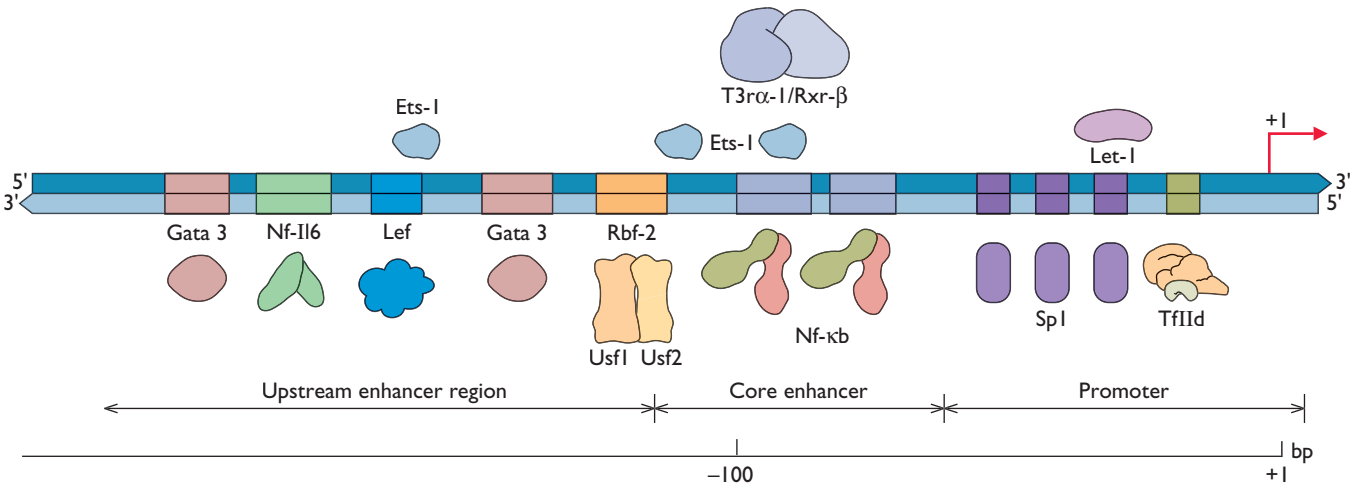
Tat and p-Tefb bind **cooperatively** to the TAR RNA stem-loop, that is, with higher affinity than either protein alone, and with greater specificity. This property is the result of the

Table 8.6 Some viral transcriptional regulators that bind to specific DNA sequences

Virus	Protein	Properties	Functions
Adenovirus			
Human adenovirus type 2	IVa_2	Binds to intragenic sequence in the major late promoter; late-phase specific	In conjunction with a viral L4 protein, stimulates transcription from major late promoter
Herpesviruses			
Herpes simplex virus type 1	ICP_4	Typical domain organization; one domain binds to a degenerate consensus sequence	Stimulates transcription from early and late promoters; represses transcription from all IE promoters by binding to promoter sequences
Epstein-Barr virus	Zta	bZIP protein; synthesis and activity regulated by multiple mechanisms (see the text)	Essential activator of early gene transcription; commits to lytic infection (see the text)
Papillomavirus			
Bovine papillomavirus type 1	E2	Typical domain organization; binds as dimers; can bind TfIIb and TfIId	Stimulates transcription from several promoters as enhancer-binding protein; necessary for replication of viral DNA *in vivo*
Poxvirus			
Vaccinia virus	VETF	Binds to TA-rich early promoter sequences as heterodimer; DNA-dependent ATPase	Essential for recognition of early promoters by the viral RNA polymerase

interaction of the cyclin T1 subunit of p-Tefb with nucleotides within the TAR RNA loop (Fig. 8.12B) that are not contacted by Tat, but are nevertheless crucial for stimulation of transcription. Assembly of the ternary complex containing TAR RNA, Tat, and p-Tefb induces conformational changes that activate the Cdk9 kinase of the latter protein. Once associated with transcription complexes, this enzyme phosphorylates the C-terminal domain of RNA polymerase II to stimulate elongation of transcription (Fig. 8.13A). This model was developed using *in vitro* assays and other simplified experimental systems. However, the results of experiments in which p-Tefb was inhibited in infected cells, as well as of genetic experiments, have established that p-Tefb is essential for stimulation of viral transcription by Tat *in vivo*.

Figure 8.10 The transcriptional control region of human immunodeficiency virus type 1. The organization of the U3 region of the proviral long terminal repeat is shown to scale. Proteins that bind to the enhancer or promoter sequences indicated are shown above or below the DNA. The upstream enhancer, which stimulates viral transcription in infected cells, contains binding sites for several proteins that are enriched in cells in which the virus reproduces. For example, the Lef protein is produced primarily in lymphocytes. Upon binding, this protein induces a large (130°) bend in DNA. The cellular enhancer-binding proteins listed have been shown to modulate the efficiency of transcription from the viral promoter in experimental situations. However, not all their binding sites are well conserved in different viral isolates, and only a few have been shown to contribute to transcription in infected cells.



BOX 8.9

WARNING

Caution: transient-expression assays do not reproduce conditions within virus-infected cells

Transient-expression assays (Box 8.3, panel B of the figure) provide a powerful, efficient way to investigate regulation of transcription. Advantages include the following:

- simplicity and sensitivity of assays for reporter gene activity
- ready analysis of mutated promoters to identify DNA sequences needed for the action of the regulatory protein
- application with chimeric fusion proteins and synthetic promoters to avoid transcriptional responses due to endogenous cellular proteins
- simplification of complex transcriptional regulatory circuits to focus on the activity of a single protein

Despite these advantages, transient-expression assays do not necessarily tell us how transcription is regulated in virus-infected cells, for they do not reproduce normal intracellular conditions. Important differences include the following:

- association of transcriptional templates with different proteins: the exogenous DNA may associate with cellular histones, but viral DNA may be packaged by virus-specific proteins
- abnormally high concentrations of exogenous template DNA: concentrations of reporter genes as high as 10^6 copies per cell are not unusual; this value is significantly greater than even the maximal concentrations of viral DNA molecules attained toward the end of an infectious cycle
- abnormally high concentrations of the regulatory protein as a result of its deliberate overproduction
- because of these high concentrations of template and protein, potential interactions of the viral regulator with template, or components of the cellular transcriptional machinery, that would not take place in an infected cell
- absence of viral components that might negatively or positively modulate the activity of the protein under study

The last three caveats apply to any experiment in which a viral protein is overproduced, for example, for investigation of its interactions with other proteins.

Human cells contain several proteins that stimulate elongation by RNA polymerase II, and others that inhibit this process. There have been several reports of Tat-dependent phosphorylation of such proteins, suggesting that additional mechanisms contribute to efficient elongation of viral transcription.

Tat also facilitates nucleosome remodeling. As discussed previously, integrated proviral DNA templates for transcription are organized in chromatin, by association with nucleosomes (and many other host cell proteins). Although proviral DNA is integrated preferentially into transcriptionally active genes of the host cell (Chapter 7), efficient transcription requires reorganization of nucleosomes. Nucleosomes are located at specific positions on the LTR promoter of integrated DNA. The promoter and enhancers are nucleosome free, and hence accessible to the transcriptional activators described above. In contrast, the nucleosome that is located immediately downstream of the site of initiation of transcription must be repositioned, or otherwise reorganized, to allow elongation of proviral DNA transcription. The results of recent experiments implicate Tat in inducing remodeling of this nucleosome.

In addition to binding to the cyclin T1 subunit of p-Tefb, Tat can bind to specific subunits of ATP-dependent chromatin-remodeling enzymes of the Swi/Snf family, as well as to several histone acetyltransferases, including p300/Cbp and pCaf. The data currently available are consistent with a model in which binding of Tat to TAR RNA recruits not only p-Tefb, but also Swi/Snf enzymes, which then alter the position or structure of the downstream nucleosome to promote elongation of viral transcription (Fig. 8.13B). Although some details are not yet clear, the inhibition of Tat-dependent transcription induced by small interfering RNA-mediated knockdown of specific subunits of Swi/Snf enzymes provides compelling evidence for the important contribution of this function of Tat to transcription of integrated proviral DNA.

The ability of Tat to bind to cellular proteins is governed by posttranslational modification. For example, acetylation at Lys28 (by Pcaf) promotes binding to p-Tefb, whereas acetylation at Lys50 prevents this interaction. The latter modification also prevents association of Tat with some Swi/Snf subunits, but allows binding to others (Fig. 8.13B).

Why such unusual transcriptional regulation? At this juncture, it is difficult to appreciate the value of the intricate transcriptional program of human immunodeficiency virus type 1. Those of many other viruses are executed successfully by transcriptional regulators that operate, directly or indirectly, via specific DNA sequences, and Tat can stimulate transcription effectively when made to bind to DNA experimentally. Why, then, is efficient transcription of the proviruses mediated by this unique, RNA-dependent mechanism? Binding of Tat to nascent viral RNA close to the site at which many transcriptional

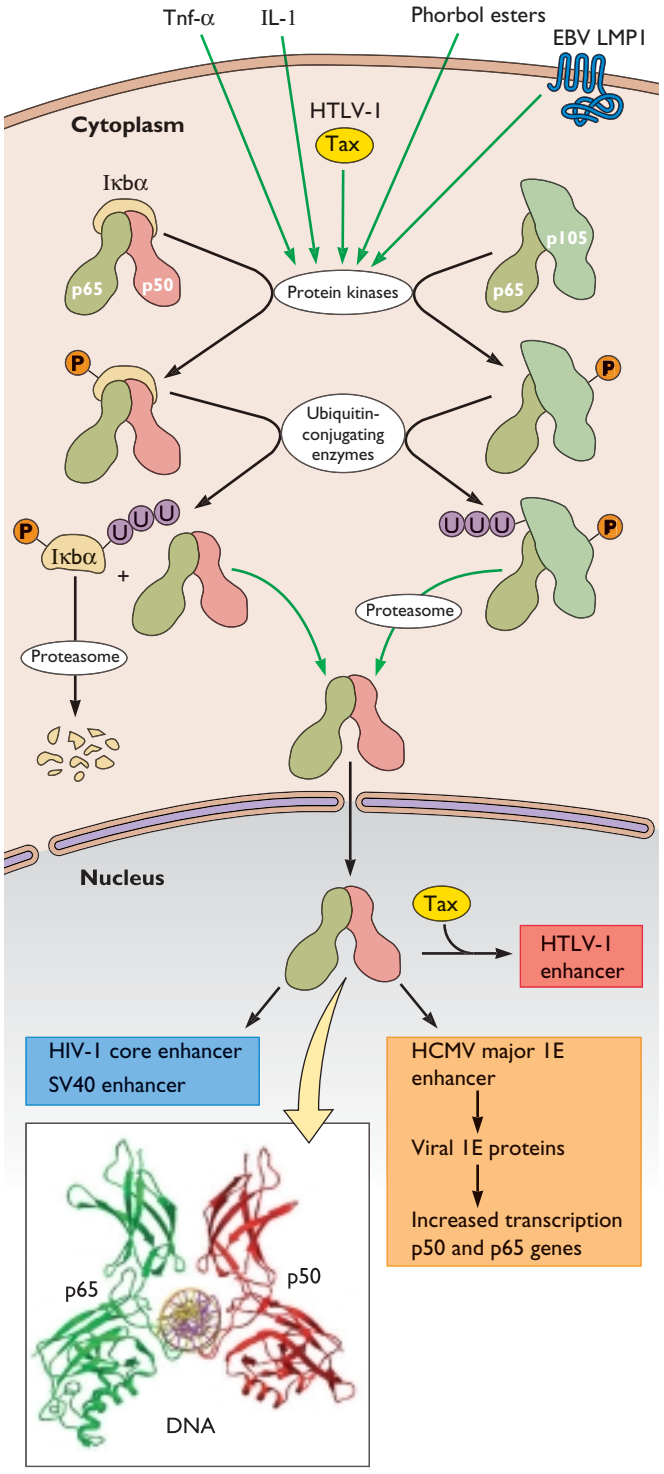


Figure 8.11 The cellular transcriptional regulator Nf-κB and its participation in viral transcription. The members of the Nf-κb Rel protein family are defined by the presence of the Rel homology region, which contains DNA-binding and dimerization motifs, and a nuclear localization signal. The p65 protein of the p50-p65 heterodimer **(left)** also contains an acidic activation domain at its C terminus. p50 is synthesized as an inactive precursor, p105 **(right)**. The p105-p65 heterodimer is one of two forms of inactive Nf-κb found in the cytoplasm (e.g., of unstimulated T cells). The second consists of mature Rel heterodimers, p50-p65, associated with an inhibitory protein such as Iκb-α (left), which blocks the nuclear localization signals of the p50 and p65 proteins. The C-terminal segment of p105 functions like Iκb, with which it shares sequences, to block nuclear localization signals and retain Nf-κb in the cytoplasm. Exposure of the cells to any of several growth factors, indicated by tumor necrosis factor alpha (Tnf-α), interleukin-1 (IL-1), and phorbol esters, results in activation (green arrows) of protein kinases that phosphorylate specific residues of Iκb or p105. Upon phosphorylation, Iκb dissociates and is recognized by the system of enzymes that adds branched chains of ubiquitin (Ub) to proteins targeted for degradation. It is then degraded by the proteasome, a multicatalytic protease that degrades polyubiquinylated proteins. Specific p105 cleavage by the proteasome also produces the p50-p65 dimer. Free Nf-κb produced by either mechanism can translocate to the nucleus because its nuclear localization signals are now accessible. In the nucleus of uninfected cells, Nf-κb binds to specific promoter sequences to stimulate transcription via the p65 activation domain. Viral transcriptional control regions to which Nf-κb binds and some viral proteins that induce activation (green arrows) of Nf-κb are indicated. The X-ray crystal structure of a p50-p65 heterodimer bound specifically to DNA is shown in the inset. The structure is viewed down the helical axis of DNA with the two strands in purple and yellow and with the p50 and p65 subunits in green and red, respectively. The dimer makes extensive contact with DNA via protein loops. HCMV, human cytomegalovirus; HIV-1, human immunodeficiency virus type 1; HTLV-1, human T-lymphotropic virus type 1; SV40, simian virus 40; EBV, Epstein-Barr virus. From F. E. Chen et al., *Nature* **391**:410–413, 1998, with permission. Courtesy of G. Ghosh, University of California, San Diego.

complexes pause or stall (Fig. 8.13A) could provide a particularly effective way to recruit the cellular proteins that stimulate processive transcription and induce nucleosome remodeling. Or it may be that regulation of transcription via an RNA sequence is a legacy from some ancestral viral-host cell interaction in an RNA world.

The Transcriptional Cascades of DNA Viruses

Common Features

The transcriptional strategies characteristic of the productive infectious cycles of viruses with DNA genomes exhibit a number of common features. The most striking is the transcription of viral genes in a reproducible and precise temporal sequence. Prior to initiation of genome replication, during **immediate-early** and **early** phases, infected cells synthesize viral proteins necessary for viral DNA synthesis, efficient gene expression, or other regulatory functions. Transcription of the **late** genes, most of which encode structural proteins, requires genome replication (Fig. 8.14). This property ensures coordinated production of the DNA genomes and the structural proteins from which progeny virus particles are assembled. Another common feature is the control of the transitions from one transcriptional stage to the next by both viral proteins

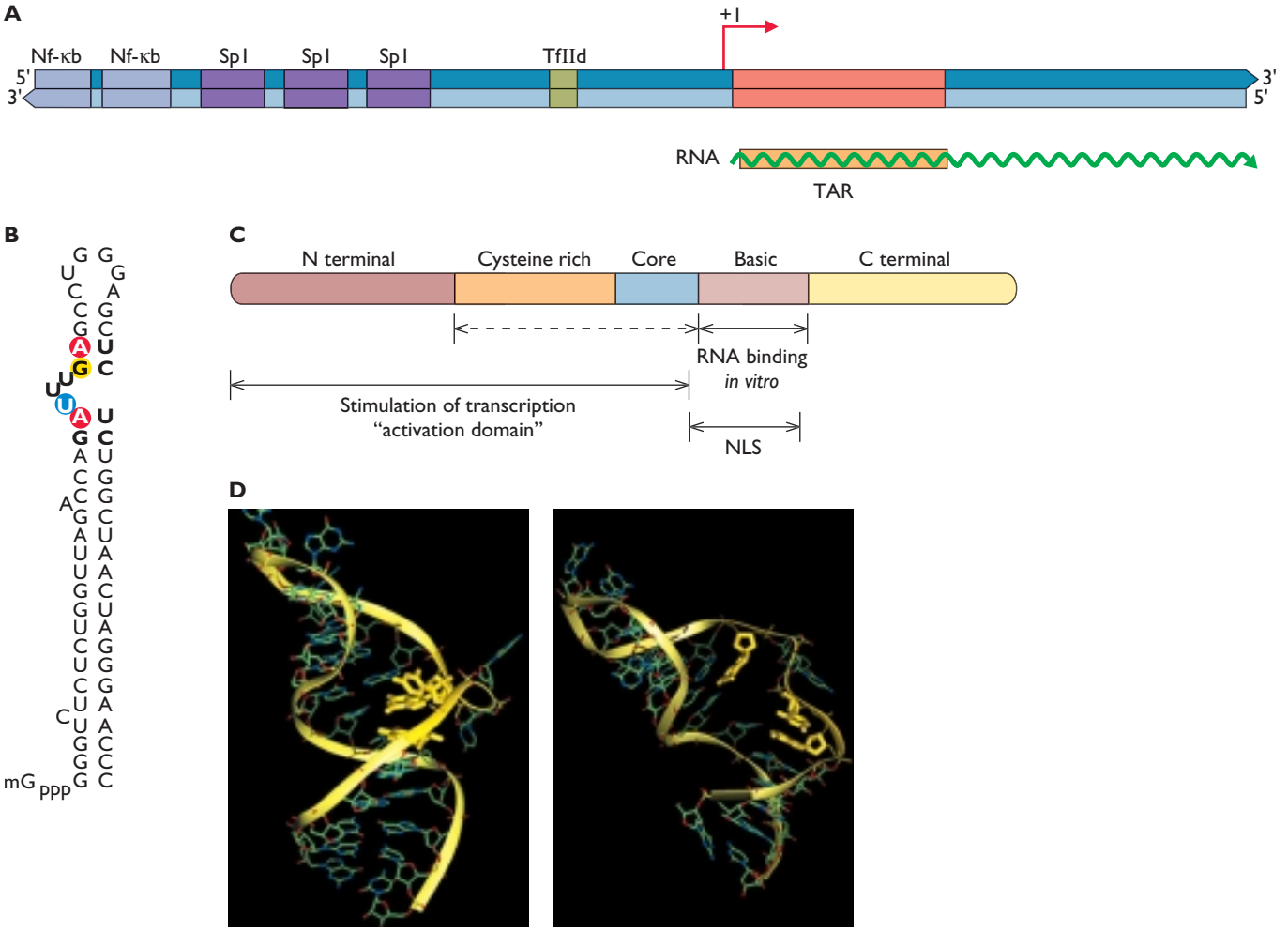


Figure 8.12 Human immunodeficiency virus type 1 TAR and the Tat protein. (A) The region of the viral genome spanning the site of transcription initiation is drawn to scale, with the core enhancer and promoter depicted as in Fig. 8.10. The DNA sequence lying just downstream of the initiation site (pink) negatively regulates transcription. Transcription of the proviral DNA produces nascent transcripts that contain the TAR sequence (tan box). **(B)** The TAR RNA hairpin extends from position +1 to position +59 in nascent viral RNA. Sequences important for recognition of TAR RNA by the Tat protein are colored. Optimal stimulation of transcription by Tat requires not only this binding site in TAR but also the terminal loop. **(C)** The Tat protein is made from several different, multiply spliced mRNAs (Appendix, Fig. 21B) and therefore varies in length at its C terminus. The regions of the protein are named for the nature of their sequences (basic, cysteine rich) or greatest conservation among lentiviral Tat proteins (core). Experiments with fusion proteins containing various segments of Tat and a heterologous RNA-binding domain identified the N-terminal segment indicated as sufficient to stimulate transcription. The basic region contains the nuclear localization signal (NLS). The basic region alone can bind specifically to RNA containing the bulge characteristic of TAR RNA. However, high-affinity binding, effective discrimination of wild-type TAR from mutated sequences *in vitro*, and RNA-dependent stimulation of transcription within cells require additional, N-terminal regions of the protein, shown by the dashed arrow. The sequences required for stimulation of transcription and specific recognition of TAR RNA in cells do not form discrete domains of the protein. **(D)** Major groove views of structures of a free TAR RNA corresponding to the apical stem and loop regions (but with a truncated stem) (left) and of the same RNA when bound to the Tat peptide (right) were determined by nuclear magnetic resonance methods. The residues shown in yellow are A22, U23, and G26, which are colored red, blue, and yellow, respectively, in panel B. Note the substantial conformational change in the trinucleotide bulge region on binding of the Tat peptide. From F. Aboul-ela et al., *J. Mol. Biol.* **253:**313–332, 1995, and F. Aboul-ela et al., *Nucleic Acids Res.* **24:**3974–3981, 1996, with permission. Courtesy of M. Afshar, RiboTargets, and J. Karn, MRC Laboratory of Molecular Biology.

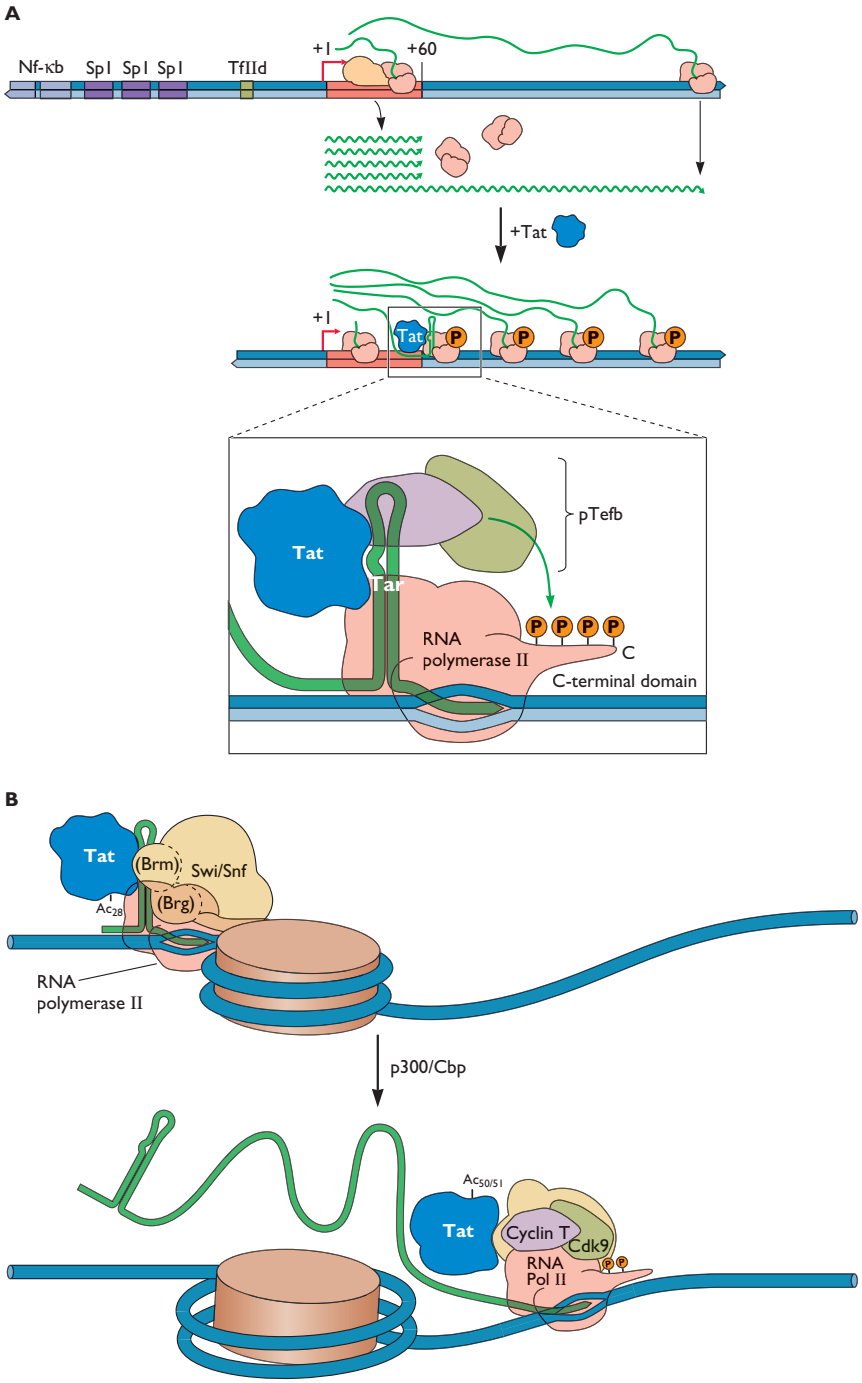


Figure 8.13 Mechanisms of stimulation of transcription by the human immunodeficiency virus type 1 Tat protein. (A) Model for the stimulation of elongation. The regulatory sequences flanking the site of initiation of transcription are depicted as in Fig. 8.10. In the absence of Tat, transcription complexes are poorly processive, and the great majority (9 of 10) terminate within 60 bp of the initiation site, releasing transcription components and short transcripts. Production of the Tat protein upon translation of mRNAs spliced from rare, full-length transcripts allows all transcriptional complexes to pass through the elongation blocks to synthesize full-length viral RNA. Binding of Tat to TAR in conjunction with the cyclin T subunit of p-Tefb leads to phosphorylation (P) of the C-terminal domain of the largest subunit of RNA polymerase II by the Cdk9 kinase subunit. As a result of this modification, transcriptional complexes become competent to carry out highly processive transcription. **(B)** Model for nucleosome remodeling. The initial transcript of proviral DNA (blue line) is depicted as in panel A. The nucleosome located a short distance downstream of the initation site blocks transcriptional elongation. When Tat bound to TAR is acetylated at Lys28, it binds to a particular subunit (Brm) of the Swi/Snf chromatin remodeling protein. Acetylation of Tat at Lys50 and Lys51 by the histone acetylases p300/Cbp induces dissociation of Tat from TAR, presumably the result of conformational change, and binding of Tat to a different subunit (Brg-1) of Swi/Snf. Remodeling of the nucleosome by Swi/Snf relieves the block to transcriptional elongation.

and genome replication (Fig. 8.14). Such viral programs closely resemble those that regulate many developmental processes in animals, in both the transcription of individual genes in a predetermined sequence, and the sequential action of proteins that regulate the transcription of different sets of genes.

The transcriptional activating proteins of different viruses exhibit distinctive properties. However, all cooperate with cellular transcriptional components to stimulate transcription of specific sets of viral genes expressed inefficiently in their absence. They can also induce transcription of cellular genes, and many repress transcription of their own genes (e.g., simian virus 40 large T antigen and the herpes simplex virus type 1 ICP4 protein). Such autoregulation presumably circumvents unfavorable consequences of excess concentrations of these viral proteins, for

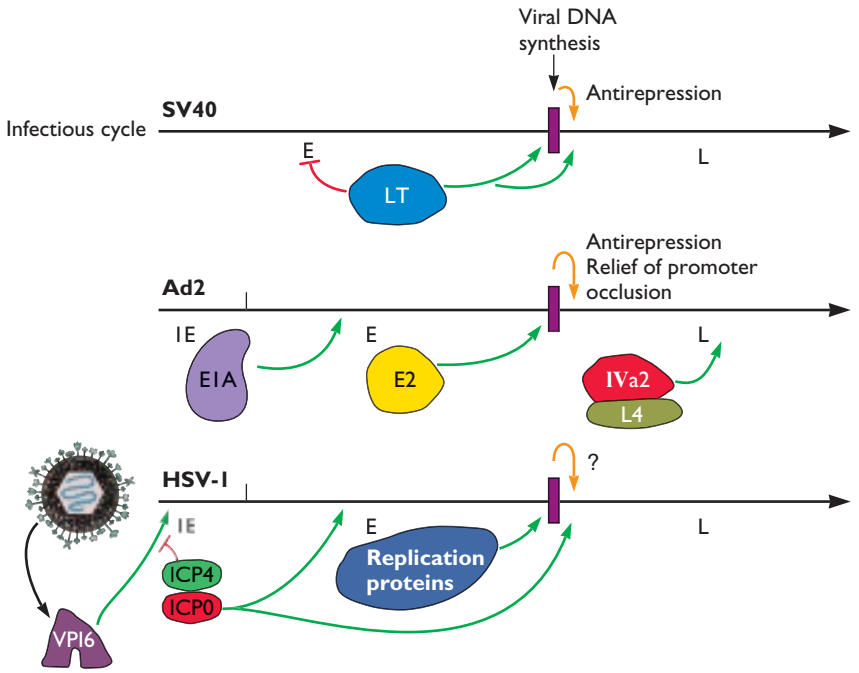


Figure 8.14 Important features of the simian virus 40 (SV40), human adenovirus type 2 (Ad2), and herpes simplex virus type 1 (HSV-1) transcriptional programs. The transcriptional programs of these three viruses are depicted by the horizontal time lines, on which the onset of viral DNA synthesis is indicated by the purple boxes. For comparative purposes only, the three reproductive cycles are represented by lines of equal length. The immediate-early (IE), early (E), and late (L) transcriptional phases are indicated, as are viral proteins that participate in regulation of transcription. Stimulation of transcription by these proteins and effects contingent on viral DNA synthesis in infected cells are indicated by green and orange arrows, respectively. Red bars indicate negative regulation of transcription.

example, unnecessary use of the resources of the infected cell, or inappropriate transcription of cellular genes.

Simian Virus 40

The transcriptional programs established in cells infected by viruses with small DNA genomes are quite simple: they comprise only two phases regulated by a single viral protein. For example, the genome of simian virus 40 contains only one early and one late transcription unit (Appendix, Fig. 13B), each of which encodes more than one protein. This type of organization reduces the genetic information that must be devoted to transcription punctuation marks and regulatory sequences, a significant advantage when genome size is limited by packaging constraints. The price for such a transcriptional strategy is heavy dependence on the host cell's RNA-processing systems to generate multiple mRNAs by differential polyadenylation and/or splicing of a single primary transcript (Chapter 10). Expression of the simian virus 40 early transcription unit by cellular enhancer- and promoter-binding proteins (Fig. 8.5) leads to the synthesis of large T antigen in infected cells. This multifunctional viral protein both induces initiation of viral DNA synthesis and activates late transcription (Fig. 8.14). Consequently, T-antigen synthesis in a permissive host cell leads to progression to the late phase of infection, when the segment of the viral genome encoding structural proteins is transcribed.

Human Adenovirus

Adenoviral genomes contain eight RNA polymerase II transcription units, which encode more than 40 proteins and are transcribed in four discrete phases (Appendix, Fig. 1B). At least three viral regulators of transcription, as well as viral DNA synthesis, govern the transitions from one phase to the next (Fig. 8.14). Upon entry of the genome into the nucleus, a single viral gene (E1A) is transcribed under the control of typical enhancers. If functional E1A proteins cannot be made, progression of the infectious cycle beyond this immediate-early phase is severely impaired. The E1A proteins, which regulate transcription by multiple mechanisms, are necessary for efficient transcription of all early transcription units. Among this set is the E2 gene, which encodes the proteins required for viral DNA synthesis and entry into the late phase of infection (Fig. 8.14). The adenoviral late phase is marked not only by transcription of late phase-specific genes, but also by an increased rate of initiation from the major late promoter

that is active during the early phase of infection. Late genes of the more complex DNA viruses therefore are defined as those that attain their maximal rates of transcription following viral DNA synthesis. No known activator of late transcription is included among the adenoviral early gene products. Instead, the synthesis of progeny adenoviral DNA molecules is indirectly coordinated with production of the protein components that will encapsidate them in virions (Fig. 8.14).

Herpes Simplex Virus Type 1

The organization of the herpes simplex virus type 1 genome demands an entirely different transcriptional strategy. The more than 80 known genes of this virus are, with few exceptions, expressed as individual transcription units. Furthermore, splicing of primary transcripts is the exception, rather than the rule. In contrast to those of simian virus 40 or human adenoviruses, herpes simplex virus type 1 genes encoding proteins that participate in the same process are not clustered but are scattered throughout the genome (Appendix, Fig. 5B). The basic distinction of early and late phases is maintained in the herpesviral transcriptional program (Fig. 8.14), but temporal control of the activity of more than 80 viral promoters is obviously more complicated. In fact, the potential for finely tuned regulation is much greater when the viral genome comprises a large number of independent transcription units.

Initial expression of both polyomaviral and adenoviral genes is directed by enhancers that operate efficiently with cellular proteins. In contrast, a viral activating protein is imported into cells infected by herpes simplex virus type 1 (Fig. 8.14). This virion structural protein, VP16, is necessary for efficient transcription of viral immediate-early genes. This simple device might seem to guarantee transcription of these genes in **all** infected cells. However, this is not the case, because VP16 functions only in conjunction with specific cellular proteins. Stimulation of immediate-early gene transcription by VP16 is of considerable importance for the success of the viral reproductive cycle.

Herpesviral immediate-early gene products resemble adenoviral E1A proteins in performing a number of regulatory functions. The regulatory scheme of herpes simplex virus type 1 is, however, considerably more complex, for this viral genome carries five immediate-early genes (Appendix, Fig. 5B). The products of two, ICP4 and ICP0, are transcriptional regulators. The ICP4 protein, which is essential for progression beyond the immediate-early phase of infection, is regarded as the major transcriptional activator. It stimulates transcription of both early and late genes, and also acts as a repressor of immediate-early transcription. As for the simpler DNA viruses, transcription of herpesviral late genes is governed by viral DNA replication (Fig. 8.14). As noted previously, two classes of late genes can be distinguished: some are transcribed only following synthesis of progress genomes and others are transcribed at maximal rates during the late phase. More subtle distinctions among the large number of late genes may be made as their transcriptional regulation becomes better understood (Box 8.10).

This summary of three DNA virus transcriptional programs illustrates the increasing sophistication of the circuits that control transcription with increasing genome size, a feature of little surprise. Underlying such diversity are the common themes of the central role of virus-encoded transcriptional regulators and the coordination of transcriptional control with viral DNA synthesis. Regulation of initiation is the mainstay of the transcriptional control programs of these DNA viruses. Nevertheless, expression of some genes is controlled at other steps in the transcription cycle, such as termination.

Viral Proteins That Stimulate Transcription Are Linchpins of Transcriptional Cascades

In this section, we focus on a few well-studied viral regulators to illustrate general principles of their operation, or fundamental insights into cellular processes that have been gained through their study.

Some viral transcriptional regulators are close relatives of well-studied cellular proteins that bind to specific DNA sequences in promoters or enhancers (Table 8.6). These viral proteins possess discrete DNA-binding domains, some with sequences that conform to motifs characteristic of cellular DNA-binding proteins, and activation domains that interact with cellular initiation proteins (Table 8.6). These properties are described in more detail for one such protein, the Epstein-Barr virus Zta protein, in the next section.

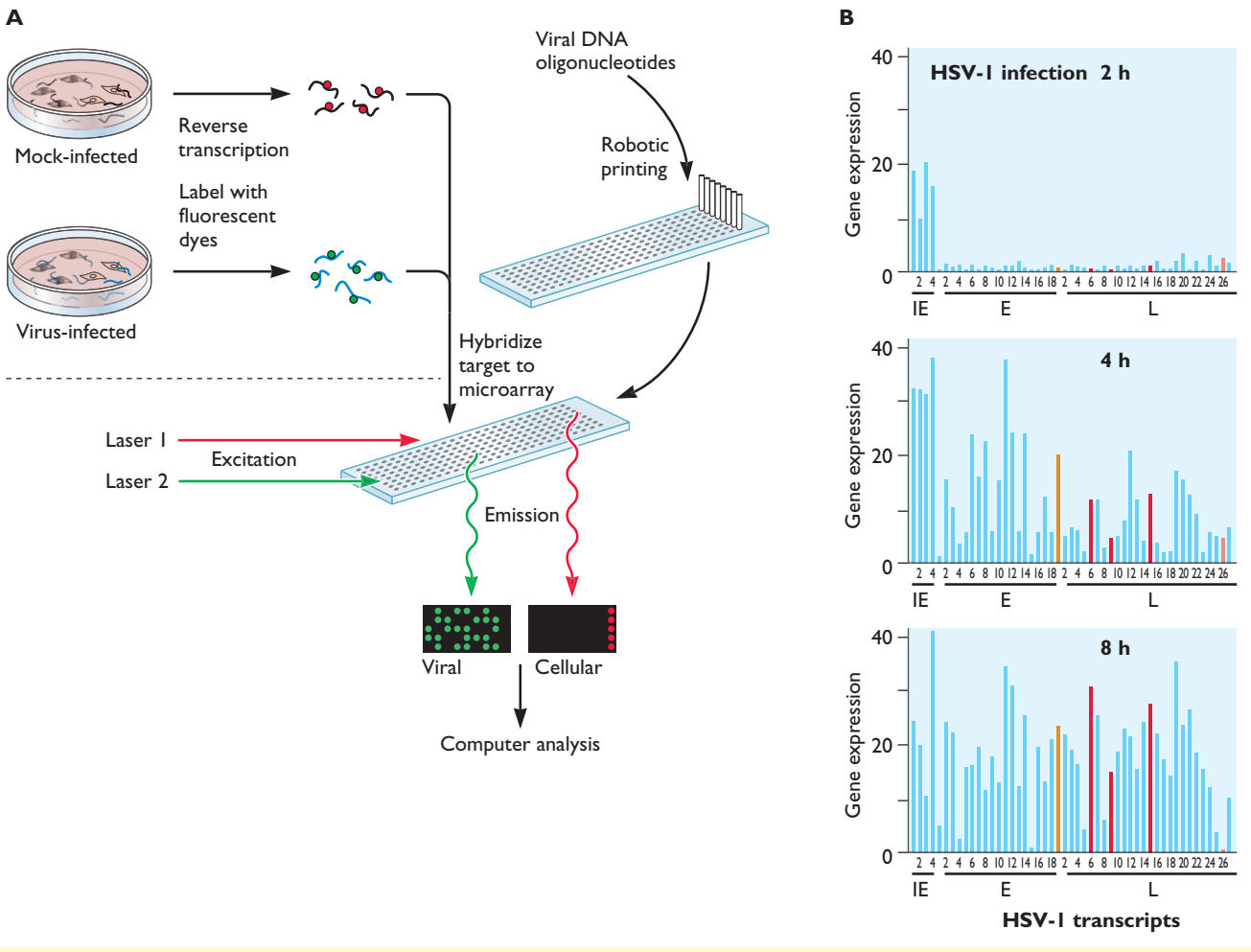
Sequence-specific DNA-binding proteins play ubiquitous roles in the transcription of cellular genes by RNA polymerase II, so it is not surprising that viral DNA genomes transcribed by this enzyme encode analogous proteins. However, viral transcriptional regulators that possess no intrinsic ability to bind specifically to DNA are equally common (Table 8.7). The preponderance of such viral proteins, quite unexpected when they were first characterized, was a strong indication that host cells also contain proteins that control transcription without themselves binding to DNA. Many such proteins (e.g., coactivators) have now been recognized. Two examples, the herpes simplex virus type 1 VP16 and adenovirus E1A proteins, illustrate the diversity of mechanisms by which this class of viral proteins regulates transcription.

BOX 8.10 EXPERIMENTS

Global analysis of herpesviral gene expression using DNA microarrays

Individual viral RNAs can be detected and quantified by several methods that exploit hybridization to DNA representing specific viral sequences, such as RNase protection or primer extension. Application of these methods to **every** RNA encoded in a large DNA genome, like that of herpes simplex virus type 1, would be extremely laborious, time-consuming, and expensive. Furthermore, it is difficult to examine more than a few RNAs simultaneously. Hybridization to DNA microarrays provides a powerful tool with which to determine and quantify expression of all viral genes in a single experiment, and to compare expression under different conditions.

In this method, illustrated in panel A of the figure, RNA isolated from infected cells is converted to complementary DNA (cDNA) by reverse transcription in the presence of a fluorescently labeled deoxyribonucleoside triphophate (dNTP). Dyes that fluoresce at different wavelengths are generally incorporated into different samples. For example, mock-infected-cell cDNA could be labeled with a cyanine 5-labeled dNTP (red) and infected-cell cDNA could be labeled with cyanine-3 dNTP (green), or cDNAs synthesized from wild-type and mutant virus-infected cell RNA could be labeled with the different dyes. The labeled cDNAs are then mixed and hybridized to a DNA micorarry, which contain DNAs representing numerous genes. Microarrays for cellular genomes are made using cDNAs or synthetic oligonucleotides, and contain DNA representing tens of thousands of genes. Microarrays for viral genomes, such as those used in the experiments described below, typically are made with oligonucleotides, and each viral gene is represented by multiple sequences. After hybridization, the microarrays are washed and exposed to



(continued)

BOX 8.10 EXPERIMENTS

Global analysis of herpesviral gene expression using DNA microarrays (continued)

laser light of appropriate wavelengths. The fluorescent signals of each DNA spot are detected, quantified and recorded by an automatic scanner. After subtraction of background and application of various other quality control procedures, they can then be analyzed in a number of different ways.

The results of an experiment in which microarrays were used to compare expression of herpes simplex virus type 1 genes at various times after infection are illustrated in panel B. In this experiment, each viral gene was represented by multiple spots on the array and a number of independently prepared samples was analyzed for each condition of interest, so that levels of expression of individual genes could be quantified reliably. The median signals for each gene examined at immediate-early (IE), early (E), and late (L) times of infection were then compared.

As expected, only IE genes were expressed efficiently 2 h after infection, whereas late RNAs predominated by 8 h. However, this global comparison revealed considerable variation in the kinetics and efficiency of expression of late genes. The concentrations of some late RNAs increased significantly between 4 and 8 h after infection (red), whereas other late RNAs reached close to maximal levels by 4 h (orange) or actually decreased in concentration between 4 and 8 h (pink).

In this experiment (as is currently typical), total RNA was isolated for microarray analysis. Consequently, the results do not establish whether changes in viral RNA concentration are the result of alterations in rates of transcription, mRNA processing, or degradation of the RNA. Nevertheless, the results shown indicate that there are several distinct patterns of expression of herpesviral late genes, and hence multiple mechanisms that govern production of late mRNAs.

The concentrations of RNA encoded by herpes simplex virus type 1 IE, E and L genes are shown in arbitrary units. Adapted from W.C. Yang et al., *J. Virol.* **76:**12758–12774, 2002, with permission.

Stringley, S. W., J. J. Garcia Ramirez, S. A. Aguilar, K. Simmen, R. M. Sandri-Goldin, P. Ghazal, and E. K. Wagner. 2000. Global analysis of herpes simplex virus type 1 transcription using an oligonucleotide-based DNA microarray. *J. Virol.* **74:**9916–9927.

Table 8.7 Some viral transcriptional regulators that lack sequence-specific DNA-binding activity

Virus	Protein	Functions and properties
Adenovirus		
Human adenovirus type 2	289R and 243R E1A proteins	Larger one stimulates early gene transcription in infected cells; both bind to the Rb protein releasing cellular E2f, and to the cellular coactivator p300
Hepadnavirus		
Hepatitis B virus	X	Required for efficient virus reproduction *in vivo*; stimulation of RNA polymerase II transcription depends on cellular, sequence-specific activators; may also stimulate transcription indirectly via activation of cellular signaling pathways
Herpesviruses		
Herpes simplex virus type 1	VP16	Stimulates transcription from IE promoters via potent acidic activation domain; achieves promoter specificity by interaction with the cellular Oct-1 protein
	ICP0	Cooperates with ICP4 to stimulate transcription of E and L genes in infected cells; important for reactivation from latency
Epstein-Barr virus	EBNA-2	Essential for B-cell transformation; induces transcription of viral LMP-1 and LMP-2, and of some cellular genes via specific DNA sequence; sequence recognition achieved by interaction with the cellular recombination binding protein Jκ
Polyomavirus		
Simian virus 40	Large T antigen[a]	Stimulates late-gene transcription; can bind to Tbp, TfIIb, and Tef-1 via different segments; contains no activation domain; may stabilize initiation complexes
Retrovirus		
Human T-lymphotropic virus type 1	Tax	Stimulates transcription from the viral LTR transcriptional control region; can bind directly to the basic region of bZIP proteins to stabilize dimers and increase their affinity for DNA; can regulate transcription indirectly by inducing degradation of IκB

[a]The sequence-specific DNA-binding activity of large T antigen (see Fig. 9.3) is not required for stimulation of transcription by this protein.

The Epstein-Barr virus Zta protein: a sequence-specific DNA-binding protein that induces entry into the productive cycle. When the gammaherpesvirus Epstein-Barr virus infects B lymphocytes, only a few viral genes are transcribed (the latent state). The products of these genes maintain the viral genome via replication from a latent phase-specific origin of replication (Fig 8.15A), modulate the immune system, and alter the growth properties of the cells. Treatment of such latently infected lymphocytic cells in culture with a variety of agents induces entry into the productive cycle. Epstein-Barr virus also enters this cycle upon infection of epithelial cells. Virus replication begins with synthesis of three viral proteins that regulate gene expression. However, just one of these, the transcriptional regulator Zta (also known as ZEBRA, Z, or EB-1), is sufficient to interrupt latency and induce entry into the productive cycle.

The Zta protein exhibits many properties characteristic of the cellular proteins that recognize local promoter sequences: it is a modular, sequence-specific DNA-binding protein that belongs to the basic-leucine zipper family (Table 8.6). This dimerization and DNA-binding domain mediates binding of Zta to viral promoters that contain its recognition sequence. The discrete activation domain, which can bind directly to cellular initiation proteins, such as subunits of TfIId, is thought to facilitate the assembly of preinitiation complexes.

The availability or activity of Zta is regulated by numerous mechanisms. Expression of the viral gene encoding Zta is controlled by cellular DNA-binding proteins that stimulate or repress transcription (Fig. 8.15B). Many of these cellular proteins are targets of signal transduction cascades that induce entry of Epstein-Barr virus into the productive cycle in latently infected B cells. In addition, the Zta protein itself is a positive autoregulator of transcription. The availability of Zta mRNA for translation is also regulated, in part, by annealing of Zta pre-mRNA to the complementary transcripts of the viral EBNA-1 gene (Fig. 8.15A). Finally, phosphorylation of Zta modulates its DNA-binding activity, whereas binding of cellular proteins, including the p53 tumor suppressor protein and the p65 subunit of Nf-κb, prevents Zta from stimulating transcription.

The net effect of these regulatory mechanisms, which depends on the type and on the proliferation and differentiation states of the Epstein-Barr virus-infected cell, determines whether active Zta protein is available, and consequently whether infection leads to latent or productive replication. Entry into the latter cycle appears to be an inevitable consequence of production of active Zta: this protein stimulates transcription from the promoters of its own gene and other early genes and plays an important role in replication from the lytic origins.

The herpes simplex virus type 1 VP16 protein: sequence-specific activation of transcription via a cellular DNA-binding protein. The herpesviral VP16 protein enters infected cells in the virion (Fig. 8.14). It has taught us much about mechanisms by which transcription by RNA polymerase II can be stimulated. Furthermore, its unusual mode of promoter recognition illustrates the importance of conformational change in proteins during formation of DNA-bound complexes.

The VP16 protein lacks a DNA-binding domain. Its acidic activation domain is one of the most potent known and has been exploited to investigate mechanisms of stimulation of transcription. Chimeric proteins in which this activation domain is fused to heterologous DNA-binding domains strongly stimulate transcription from promoters that contain the appropriate binding sites. When part of such fusion proteins, the VP16 acidic activation domain can stimulate several reactions required for initiation of transcription (Fig. 8.16A). It can also increase the rate of transcriptional elongation and stimulate transcription from chromatin templates (Fig. 8.16B). These properties established that a single protein can regulate RNA polymerase II transcription by multiple molecular mechanisms.

The VP16 protein is the founding member of a class of viral regulators that activate transcription from promoters that contain a specific consensus sequence, yet possess no sequence-specific DNA-binding activity. The 5′-flanking regions of viral immediate-early genes contain at least one copy of a consensus sequence, 5′TAATGARAT3′ (where R is a purine), which is necessary for VP16-dependent activation of their transcription. This sequence is bound by VP16 only in association with at least two cellular proteins, octamer-binding protein 1 (Oct-1) and host cell factor (Hcf) (Fig. 8.17). The Oct-1 protein is a ubiquitous transcriptional activator named for its recognition of a consensus DNA sequence termed the octamer motif. This protein and VP16 can associate to form a ternary (three-component) complex on the 5′TAATGARAT3′ sequence, but the second cellular protein, Hcf, is necessary for stable, high-affinity binding. Hcf is thought to regulate transcription, as it can bind to several sequence-specific transcriptional regulators and proteins that modulate chromatin structure. The VP16 protein and Hcf form a heteromeric complex in the absence of Oct-1 or DNA. An important function of Hcf therefore appears to be induction of a conformational change in VP16, to allow its stable binding to Oct-1 on the immediate-early promoters (Fig. 8.17).

One of the most remarkable features of the mechanism by which the VP16 protein is recruited to immediate-early promoters is its specificity for Oct-1. This protein is a member of a family of related transcriptional regulators defined by a common DNA-binding motif called the POU-homeodomain.

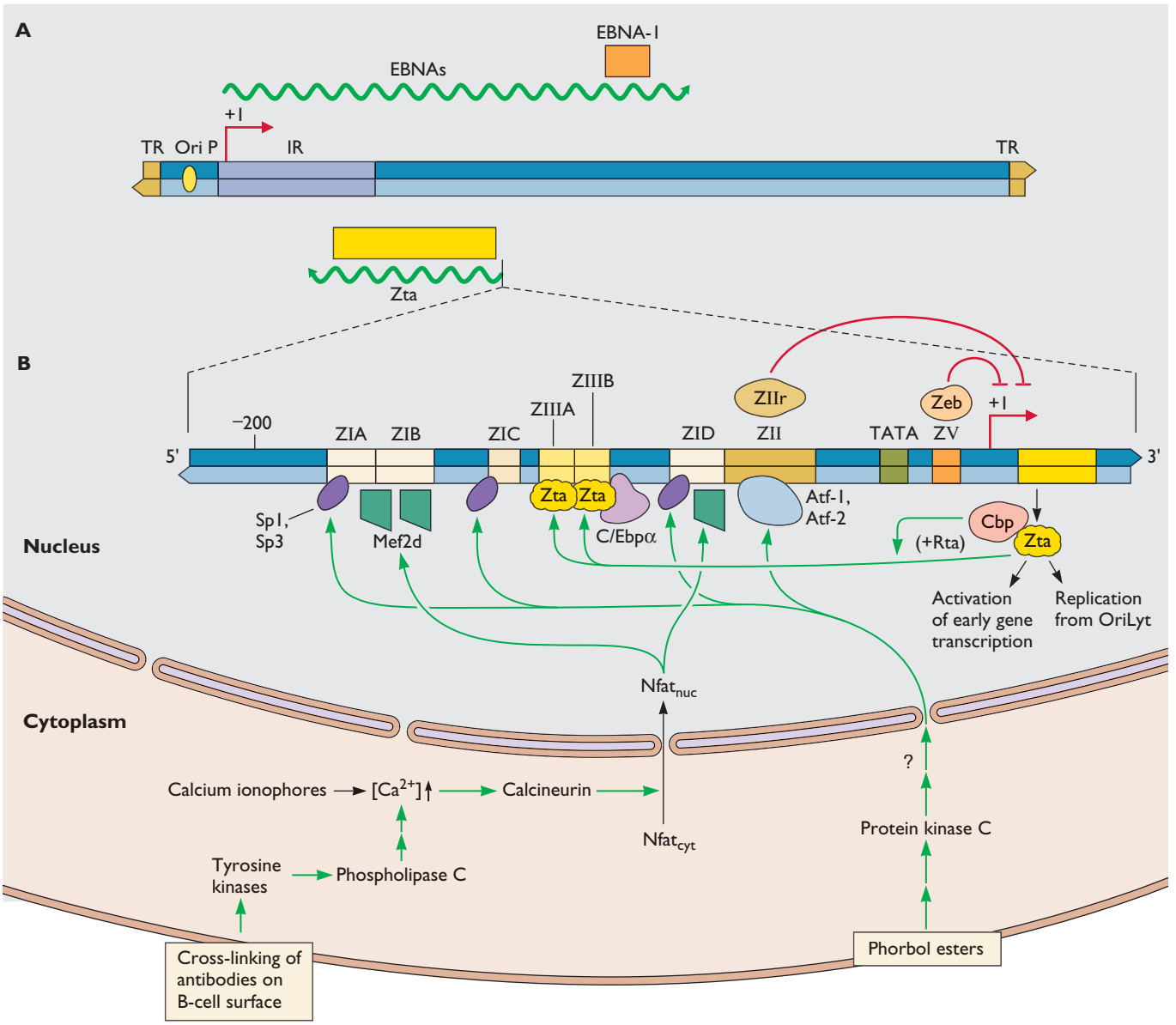


Figure 8.15 Organization and regulation of the Epstein-Barr virus Zta gene promoter. (A) Organization of the transcription units that contain the coding sequence for the Epstein-Barr virus nuclear antigen (EBNA) proteins (an ~100-kb transcription unit), Zta, and Rta. The locations of the genomic terminal (TR) and internal (IR) repeat sequences, the origin of replication for plasmid maintenance (OriP), and the coding sequences for the EBNA-1 and Zta proteins are indicated. The Zta protein is synthesized from spliced mRNAs processed from the primary transcripts shown. **(B)** Sequences that regulate transcription from the Zp promoter of the Zta gene are shown to scale and in the conventional 5′→3′ direction. The cellular or viral proteins that operate via these sequences are shown below. The Atf and Mef2d proteins are members of the basic-leucine zipper and Mads families, respectively. During latent infection, binding of cellular transcriptional repressors (for example, to the ZII and ZV sequences) prevents transcription from the Zp promoter (red bars). Signals that induce entry into the productive cycle activate transcription of the Zta gene via effects on cellular transcriptional activators (green arrows). Such pathways are illustrated with signaling initiated by cross-linking of antibodies of B cell surfaces, and treatment of cells with phorbol esters. Once Zta protein is produced, it stimulates transcription further by binding to the ZIII sequences in association with the cellular coactivator Cpb. It also stimulates transcription from the Zp promoter indirectly, by increasing the intracellular concentration of another cellular activator C/Ebpα.

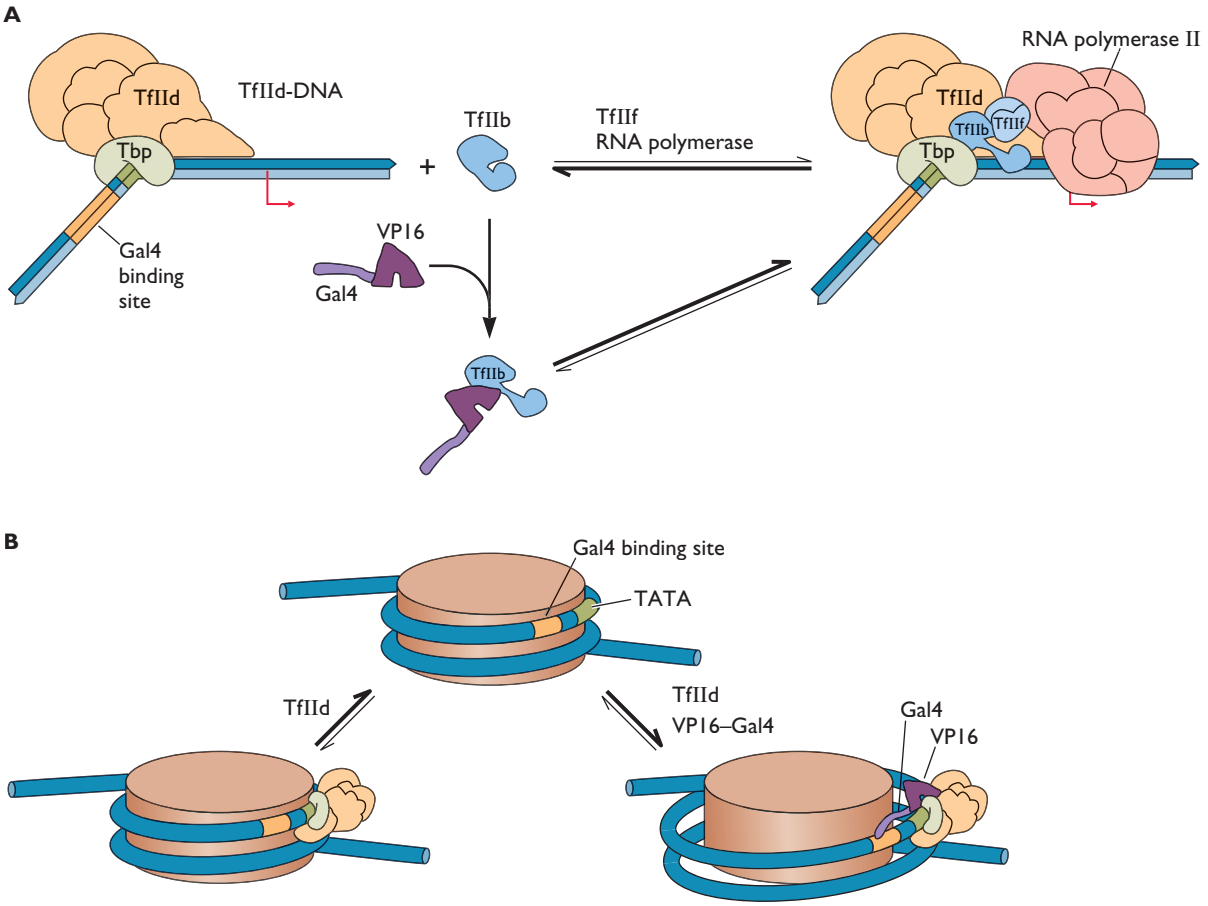


Figure 8.16 Models for transcriptional activation by the herpes simplex virus type 1 VP16 protein. **(A)** Induction of conformational change in TfIIb. In native TfIIb, the N- and C-terminal domains associate with one another such that internal segments of the protein that interact with TfIIf-RNA polymerase II are inaccessible. Binding of the acidic activation domain of VP16, for example as a chimera with the DNA-binding domain of the yeast protein Gal4, disrupts this intermolecular association of TfIIb domains, exposing its binding sites for TfIIf and RNA polymerase II. Consequently, formation of the preinitiation complex containing TfIIb, TfIIf, and RNA polymerase II is now a more favorable reaction. The VP16 activation domain can also bind directly to subunits of TfIId, TfIIf, and TfIIh. These interactions may also favor formation of the closed initiation complex, or they may be related to the ability of the activation domain to stimulate other reactions, such as promoter clearance. **(B)** Alleviation of transcriptional repression by nucleosomes. Many activators, including the acidic activation domain of VP16, stimulate transcription from nucleosomal DNA templates to a much greater degree than they do transcription from naked DNA. This property is the result of their ability to alleviate repression of transcription by nucleosomes, an activity therefore termed antirepression. Organization of DNA into a nucleosome can block access of proteins to their DNA-binding sites, as illustrated for binding of TfIId to a TATA sequence (left). Association of the acidic activation domain of VP16 with the template alters the interaction of the DNA with the nucleosome to allow TfIId access to the TATA sequence (right), presumably as a result of recruitment of ATP-dependent chromatin remodeling enzymes and/or histone acetyltransferases.

The VP16 protein distinguishes Oct-1 from all other members of this family, including Oct-2, which binds to exactly the same DNA sequence as Oct-1. In fact, VP16 detects a **single** amino acid difference in the exposed surfaces of DNA-bound Oct-1 and Oct-2 homeodomains. Such discrimination is not only remarkable but also of biological importance, because only the Oct-1 protein is synthesized ubiquitously.

The GARAT segment of TAATGARAT-containing elements of immediate-early genes is indispensable for the assembly of VP16 into the DNA-bound complex and therefore defines promoters that are recognized by the viral protein. Nevertheless, the DNA-binding domain of Oct-1 recognizes the GARAT motif (Fig. 8.17). This DNA sequence is therefore the crucial effector of assembly of

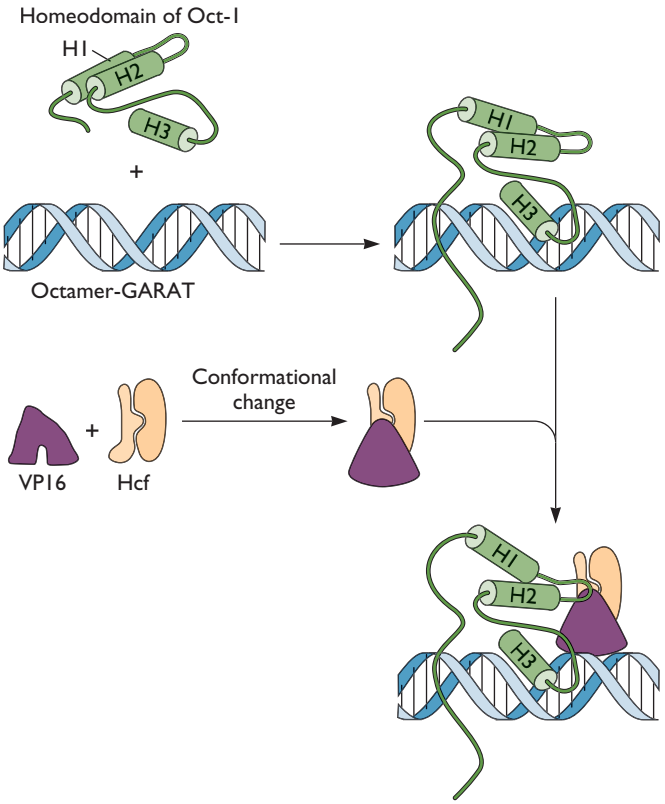


Figure 8.17 Conformational changes and recruitment of VP16 to herpes simplex virus type 1 promoters. Binding of the Oct-1 homeodomain to DNA containing the GARAT sequence and of VP16 to Hcf induces conformational changes that allow specific recognition of GARAT-bound Oct-1 by VP16. This mechanism ensures that the VP16 protein is recruited only to promoters that contain the GARAT sequence, that is, viral immediate-early promoters.

the quaternary complex. The mechanism by which VP16, in conjunction with Hcf and Oct-1, recognizes herpesviral immediate-early promoters illustrates the important contributions of induced conformational changes to transcriptional specificity.

The VP16 protein interacts with cellular Hcf and Oct-1 proteins via its N-terminal region. Its C-terminal region contains the acidic activation domain discussed previously. The results of chromatin immunoprecipitation experiments suggest that stimulation of IE gene transcription in infected cells is mediated by several of the biochemical activities exhibited by the acidic activation domain in simplified experimental systems (Box 8.11).

The incorporation of the VP16 protein into virions at the end of one infectious cycle is an effective way to ensure transcription of viral genes and initiation of a new cycle in a new host cell. Nevertheless, some features of this mechanism are not fully appreciated, in particular the benefits conferred by the indirect mechanism by which VP16 recognizes immediate-early promoters. One advantage over direct DNA binding may be the opportunity to monitor the growth state of the host cell that is provided by the requirement for binding to Hcf, a protein that is important for proliferation of uninfected cells. The dependence on Hcf may also contribute to the establishment of latent infections in neurons (see "Entry into one of two alternative transcriptional programs" below).

The VP16 activation domain (as well as Hcf) is necessary for efficient virus reproduction when infected cells contain one or a few viral genomes. However, the need for VP16 is circumvented, at least partially, when a large number of genomes enter infected cell nuclei (Box 8.12). Adenovirus mutants lacking the coding sequence for E1A CR3 (see the next section) exhibit the same phenotype.

Adenoviral E1A proteins: regulation of transcription of multiple mechanisms. Two E1A proteins, synthesized from differentially spliced mRNAs (Fig. 8.18), are synthesized during the immediate-early phase of adenovirus infection. These two proteins share all sequences except for an internal segment (conserved region 3 [CR3]) that is unique to the larger protein. Nevertheless, they differ considerably in their regulatory potential, because the CR3 segment is primarily responsible for stimulation of transcription of viral early genes. As the larger E1A protein neither binds specifically to DNA nor depends on a specific promoter sequence, it is often considered the prototypical example of viral proteins that stimulate transcription by indirect mechanisms, some of which are listed in Table 8.7.

The CR3 segment of the larger E1A protein comprises an N-terminal zinc finger motif followed by 10 amino acids that are highly conserved (Fig 8.19A). The latter mediate binding of the E1A protein to such cellular, sequence-specific activators as Atf-2, and Sp1, and hence association with the viral promoters. The zinc finger motif is essential for stimulation of transcription by the E1A protein in infected cells. It binds with exceptionally high affinity to a single component (Med23) of the human mediator complex, which contains at least 20 different subunits and is essential for regulation of transcription by RNA polymerase II. This interaction stimulates assembly of preinitiation complexes (see Fig. 8.2) in *in vitro* reactions, and is required for stimulation of transcription in infected cells: CR3-dependent stimulation of viral E2 transcription is observed in mouse embryonic stem cells, but not in mutant cells, homozygous for deletion of the *Med23* gene.

Adenoviral E1A proteins activate transcription by a second mechanism, which is mediated by the conserved regions of the N-terminal exon, CR1 and CR2 (Fig. 8.18). This function was elucidated through studies of

BOX 8.11 EXPERIMENTS

In vivo *functions of the VP16 acidic activation domain*

The chromatin immunoprecipitation assay (Box 8.1) has been used to compare the proteins associated with herpes simplex virus type 1 immediate-early (IE) promoters in cells infected by the wild-type virus, or a mutant encoding VP16 that lacks the acidic activation domain. Cross-linked DNA was immunoprecipated with antibodies to VP16, RNA polymerase, or several other cellular proteins. The concentrations of viral promoter DNA present in such immunoprecipates was then assessed by using PCR. The results summarized in the table suggest that the activation domain stimulates IE gene transcription in infected cells by mechanisms observed in simplified experimental systems.

Association of RNA polymerase II and Tbp with the viral promoters depended on synthesis of VP16 containing an activation domain, consistent with stimulation of assembly of initiation complexes. This domain is also necessary for efficient recruitment of histone acetyltransferases (Cbp) and ATP-dependent remodeling proteins (Brg-1), as well as loss of histone H3, suggesting that it also allows remodeling of chromatin templates in infected cells.

Herrera, F. J., and S. J. Triezenberg. 2004. VP16-dependent association of chromatin-modifying coactivators and underpresentation of histones at immediate-early gene promoters during herpes simplex virus infection. *J. Virol.* **78:**9689–9696.

Promoter-bound proteins	VP16 acidic activation domain	
	Not present	Present
VP16	++	++
Oct-1	++	++
RNA polymerase II	–	++
Tbp	–	++
Cbp	+	++
Brg-1	–	++
Histone H3	++	–

transformation and immortalization by E1A gene products. The CR1 or CR2 segments interact with several cellular proteins. Two of these, Rb and p300 (Fig. 8.18), are of special relevance to transcriptional regulation. The Rb protein is the product of the cellular retinoblastoma susceptibility gene, a tumor suppressor that plays a crucial role in cell cycle progression (Volume II, Chapter 7). The discovery that Rb binds to adenoviral E1A proteins, as well as to transforming proteins of papillomaviruses and polyomaviruses, led to elucidation of the mechanism of action of this important cellular protein.

In uninfected cells, Rb binds to cellular E2f proteins, sequence-specific transcriptional activators originally discovered because they bind to the human adenovirus type 2 E2 early promoter (Fig. 8.4). Such E2f-Rb complexes possess the specific DNA-binding activity characteristic of E2f, but Rb represses transcription (Fig. 8.19B). The CR1 and CR2 regions of the E1A proteins bind to the same

BOX 8.12 DISCUSSION

How does high multiplicity of infection overcome the need for viral activators of early transcription?

Mutants of human adenovirus type 5 (Ad5) that do not encode the larger (289R) E1A protein and of herpes simplex virus type 1 deleted for the coding sequence for the VP16 activation domain grow very poorly in cells infected at a low multiplicity of infection, e.g., 1 PFU/cell. However, when the multiplicity of infection is increased, these mutants do replicate, albeit more slowly and to lower yields than the wild-type viruses. How does increasing the concentration of infecting virus overcome the need for the viral activators of transcription?

The answer is thought to lie in the number of genomes that reach infected cell nuclei. Because of the high particle-to-PFU ratio typical of these viruses (see Chapter 2), we cannot infer that a single genome enters the nucleus when the multiplicity of infection is 1 PFU/cell. Nevertheless, increasing the multiplicity of infection, for example to 100 PFU/cell, obviously results in entry of a much larger number of viral DNA molecules. Such high DNA template concentrations presumably compensate for the low affinity with which cellular transcriptional regulators bind to viral proteins in the absence of the E1A or VP16 proteins. Consequently, early or immediate-early genes **are** transcribed, although less efficiently than in cells containing the viral activators.

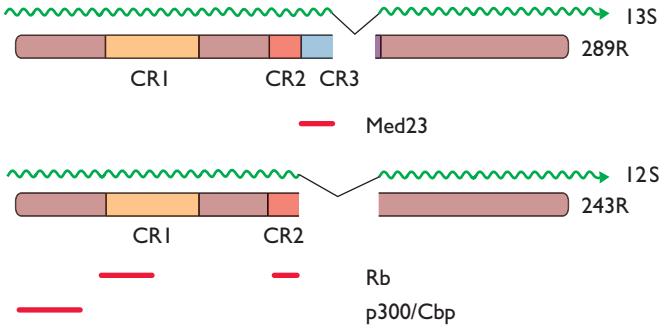


Figure 8.18 Features of the adenovirus type 2 E1A proteins. Primary transcripts of the immediate-early E1A gene are alternatively spliced to produce the abundant 13S and 12S mRNAs. As such splicing does not change the translational reading frame, the E1A proteins are identical, except for an internal segment of 46 amino acids unique to the larger protein. The three most highly conserved regions are designated CR1, CR2, and CR3. The regions of the E1A proteins necessary for interaction with the Rb protein, p300 Cbp, and Med23 are indicated (red lines).

segments of Rb as does E2f. Binding of the E1A proteins to Rb therefore releases E2f proteins from association with this repressor to allow transcription from E2f-dependent promoters. During the early phase of infection, E2f proteins are essential for efficient transcription of the gene that encodes the proteins required for viral DNA synthesis. Sequestration of Rb by the E1A proteins therefore ensures production of replication proteins and progression into the late phase of the infectious cycle (Fig. 8.14).

The N-terminal sequences common to the two E1A proteins also bind directly to the cellular coactivators p300 and Cbp (Fig. 8.18). These proteins are coactivators that interact with, and mediate stimulation of transcription by, the basic-leucine zipper protein Creb and steroid/thyroid hormone receptors. p300/Cbp are histone acetylases and bind to other histone acetylases. Modification of histones by these enzymes alters the structure of transcriptionally active chromatin and stimulates multiple reactions in transcription. The E1A proteins disrupt the interaction of p300/Cbp with Creb, and compete for their binding to other histone acetylases. This activity of E1A proteins has been implicated in repression of enhancer-dependent transcription, and is required for induction of cell proliferation and transformation.

The multiplicity of mechanisms by which the E1A proteins engage with components of the cellular transcriptional machinery is one of their most interesting features. Regulation by multiple mechanisms may prove to be a definitive property of viral proteins that cannot bind directly to DNA (Table 8.7). For example, the human T-lymphotropic virus type 1 Tax protein stimulates transcription by binding to specific cellular members of the basic-leucine zipper family, and by activating Nf-κb. Tax can activate the signaling cascade that induces degradation of Iκb and translocation of Nf-κb from the cytoplasm to the nucleus (Fig. 8.11). This mechanism illustrates the potential for intervention of viral regulators in cellular pathways well upstream of any transcriptional activator.

Coordination of Transcription of Late Genes with Viral DNA Synthesis

Large quantities of viral structural proteins must be made to ensure efficient assembly of virions. Such a high concentration of foreign proteins is undoubtedly injurious to the host cell, and could therefore interfere with cellular processes needed for virus replication. In the case of the viruses under consideration here, this potential problem is avoided, because structural proteins are synthesized only during the late phase of infection. As replication of viral DNA genomes is in full swing by this time, virion assembly is the only step then necessary to complete the reproductive cycle. This pattern of viral structural protein synthesis results from the dependence of late-gene transcription on viral DNA replication: drugs or mutations that inhibit viral DNA synthesis in infected cells also block efficient expression of late genes. In fact, late genes are defined experimentally as those that are not transcribed when DNA synthesis is blocked. Despite their importance, the mechanisms by which activation of transcription can be integrated with viral DNA synthesis remain incompletely understood.

Titration of cellular repressors. The most obvious consequence of genome replication in cells infected by DNA viruses is the large increase in concentration of viral DNA molecules. Even in experimental situations, infected cells contain a relatively small number of copies of the viral genome during the early phase of infection, typically 1 to 100 copies per cell depending on the multiplicity of infection. As soon as viral DNA synthesis begins, this number rises geometrically to values as high as hundreds of thousands of viral DNA molecules per infected cell nucleus. At such high concentrations, viral promoters can compete effectively for components of the cellular transcription machinery.

The increase in DNA template concentration also titrates cellular transcriptional repressors that bind to specific sequences of certain viral late promoters. For example, the simian virus 40 major late promoter contains multiple binding sites for a cellular repressor that belongs to the steroid/thyroid hormone receptor superfamily (Fig. 8.20). Viral DNA replication increases the concentration of the late promoter above that at which every copy can be bound by the repressor, and therefore allows this promoter to become active (Fig. 8.20). This "antirepression" mechanism directly coordinates activation of transcription of late genes with

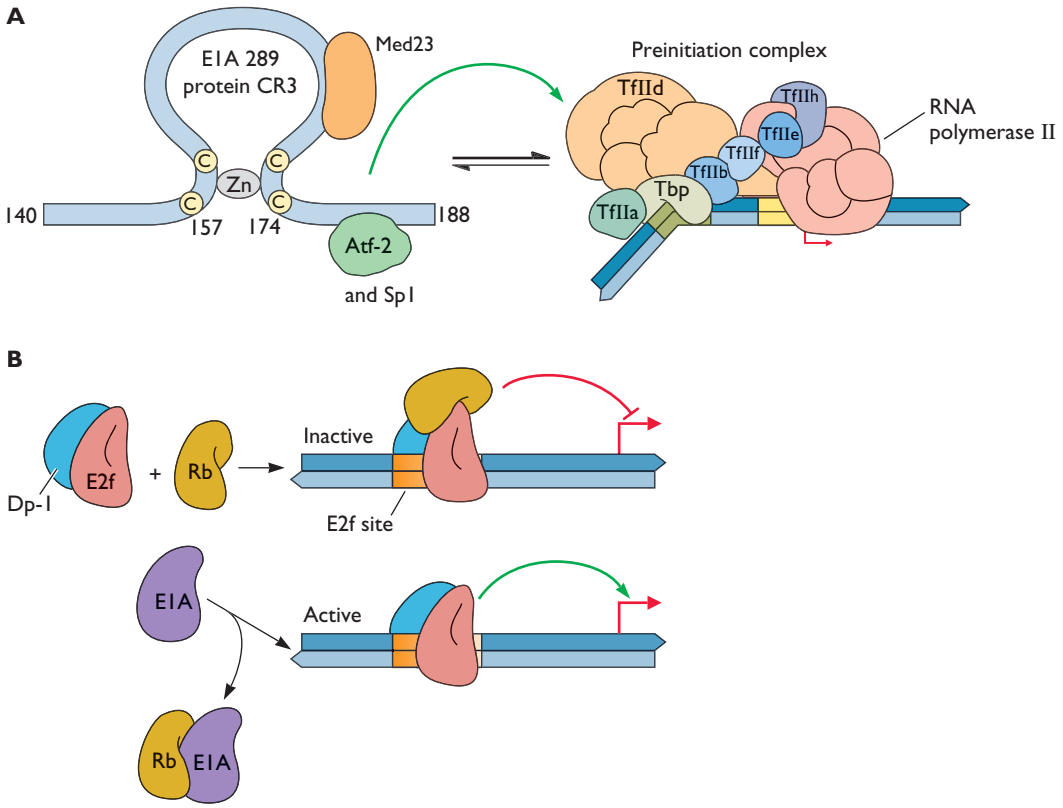


Figure 8.19 Indirect stimulation of transcription by adenoviral E1A proteins. (A) Interactions of the E1A CR3 sequences with components of the RNA polymerase II transcriptional machinery. The C-terminal segment of CR3 interacts with several cellular activators that bind to specific DNA sequences, as indicated, as well as with particular Taf subunits of TfIId. The Zn finger motif is required for tight binding to the Med23 subunit of the Mediator complex. This interaction stimulates assembly of closed preinitiation complexes. The exceptionally high affinity binding of CR3 to Med23 may also facilitate reinitiation. **(B)** Model of competition between E1A proteins and E2f for binding to Rb protein. The E2f transcriptional activators are heterodimers of a member of the E2f protein family (described in Chapter 9) and a related differentiation-regulated transcription factor protein, such as Dp-1. The binding of E2f to its recognition sites in specific promoters is not inhibited by association with the Rb protein, but Rb represses transcription from E2f-dependent promoters (top). Adenoviral E1A proteins made in infected (or transformed) cells bind to Rb and disrupt the E2f-Rb interaction. Consequently, Rb is removed from association with E2f, which can then stimulate transcription.

viral DNA synthesis, and is highly efficient. Consequently, it is not surprising that the same mechanism regulates transcription of the adenoviral IVa_2 gene, and hence the switch to the late phase transcriptional program (Fig. 8.14).

Viral activators of late-gene transcription. Although viral DNA synthesis is sufficient for activation of transcription of some viral late genes (e.g., the adenoviral IVa_2 gene), in most cases efficient transcription from late promoters also requires one or more viral proteins. For example, maximally efficient transcription from the simian virus 40 major late promoter depends on the viral early gene product large T antigen. This protein controls simian virus 40 late transcription both directly, as an activating protein (Fig. 8.14; Table 8.7), and indirectly, as a result of its essential functions in viral DNA synthesis (Chapter 9). Among the more complex DNA viruses, the coupling of replication to transcription of late genes can be more indirect. As noted above, late phase-specific transcription of the adenoviral IVa_2 gene is controlled by viral DNA synthesis-dependent titration of a cellular repressor. However, the IVa_2 protein is itself a sequence-specific activator of transcription. Once synthesized in infected cells, it cooperates with a viral L4 protein that also binds to specific promoter sequences, and stimulates the rate of initiation of transcription from the major late promoter at least 20-fold. Synthesis of adenoviral DNA therefore initiates a transcriptional cascade in which late promoters are activated sequentially (Fig. 8.14).

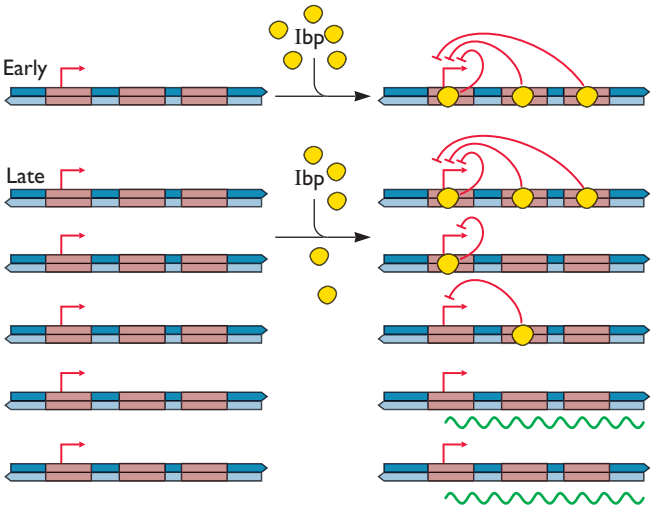
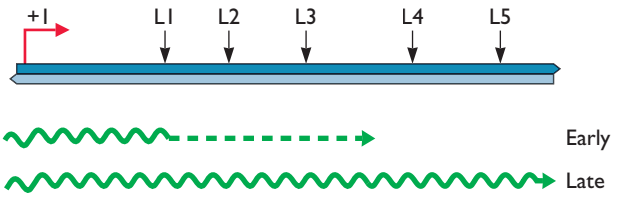


Figure 8.20 Cellular repressors regulate of the activity of the simian virus 40 late promoter. The sequence surrounding the simian virus 40 major late initiation site (the thickest arrow in Fig. 8.3D) contains three binding sites for the cellular repressor termed initiator-binding protein (Ibp), which contains members of the steroid/thyroid receptor superfamily. During the early phase of infection, the concentration of Ibp relative to that of the viral major late promoter is sufficiently high to allow all Ibp-binding sites in the viral genomes to be occupied. The concentration of Ibp does not change during the course of infection. However, as viral DNA synthesis takes place in the infected cell, the concentration of the major late promoter becomes sufficiently high that not all Ibp-binding sites can be occupied. Consequently, the major late promoter becomes accessible to cellular transcription components. Although we generally speak of "activation" of late gene transcription, this DNA replication-dependent mechanism is, in fact, one of escape from repression.

Transcription from simple promoters. Transcription of herpesviral late genes also requires viral DNA replication and synthesis of viral activators of transcription, for example, ICP4 and ICP0 in the case of herpes simplex virus type 1. However, the promoters of true late genes (those that are not expressed during the earlier phases) also share an unusually simple structure: they comprise typical TATA and initiator sequences and one additional element located between positions +10 and +40. How this simple promoter structure facilitates transcription of late genes has not been established. Nevertheless, this property might relate to the presence of a unique form of RNA polymerase II during the late phase of infection. The hypophosphorylated and hyperphosphorylated forms of the enzyme present in uninfected cells are replaced by a species in which the C-terminal domain of the large subunit is phosphorylated only on the second serine of the repeated YSPTSPS sequence. This modification has been implicated in efficient promoter clearance, whereas phosphorylation of the first serine facilitates transcriptional elongation by pTEFb. The herpesviral late genes that are transcribed by this infected cell-specific form of RNA polymerase II lack introns and are considerably shorter than typical cellular genes. Moreover, they are probably not associated with nucleosomes. Consequently, pTefb and regulators that modify nucleosome structure may be dispensable for transcription of these genes, and the simple structure of the promoters sufficient for recruitment of the components that mediate their transcription.

Regulation of termination. Viral DNA synthesis can also affect transcriptional termination, a regulatory mechanism illustrated by the human adenovirus type 2 major late transcription unit. During the early phase of infection, major late transcription terminates within a region in the middle of the transcription unit, such that no elongating complexes reach the L4 poly(A) addition site (Fig. 8.21). As discussed in Chapter 10, such restricted transcription is coupled with preferential utilization of specific RNA-processing signals to produce a single major late mRNA and protein during the early phase. Viral DNA synthesis is necessary to induce transcription of distal segments of the transcription unit to a termination site close to the right-hand end of the viral genome (Fig. 8.21). The inefficient termination of early transcription at many sites spread over about 12 kbp of DNA, and the fact that only replicated viral DNA molecules can support complete transcription, are features that suggest an unusual regulatory mechanism. One hypothesis is that alterations in template structure upon viral DNA synthesis may contribute to the regulation of termination of major late transcription (Box 8.13).

Figure 8.21 DNA replication-dependent alterations in termination of human adenovirus type 2 major late transcription. The segment of the viral genome from 15 to 100 map units containing the major late transcription unit is shown to scale **(top)**. The five sites at which primary major late transcripts are polyadenylated, designated L1 to L5, are indicated by the vertical arrows. During the early phase, major late transcripts terminate at multiple sites beyond the L1 site, as indicated by the dashed line, and none extend beyond the position indicated by the green arrowhead. The mechanisms responsible for this termination, which takes place within a DNA segment of 11 to 12 kb, are not understood. Following synthesis of both viral DNA and late proteins, all regions of the major late transcription unit are transcribed with equal efficiency to a termination site near the right-hand end of the genome.



BOX 8.13 DISCUSSION

Unusual viral templates for RNA polymerase II transcription?

In contrast to the genomes of papillomaviruses and polyomaviruses, adenoviral and herpesviral DNAs are not packaged by cellular nucleosomes to form regular, repeating structures in either virions or infected cells. The significance of the unusual nature of these transcriptional templates is not fully appreciated, but there are hints that this property might contribute to regulation of viral gene expression.

In virions, adenoviral DNA is packaged within the core by specialized viral DNA-binding proteins (Appendix, Fig. 1A). The major core protein, protein VII, is retained when the genome enters the infected cell nuclei and remains associated with viral DNA throughout the early phase. The results of recent experiments indicate that transcription of viral DNA is necessary and sufficient to induce dissociation of protein VII from DNA by the end of the early phase. Furthermore, because protein VII is the product of a late gene, viral DNA synthesis must necessarily be accompanied by reorganization of the viral nucleoproteins. Indeed, the templates for major late transcription during the late phase of infection appear protein free in the electron microscope, and by application of other assays. It is therefore possible that production of protein-free viral DNA molecules contributes to alterations in termination of major late transcription that accompany entry into the late phase (see "Coordination of Transcription of Late Genes with Viral DNA Synthesis").

Herpes simplex virus DNA is not associated with dedicated viral packaging proteins in virions. It has been reported that viral DNA in infected cells is associated with histones, but not organized in the form of a regular array. This conclusion is based largely on results of chromatin immunoprecipitation assays (Box 8.1). At this juncture, it remains unclear whether such histone-associated viral DNA molecules serve as templates for transcription. For example, the chromatin immunoprecipitation assay does not establish the fraction of viral molecules that are associated with a particular histone. Furthermore, experiments to investigate whether RNA polymerase II and histones are present on the same or different herpesviral DNA molecules have not been reported. Consequently, whether changes in the structure of DNA templates for transcription accompany viral DNA synthesis and entry into the late phase of infection is not known. On the other hand, there is accumulating evidence that changes in the nucleoprotein organization of herpesviral DNA are important in establishment of latency, and reactivation from this state (see the text).

Chatterjee, P. D., M. E. Vayda, and S. J. Flint. 1986. Adenoviral protein VII packages intracellular viral DNA throughout the early phase of infection. *EMBO J.* **5:**1633–1644.

Chen, J., N. Morral, and D. A. Engel. 2007. Transcription releases protein VII from adenovirus chromatin. *Virology* **369:**411–422.

Xue, Y., J. S. Johnson, D. A. Ornelles, J. Libberman, and D. A. Engel. 2005. Adenovirus protein-VII functions throughout early phase and interacts with cellular proteins SET and PP32. *J. Virol.* **79:**2474–2483.

Availability and structure of templates. Newly replicated DNA molecules must be partitioned for such fates as entry into additional replication cycles, service as templates for transcription, or assembly into virions. This process must be regulated so that different fates predominate at different times in the infectious cycle. Although transcription of all viral DNA molecules made in infected cells seems to be a simple mechanism to ensure efficient transcription of late genes, no more than 5 to 10% of the large numbers that accumulate are transcriptionally active. In the case of simian virus 40, synthesis of viral DNA molecules is coordinated with assembly into nucleosomes, and transcriptional activity can be ascribed to establishment of the open chromatin region in minichromosomes described above. Analogous mechanisms do not appear to be operating in cells infected by adenoviruses or herpesviruses (Box 8.13). It is not known whether a subset of these viral DNA molecules is also marked for transcriptional activity by some unknown molecular device. However, one important parameter governing the concentration of transcriptional templates must be the relative concentrations of viral DNA molecules and the proteins that package them for assembly into virions, because packaging and transcription are mutually exclusive.

Entry into One of Two Alternative Transcriptional Programs

As discussed in Chapter 1, studies of bacteriophage lambda led to the discovery that some viral infections result in maintenance of the viral genome for long periods in infected cells, rather than in viral replication. Whether lambda enters this lysogenic state or the lytic cycle is determined by the outcome of the opposing actions of two viral proteins that repress transcription (Box 8.14). This regulatory mechanism, which was among the first to be elucidated in detail, established the importance of repression of transcription of specific genes, and general paradigms for transcriptional switches. Several animal viruses can establish a similar pattern of infection. For example, **latent infection** is a characteristic feature of herpesvirus infection of specific types of host cell. As in bacteriophage lambda lysogeny, latent infections are characterized by both lack of efficient expression of many viral genes that are transcribed during productive infection, and activation of a unique, latent-phase

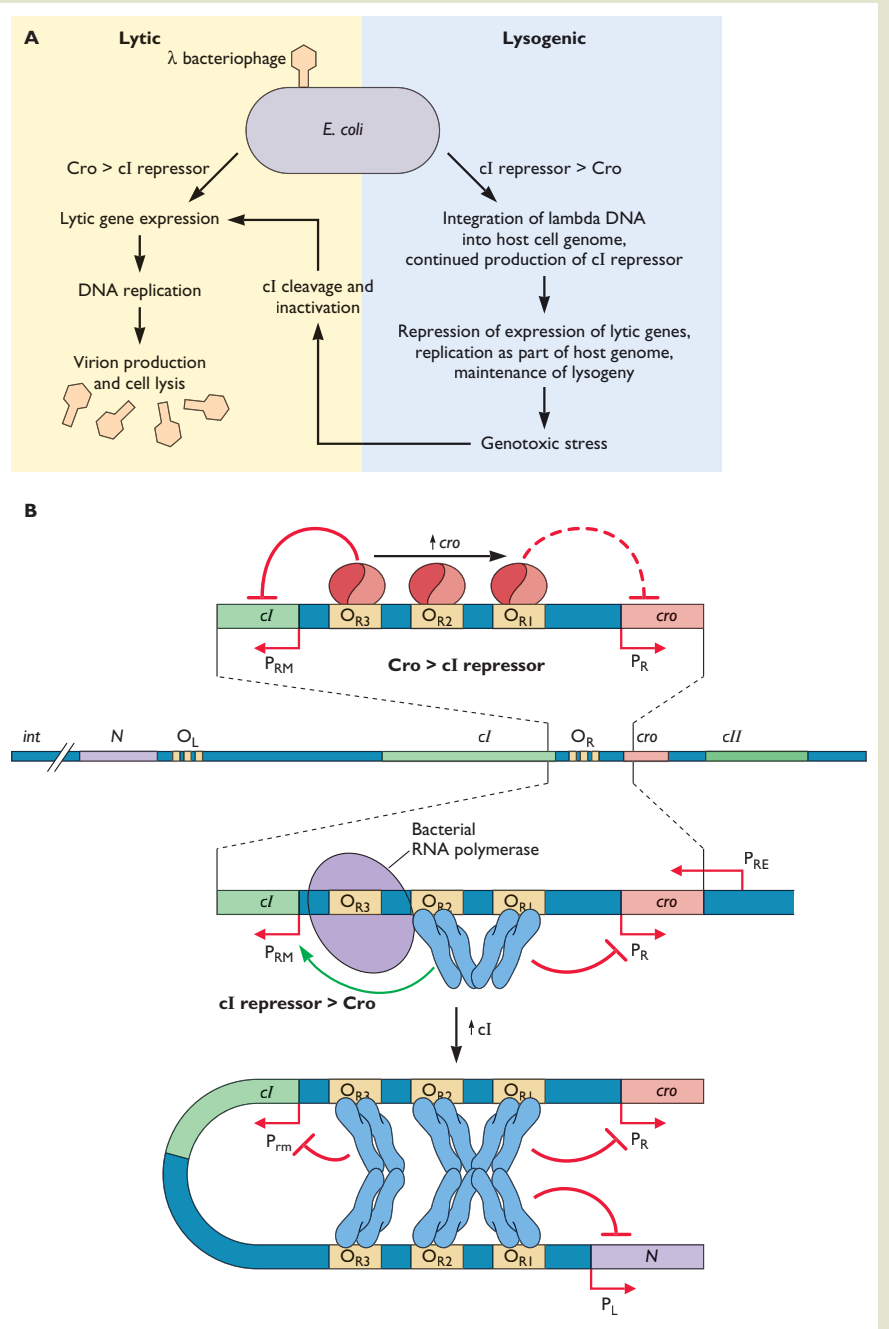
BOX 8.14 DISCUSSION

Two bacteriophage lambda repressors govern the outcome of infection

As summarized in panel A of the figure, infection of *Escherichcia coli* by bacteriophage lambda leads to either synthesis of progeny virions and lysis of the host cell (lytic infection) or stable integration of the viral genome into that of the host cell (lysogenic infection). During lysogeny, lytic genes are not expressed. The actions of two repressors of transcription encoded within the viral genome, the cI (lambda) repressor and Cro, make a major contribution to the lytic/lysogeny "decision." The regulatory circuits by which these proteins govern expression of lambda lytic and lysogeny genes were among the first to be understood in detail.

The region of the lambda genome containing the *cI* repressor and *cro* genes is illustrated at the top of panel B. These coding sequences are flanked by genes encoding proteins that regulate transcription during lytic infection (e.g., N), or that are required during establishment of lysogeny (e.g., *int*, which encodes an integrase). Although both repressors bind to the operator sequences O_R and O_L adjacent to the right (P_R) and left (P_L) promoters, respectively, events at O_R are critical in determining the outcome of infection. The expanded view of the region of the genome containing O_R and P_R indicates the three binding sites for the repressors, and the two promoters from which the *cI* gene is expressed, the promoters for repressor establishment and for repressor maintenance, P_{RE} and P_{RM}, respectively.

When the lambda genome enters a host cell, transcription from the P_R and P_{RE} promoters by the bacterial RNA polymerase leads to synthesis of the cI repressor and Cro. The highest-affinity binding site for the cI repressor in O_R is O_{R1}, but this dimeric protein binds cooperatively to O_{R1} and O_{R2}. As these binding sites overlap sequences of the P_R promoter essential for binding of *E. coli* RNA polymerase, transcription of *cro* (and other rightward lytic genes) is repressed (red bar). Transcription from P_L is blocked in the same way by binding of the cI repressor to O_{L1} and O_{L2}. The N-terminal domain of cI repressor bound to O_{R2} contacts the subunit of RNA polymerase that binds to the nearby P_{RM} promoter. This interaction stimulates the formation of an open initiation



(continued)

complex at the P_{RM} promoter, and hence transcription of the *cI* gene (green arrow). Consequently, as expression of lytic genes is repressed, the concentration of cI repressor is increased to a value some 10-fold higher than that compatible with expression of lytic genes. The cI repressor has only low affinity for the O_{R3}-binding site. However, cooperative interactions occur between dimers bound to the O_L and O_R sites, to facilitate binding to O_{R3} and repression of transcription from P_{RM}. Because of such cooperative binding, whether cI repressor stimulates or blocks its own synthesis is very sensitive to cI concentration, and repressor concentration is maintained within a narrow range.

Although Cro binds to the same O_R sites as the cI repressors, it has the highest affinity for O_{R3}. It therefore occupies this site preferentially, and then binds to O_{R2}, to block binding of RNA polymerase to the P_{RM} promoter. Consequently, the cI repressor does not attain the concentrations necessary for establishment (and maintenance) of lysogeny. Binding of Cro to O_{R2} and O_{R1} leads to weak repression of transcription from P_R (and from P_L by an analogous mechanism). This function of Cro favors lytic infection, for example, by reducing production of the cII protein, a transcriptional regulator that promotes lysogeny by activating transcription of the *cI* gene from P_{RE}, and of the integrase gene.

It has been known for many years that environmental conditions and the activities of particular host cell gene products influence the outcome of lambda infection. How these cues are integrated with one another and into the regulatory network comprising the cI repressor/Cro switch and other lambda regulators remains incompletely understood.

Dodd, B., K. E. Shearwin, and J. B. Egan. 2005. Revisited gene regulation in bacteriophage λ. *Curr. Opin. Genet. Dev.* **15:**145–152.

Ptashne, M., A. Jeffrey, A. D. Johnson, R. Maurer, B. J. Meyer, C. O. Pabo, T. M. Roberts, and R. T. Sauer. 1980. How λ repressor and Cro work. *Cell* **19:**1–11.

transcriptional program. Furthermore, whether a herpesvirus infection is latent or lytic, as well as reentry into the productive cycle from latency (**reactivation**), is governed by mechanisms that regulate transcription.

In the case of the gammaherpesvirus Epstein-Barr virus, the availability and activity of a single viral protein determine whether an infection is latent or lytic. As described in a previous section, transcription of the gene encoding the transcriptional activator Zta is repressed by cellular proteins when B cells are initially infected. This protein is necessary for transcription of viral early genes, as well as viral DNA replication during the lytic cycle. Consequently, a latent infection ensues until the infected cell is exposed to conditions that activate transcription of the Zta gene. As Zta also represses transcription of the genes expressed in latently infected cells, it can be viewed as a simple regulatory switch. In contrast, more complex mechanisms appear to determine the outcome of infection by the alphaherpesviruses, which establish latent infections in neurons.

During latent infection of neurons by herpes simplex virus type 1, a set of specific RNAs, termed the **latency-associated transcripts (LATs)**, are the only viral products synthesized in large quantities (Fig. 8.22). The 2.0- and 1.5-kb LATs accumulate to 40,000 to 100,000 copies within the nucleus, lack poly(A) tails, and are not linear molecules. Indeed, all properties established to date indicate that they are stable introns produced by splicing of precursor RNA, as discussed in Chapter 10. These noncoding RNAs have been reported to inhibit apoptosis, interfere with expression of interferon genes, and stimulate expression of the protein chaperone Hsp70, by mechanisms that are not yet well understood. Such functions presumably promote the survival of latently infected neurons.

The LATs are also important for establishing a latent infection. Their stable production in neuronal cells suppresses replication of the virus, and synthesis of the immediate-early gene products needed for progression through the productive infectious cycle. These observations raise the intriguing question of how viral RNAs with the properties of introns excised from pre-mRNAs repress expression of these viral genes. The 2.0-kb LAT RNA has been reported to be associated with cellular ribosomes and splicing proteins, suggesting that it might alter splicing of immediate-early pre-mRNAs or production of cellular proteins that modulate viral gene expression. However, the ability of LATs to modulate chromatin structure is likely to make a major contribution to inhibition of transcription of lytic genes. As a latent infection is established, lytic genes become organized by nucleosomes that carry modifications associated with repression of transcription. In contrast, the LAT gene is associated with nucleosomes containing histones with modifications characteristic of actively transcribed genes, at least in part because it is bounded by specialized DNA sequences (insulators) that serve as boundaries between different types of chromatin. When the LAT region is deleted, repressive chromatin does not form efficiently on lytic genes. The viral LATs may function like the growing list of cellular RNAs

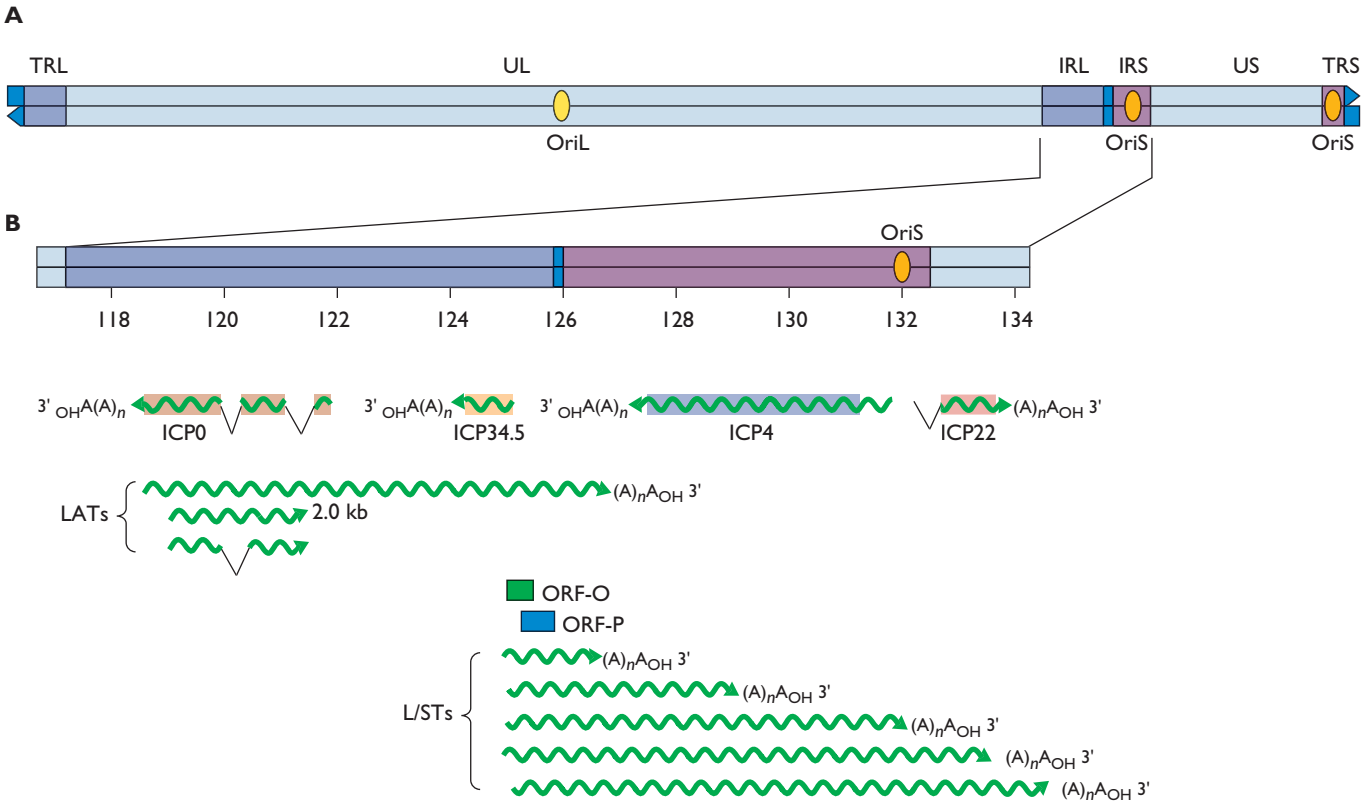


Figure 8.22 The latency-associated transcripts of herpes simplex virus type 1. (A) Diagram of the herpes simplex virus type 1 genome, showing the unique long and short segments, UL and US, respectively, the terminal repeat (TRL and TRS) and internal repeat (IRL and IRS) sequences, and the origins of replication, OriL and OriS. **(B)** Expanded map of the region shown, with the scale in kilobase pairs. This region encodes immediate-early proteins ICP0, ICP4, and ICP22, which play important roles in establishing a productive infection. Below are shown the locations of sequences encoding the latency-associated transcripts (LATs) and the transcripts named L/STs. The arrows indicate the direction of transcription, and $(A)_n A_{OH}$ indicates 3′ poly(A) sequences where known. Open reading frames, including ORF-O and ORF-P, are shown as boxes. The evidence currently available suggests that ORF-P (and perhaps ORF-O) may control LAT RNA synthesis.

that are known to regulate chromatin structure. The products of other RNAs made in smaller quantities in latently infected neurons, such as the L/ST RNAs (Fig. 8.22), can block the binding of the immediate-early ICP4 protein to promoter sequences. These observations suggest that once latency-associated genes are expressed in an infected neuron, the transcriptional cascade characteristic of productive infection is repressed.

As we have seen, the virion protein VP16 activates transcription of viral immediate-early genes and entry into the productive cycle transcriptional cascade. This viral protein enters infected neurons, but its ability to activate transcription is blocked by at least two mechanisms. In these cells, the essential VP16 cofactor Hcf is localized largely in the cytoplasm, sequestered from viral genomes and VP16 that enter infected cell nuclei. In addition, Hcf binds to Zhangfei, a cellular protein that is a strong repressor of transcription. Zhangfei is synthesized in sensory neurons (a natural site of latency), but not in most other cell types. Inhibition of production of the immediate-early ICP0 protein is also likely to be important, particularly to prevent reactivation from latency (when VP16 is not present). This protein is a powerful activator of transcription in simplified experimental systems, and is required for efficient viral replication in cells infected at low multiplicity and for efficient reactivation from latency. Among the several functions of ICP0 (see Volume II, Chapter 3) is activation of immediate-early transcription (in cooperation with ICP4), probably mediated at least in part by changes in histone modification. Whether the effects of LATs described previously are

Table 8.8 Viral RNA polymerase III transcription units

Virus	RNA polymerase III transcript	Function
Adenovirus		
Human adenovirus type 2	VA-RNA I	Prevention of activation of RNA-dependent protein kinase
	VA-RNA II	Not known
Herpesviruses		
Epstein-Barr virus	EBER 1	Always made in latently infected cells; implicated in transformation and oncogenesis
	EBER 2	
Herpesvirus saimiri	HSVR 1–5	Degradation of certain cellular mRNAs
Retrovirus		
Moloney murine leukemia virus	Let	Stimulation of transcription of specific cellular genes (e.g., CD4 and class I major histocompatibility complex genes)

sufficient to ensure that ICP0 and ICP4 cannot activate transcription of early genes remains to be established.

Transcription of Viral Genes by RNA Polymerase III

Several of the viruses considered in this chapter contain RNA polymerase III transcription units in their genomes (Table 8.8). The first, and still best understood, example is the gene encoding human adenovirus type 2 virus-associated RNA I (VA-RNA I). The VA-RNA I gene specifies an RNA product that ameliorates the effects of a host cell defense mechanism, and has also been implicated in RNA interference (Volume II, Chapter 3). It contains a typical, intragenic promoter that has been widely used in studies of initiation of transcription by RNA polymerase III.

RNA Polymerase III Transcribes the Adenoviral VA-RNA Genes

The VA-RNA I Promoter

The human adenovirus type 2 genome contains two VA-RNA genes located very close to one another (Appendix, Fig. 1B). The VA-RNA I promoter is described here, for it is the more thoroughly characterized. Transcription of the VA-RNA I gene depends on two intragenic sequences, the A and B boxes (Fig. 8.23A). Sequences upstream of sites of initiation are not necessary for promoter activity, but do modulate the specificity or efficiency of initiation. As in the RNA polymerase II system, the essential promoter sequences are binding sites for accessory proteins necessary for promoter recognition. The internal sequences are recognized by the RNA polymerase III-specific initiation protein TfIIIc, which binds to the promoter to begin assembly of an initiation complex that also contains TfIIIb and the enzyme (Fig. 8.23B). This pathway of initiation was elucidated by using *in vitro* assays. We can be confident that this same pathway operates in adenovirus-infected cells, for there is excellent agreement between the effects of A and B box mutations on VA-RNA I synthesis *in vitro* and in mutant virus-infected cells.

Regulation of VA-RNA Gene Transcription

The two VA-RNA genes are initially transcribed at similar rates, but during the late phase of infection, production of VA-RNA I is accelerated greatly. Such preferential transcription is the result of competition of the VA-RNA I promoter with the intrinsically much weaker transcriptional control region of the VA-RNA II gene for a limiting component of the RNA polymerase III transcriptional machinery. Repression of VA-RNA I transcription may account for the similar rates at which the two genes are transcribed during the early phase. The control of transcription of VA-RNA genes emphasizes the fact that transcription by RNA polymerase III can, and must, be regulated, although the mechanisms are probably less elaborate than those that govern transcription by RNA polymerase II. The properties of other viral RNA polymerase III transcription units illustrate the kinship of the RNA polymerase II and III systems (Fig. 8.24).

Inhibition of the Cellular Transcriptional Machinery in Virus-Infected Cells

Inhibition of cellular transcription in virus-infected cells offers several advantages. Cellular resources, such as substrates for RNA synthesis, can be devoted to the exclusive

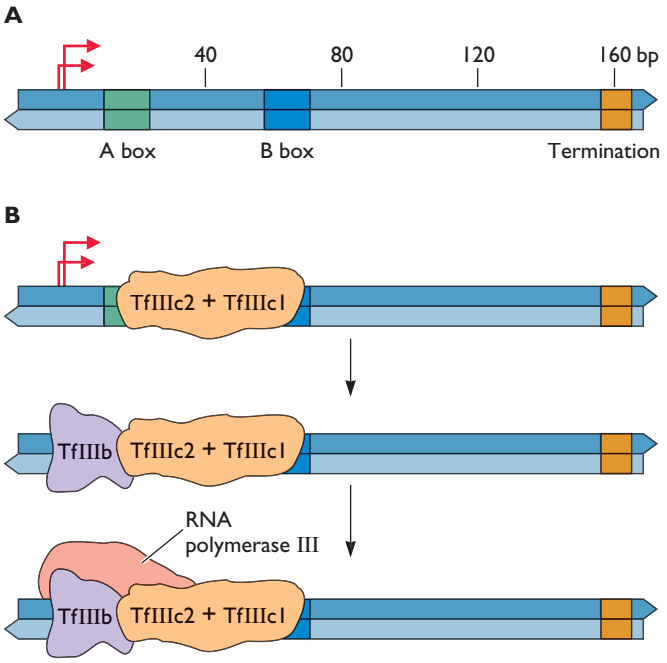


Figure 8.23 Organization and recognition of the human adenovirus type 2 VA-RNA I RNA polymerase III promoter. **(A)** The VA-RNA I gene is depicted to scale, in base pairs. The intragenic A and B box sequences are essential for efficient VA-RNA I transcription, and are closely related to the consensus A and B sequences of cellular tRNA genes. The VA-RNA termination site sequences are also typical of those of cellular genes transcribed by RNA polymerase III. **(B)** Assembly of an initiation complex on the VA-RNA I promoter is initiated by binding of TfIIIc2 to the intragenic promoter sequences. This reaction is stimulated by TfIIIc1, which makes contact with the A box. The TfIIIc-DNA complex is recognized by TfIIIb, which in turn allows recruitment of the polymerase. All mammalian RNA polymerase III initiation proteins contain multiple subunits. The TATA-binding protein is an essential component of TfIIIb.

production of viral mRNAs (and, in many cases, RNA genomes), and competition between viral and cellular mRNAs for components of the translational machinery is minimized. The essential participation of cellular transcriptional systems in the infectious cycles of most viruses considered in this chapter precludes inactivation of this machinery. However, posttranscriptional mechanisms allow selective expression of adenoviral and herpesviral genes (Chapter 10). Furthermore, herpes simplex virus type 1 infection induces inhibition of transcription of many cellular genes. Selective transcription of viral genes is accompanied by loss of the forms RNA polymerase II present in uninfected cells. Degradation of the hypophosphorylated form by the proteasome correlates with inhibition of transcription of cellular genes. As discussed previously, the unusual organization of viral late promoters probably facilitates selective transcription of late genes by the modified transcriptional machinery of the host cell. The poxviruses induce rapid inhibition of synthesis of all classes of cellular RNA in infected cells. Such inhibition requires viral proteins, but these have not been identified.

Replication of the majority of viruses with RNA genomes requires neither the cellular transcriptional machinery nor its RNA products, and is accompanied by inhibition of cellular mRNA synthesis. Among the best-characterized examples is the inhibition of transcription by RNA polymerase II characteristic of poliovirus-infected cells. The defect in RNA polymerase II transcription in extracts of poliovirus-infected cells can be explained by the fact that $3C^{pro}$ cleaves the Tbp subunit of TfIId at several sites. This modification eliminates the DNA-binding activity of Tbp and hence transcription by RNA polymerase II. The TATA-binding protein is also a subunit of initiation proteins that function with RNA polymerase III (TfIIIb) and RNA polymerase I. Its cleavage by $3C^{pro}$ in poliovirus-infected cells therefore appears to be a very efficient way to inhibit all cellular transcriptional activity. As poliovirus yields are reduced in cells that synthesize an altered form of Tbp resistant to cleavage by $3C^{pro}$, it is clear that inhibition of cellular transcription is necessary for optimal virus replication.

Two gene products of the rhabdovirus vesicular stomatitis virus have been implicated in inhibition of cellular transcription. The first is the viral leader RNA described in Chapter 6, which inhibits transcription by both RNA polymerase II and RNA polymerase III *in vitro* and is primarily responsible for the rapid inhibition of cellular RNA synthesis in infected cells. Following synthesis in the cytoplasm, the leader RNA enters the nucleus. The question of how short RNA molecules inhibit DNA-dependent RNA transcription cannot yet be answered, although *in vitro* experiments suggest that binding of a cellular protein to specific sequences within the RNA may be important. The viral M protein is also a potent inhibitor of transcription by RNA polymerase II, even in the absence of other viral gene products. This activity may become important later in infection, when replication of genome RNA predominates over transcription, such that less leader RNA is produced.

Unusual Functions of Cellular Transcription Components

In the preceding sections, we concentrated on the similarities among the mechanisms by which viral and cellular DNA are transcribed. Even though all mechanisms of regulation of expression of viral genes by the host cell's RNA polymerase II or RNA polymerase III cannot be described in molecular detail, it seems clear that the majority are not unique to viral systems. It is therefore an axiom of

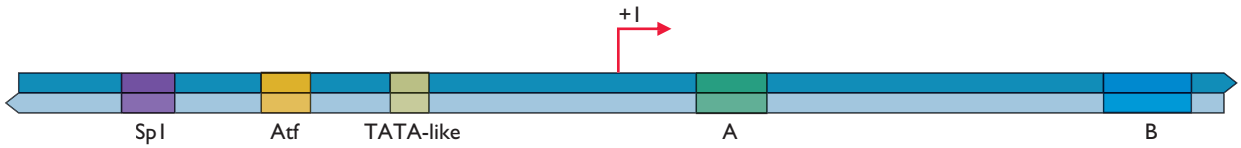


Figure 8.24 Some viral RNA polymerase III genes contain both external and internal promoter elements. The 5′ end of the Epstein-Barr virus EBER-2 transcription unit is shown to scale. This gene contains typical intragenic A and B box sequences. However, efficient transcription by RNA polymerase III also depends on the 5′ flanking sequence, which includes binding sites for the RNA polymerase II stimulatory proteins Sp1 and Atf. The TATA-like sequence is important for efficient transcription, and essential for specifying transcription by RNA polymerase III.

molecular virology that **every** mechanism by which viral transcription units are expressed by cellular components, or by which their activity is regulated, will prove to have a normal cellular counterpart. However, virus-infected cells also provide examples of novel functions or activities of cellular proteins that mediate transcription.

One example of the latter kind of mechanism is the production of hepatitis delta satellite virus RNA from an **RNA** template by RNA polymerase II, described in Chapter 6. The RNA of viroids, infectious agents of plants, is synthesized in the same manner. Such RNA-dependent RNA synthesis by RNA polymerase II from a specific RNA promoter is one of the most remarkable interactions of a viral genome with the cellular transcriptional machinery. No cellular analog of this reaction is yet known. Even more divergent functions of cellular transcriptional components in virus-infected cells are illustrated by the participation of the RNA polymerase III initiation proteins TfIIIb and TfIIIc in integration of the yeast retroid element Ty3 (see Chapter 7). Given the large repertoire of molecular and biochemical activities displayed by components of the eukaryotic transcriptional machinery, it seems likely that other unusual activities of these cellular proteins in virus-infected cells will be discovered.

A Viral DNA-Dependent RNA Polymerase

The DNA genomes of viruses considered in preceding sections replicate in the nucleus of infected cells, where the cellular transcriptional machinery resides. In contrast, poxviruses such as vaccinia virus are reproduced exclusively in the cytoplasm of their host cells. This feat is possible because the genomes of these viruses encode the components of a transcription and RNA-processing system that produces viral mRNAs with the hallmarks of cellular mRNA 5′ caps and 3′ poly(A) tails. This system, which is carried into infected cells within virions, includes a DNA-dependent RNA polymerase responsible for transcription of all vaccinia virus genes. A striking feature of the viral enzyme is its structural and functional resemblance to cellular RNA polymerases.

The vaccinia virus RNA polymerase, like cellular RNA polymerases, is a complex, multisubunit enzyme built from the products of at least eight genes. The amino acid sequences of several of these subunits, including the two largest and the smallest, are clearly related to subunits of RNA polymerase II. The viral enzyme transcribes all classes of vaccinia virus genes expressed at different times in the infectious cycle (early, intermediate, and late). Like the cellular enzymes, vaccinia virus RNA polymerase recognizes promoters by cooperation with additional proteins. For example, formation of initiation complexes on vaccinia virus early promoters is mediated by two viral proteins, VETF and RAP94, which are responsible for the recognition of promoter sequences and recruitment of the RNA polymerase, respectively (Fig. 8.25). These viral proteins are functional analogs of the cellular RNA polymerase II initiation proteins TfIId and TfIIf (Box 8.3). However, the vaccinia virus transcriptional machine is not analogous to its cellular counterpart in every respect. Cellular RNA polymerase II generally transcribes far beyond the sites at which the 3′ ends of mature cellular or viral mRNAs are produced by processing of the primary transcript, and does not terminate transcription at simple, discrete sites. In contrast, transcription of the majority of vaccinia virus early genes **does** terminate at discrete sites, 20 to 50 bp downstream of specific T-rich sequences in the template. Termination requires the viral termination protein, which is also the viral mRNA-capping enzyme (see Chapter 10). The 3′ ends of the viral mRNAs correspond to sites of transcription termination. This viral mechanism is considerably simpler than the cellular counterpart.

The viral RNA polymerase and the several other proteins necessary for transcription of early genes enter host cells within vaccinia virus particles. Subsequently, viral gene expression is controlled by the ordered synthesis of viral proteins that permit sequential recognition of intermediate and late promoters. For example, transcription of intermediate genes requires synthesis of the viral RPO30 gene product (a subunit of the viral polymerase) and a second viral protein termed VITF3, while late transcription depends on synthesis of several intermediate gene

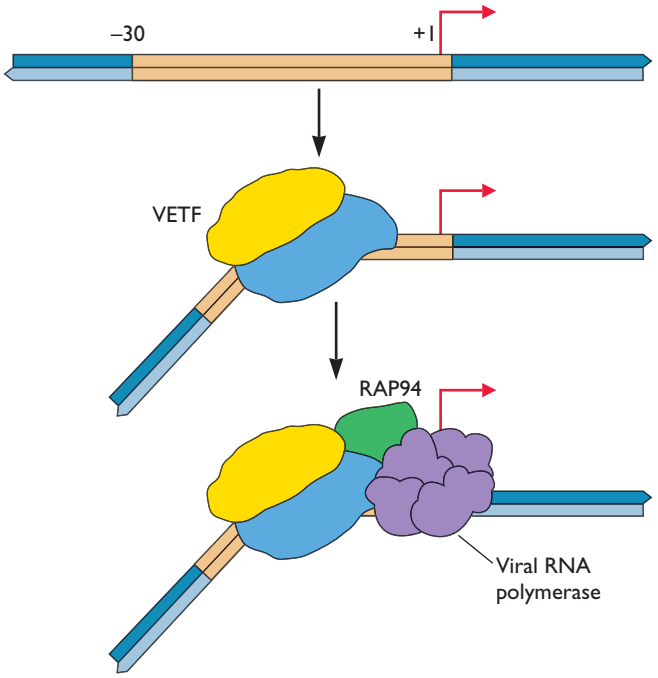


Figure 8.25 Assembly of an initiation complex on a vaccinia virus early promoter. Vaccinia virus early promoters contain of an AT-rich sequence (tan) immediately upstream of the site of initiation. Vaccinia virus RNA polymerase cannot recognize these (or any other) viral promoters in the absence of other viral proteins. The vaccinia virus early transcription protein (VETF) is necessary for early promoter recognition and must bind before the viral RNA polymerase. This heteromeric protein binds specifically to early promoters and induces DNA bending. It also possesses DNA-dependent ATPase activity. VETF and the second protein necessary for early promoter specificity, RAP94, enter infected cells in virions. The RAP94-RNA polymerase complex associates with early promoter-bound VETF to form a functional initiation complex. Assembly of these vaccinia virus initiation complexes is therefore analogous to, although simpler than, formation of RNA polymerase II initiation complexes (Box 8.4).

products. The viral genome also encodes several proteins that regulate elongation during transcription of late genes. Activation of intermediate and late gene transcription also requires viral DNA replication. Transcription of vaccinia virus genetic information is therefore regulated by mechanisms similar to those operating in cells infected by other DNA viruses, even though the transcriptional machinery is viral in origin.

Surprisingly, the vaccinia virus transcription system is not entirely self-contained: a cellular protein is essential for transcription of viral intermediate genes. This protein, termed Vitf2, is located in the nucleus of uninfected cells, but is present in both the cytoplasm and the nucleus of infected cells. As a significant number of vaccinia virus genes encode proteins necessary for transcription, such dependence on a cellular protein must confer some special advantage. An attractive possibility is that interaction of the viral transcriptional machinery with a cellular protein serves to integrate the viral reproductive cycle with the growth state of its host cell. The identification of Vitf2 as a heterodimer of proteins that are produced in greatest quantities in proliferating cells is consistent with this hypothesis.

Perspectives

It is difficult to exaggerate the contributions of viral systems to the elucidation of mechanisms of transcription and its regulation in eukaryotic cells. The organization of RNA polymerase II promoters considered typical was first described for viral transcriptional control regions, enhancers were first discovered in viral genomes, and many important cellular regulators of transcription were identified by virtue of their specific binding to viral promoters. Perhaps even more importantly, efforts to elucidate the molecular basis of regulatory circuits crucial to viral infectious cycles have established general principles of eukaryotic transcriptional regulation. These include the importance of proteins that do not recognize DNA sequences directly, and the ability of a single transcriptional regulator to modulate multiple components of the machinery. The insights into regulation of elongation by RNA polymerase II gained from studies of the human immunodeficiency virus type 1 Tat protein emphasize the intimate relationship of viral proteins with cellular components that make viral systems such rich resources for the investigation of eukaryotic transcription.

The identification of cellular and viral proteins necessary for transcription of specific viral genes has allowed many regulatory circuits to be traced. For example, the tissue distribution or the availability of particular cellular activators that bind to specific viral DNA sequences can account for the tropism of individual viruses, or conditions under which different transcriptional programs (latent or lytic) can be established. Furthermore, the mechanisms that allow sequential expression of viral genes are quite well established. Regardless of whether regulatory circuits are constructed of largely cellular or mostly viral proteins, these transcriptional cascades share such mechanistic features as sequential production of viral activators, and integration of transcription of late genes with synthesis of viral DNA. Our ability to describe these regulatory mechanisms in some detail represents enormous progress.

The models for the individual regulatory processes described in this chapter were developed by using convenient and powerful experimental systems. It is clear that such simplified systems (e.g., *in vitro* transcription reactions

and transient-expression assays) do not reproduce all features characteristic of infected cells, even in the simplest cases, and cannot address such issues as how transcription of specific genes can be coupled with replication of the viral genome. It is therefore crucial that the models be tested in virus-infected cells, even though it is more difficult to elucidate the molecular functions and mechanisms of action of transcriptional components *in vivo*. Many viral regulatory proteins perform multiple functions, a property that can confound genetic analysis. Moreover, the study of individual reactions, such as binding of a protein to a specific promoter sequence, or formation of preinitiation complexes, is technically more demanding. Nevertheless, viral *cis*-acting sequences and regulatory proteins remain more amenable to genetic analyses of their function in the natural context than do their cellular counterparts. In conjunction with increasingly powerful and sensitive methods for examining intracellular processes, such as the chromatin immunoprecipitation assay, continued efforts to exploit such genetic malleability will eventually establish how transcription of viral DNA templates is mediated and regulated within infected cells. Such information not only will address outstanding virological questions, but also should allow current models of the fundamental cellular process of transcription to be refined.

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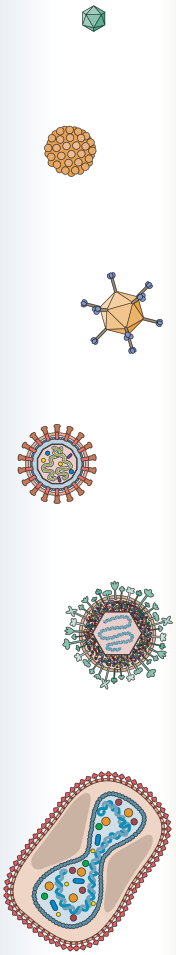


9

Genome Replication Strategies: DNA Viruses

The more the merrier.
ANONYMOUS

Introduction

The genomes of DNA viruses come in a considerable variety of sizes and shapes, from small single-stranded to large double-stranded molecules that may be linear or circular (Table 9.1). Whatever their physical nature, viral DNA molecules must be replicated efficiently within an infected cell to provide genomes for assembly into progeny virions. Such replication invariably requires the synthesis of at least one viral protein and often expression of several viral genes. Consequently, viral DNA synthesis cannot begin immediately upon arrival of the genome at the appropriate intracellular site, but rather is delayed until viral replication proteins have attained a sufficient concentration. Initiation of viral DNA synthesis typically leads to many cycles of replication, and the accumulation of very large numbers of newly synthesized DNA molecules. However, more long-lasting virus-host cell interactions, such as latent infections, are also common, both in nature and in the laboratory. In these circumstances, the number of viral DNA molecules made is strictly controlled.

Replication of all DNA, from the genome of the simplest virus to that of the most complex vertebrate cell, follows a set of universal rules: (i) DNA is always synthesized by template-directed, stepwise incorporation of deoxynucleoside monophosphates (dNMPs) from deoxynucleoside triphosphate (dNTP) substrates into the 3′-OH end of the growing DNA chain; (ii) each parental strand of a duplex DNA template is copied by base pairing to produce two daughter molecules identical to one another and to their parent (**semiconservative replication**); (iii) replication of DNA begins and ends at specific sites in the template, termed **origins** and **termini**, respectively; and (iv) DNA synthesis is catalyzed by DNA-dependent DNA polymerases, but many accessory proteins are required for initiation or elongation. In contrast to all DNA-dependent, and many RNA-dependent, RNA polymerases, **no** DNA polymerase can initiate template-directed DNA synthesis *de novo*. All require a **primer** with a free 3′-OH end to which dNMPs complementary to those of the template strand are added.

Table 9.1 Cellular and viral proteins used in synthesis of viral DNA

Virus	Genome	Viral origin-binding protein(s)	DNA polymerase(s)	Effects of infection on cellular DNA synthesis
Parvovirus				
Adeno-associated virus type 2	4,680 bp, linear, single (−) or (+) strands	Rep 78/68	Cellular	None
Papovavirus				
Simian virus 40	5,243 bp, closed, double-stranded circle	LT	Cellular, DNA polymerases α and δ	Induction of entry into S phase and cellular DNA synthesis
Adenovirus				
Human adenovirus type 2	35,937 bp, linear, double stranded	DNA polymerase preterminal protein complex	Viral	Induction of entry into S phase in quiescent cells; inhibition of cellular DNA synthesis in actively growing cells
Herpesviruses				
Herpes simplex virus type 1	~150 kbp, linear, double stranded, 4 isomers	UL9	Viral	Inhibition of cellular DNA synthesis
Epstein-Barr virus	172 kbp, linear, double stranded			Immortalization of latently infected B cells
OriP		EBNA-1	Cellular	
OriLyt		Zta	Viral	Induction of cell cycle arrest and inhibition of cellular DNA synthesis by Zta
Poxvirus				
Vaccinia virus	~200 kbp, linear, double stranded with closed terminal loops	None known	Viral	Inhibition of cellular DNA synthesis

The genomes of RNA viruses must encode enzymes that catalyze RNA-dependent RNA or DNA synthesis. In contrast, those of DNA viruses can be replicated by the cellular machinery. Indeed, replication of the smaller DNA viruses, such as parvoviruses and polyomaviruses, requires but a single viral replication protein, and the majority of reactions are carried out by cellular proteins (Table 9.1). This strategy avoids the need to devote limited viral genetic coding capacity to enzymes and other proteins required for DNA synthesis. In contrast, the genomes of all larger DNA viruses encode DNA polymerases and additional replication proteins (Table 9.1). In the extreme case, exemplified by poxviruses, the viral genome encodes a complete DNA synthesis system, and is replicated in the cytoplasm of its host cells.

There is also considerable variety in the mechanism of priming of viral DNA synthesis. In some cases, DNA synthesis is initiated with RNA primers, the mechanism by which cellular genomes are replicated. In others, unusual structural features of the genome or viral proteins provide primers. Despite such distinctions, the replication strategies of different viral DNAs are based on the only two mechanisms of double-stranded DNA synthesis known (Box 9.1) and on common molecular principles. For example, the genomes of polyomaviruses and herpesviruses, which are quite different in size and structure, are replicated by the cellular replication machinery and viral replication proteins, respectively (Table 9.1). Nevertheless, synthesis of these two DNAs is initiated by the same priming mechanism, and the herpesviral replication machinery carries out the same biochemical reactions as the host proteins that mediate synthesis of simian virus 40 DNA.

DNA Synthesis by the Cellular Replication Machinery: Lessons from Simian Virus 40

Our current understanding of the intricate reactions by which both strands of a typical double-stranded DNA template are copied in eukaryotic cells is based on *in vitro* studies of simian virus 40 DNA synthesis. In this section, we focus on the cellular replication machinery and the molecular functions of its components that have been established by using this viral system. However, we first briefly discuss general features of eukaryotic DNA replication, and why simian virus 40 proved to be a priceless resource for those seeking to understand this process.

Eukaryotic Replicons

General Features

The complete replication of large eukaryotic genomes within the lifetime of an actively growing cell depends on their organization into smaller units of replication termed **replicons** (Fig. 9.1): at the maximal rate of replication

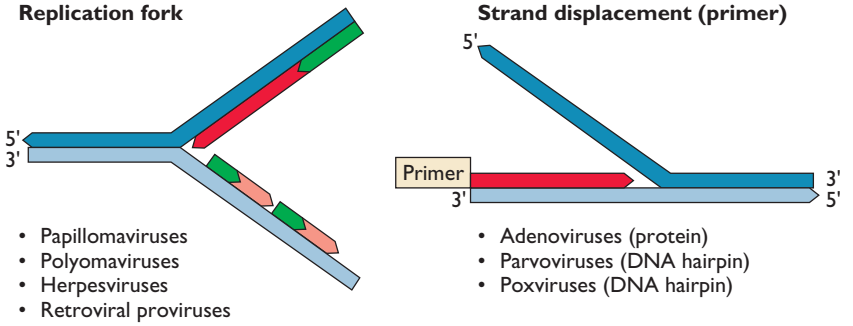
BOX 9.1 BACKGROUND

The two mechanisms of synthesis of double-stranded viral DNA molecules

Replication of double-stranded nucleic acids proceeds by **either** synthesis of daughter strands from a replication fork, **or** strand displacement. No other replication mechanisms are known.

Among viral genomes, only those of certain double-stranded DNA viruses are synthesized via a replication fork. Replication of viral double-stranded RNAs **never** proceeds via this mechanism.

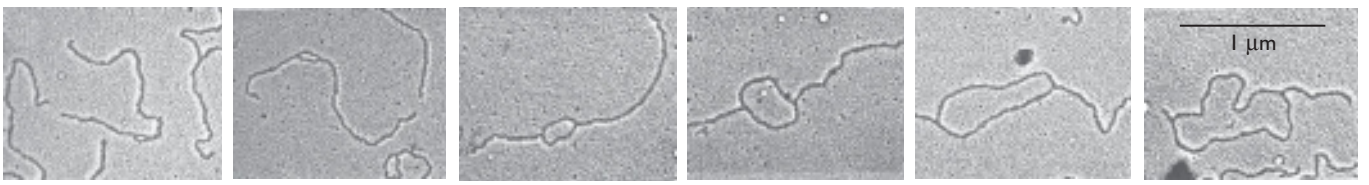
DNA synthesis via a replication fork is **always** initiated from an RNA primer. In contrast, strand displacement synthesis of viral DNA **never** requires an RNA primer.



Parental DNA, RNA primers, and newly synthesized DNA are shown in blue, green, and red/pink, respectively. The primer indicated by the tan box can be a DNA structure or a protein.

Figure 9.1 Properties of replicons. (A) Electron micrographs of replicating simian virus 40 DNA, showing the "bubbles" of replicating DNA, in which the two strands of the template are unwound. These linear DNA molecules were obtained by restriction cleavage of viral enzyme DNA that had replicated to different degrees in infected cells. They are arranged in order of increasing degree of replication to illustrate the progressive movement of the two replication forks from a single origin of replication. From G. C. Fareed et al., *J. Virol.* **10**:484–491, 1972, with permission. **(B)** Bidirectional replication from an origin. Newly synthesized DNA is shown in red and pink, a convention used throughout the text.

A



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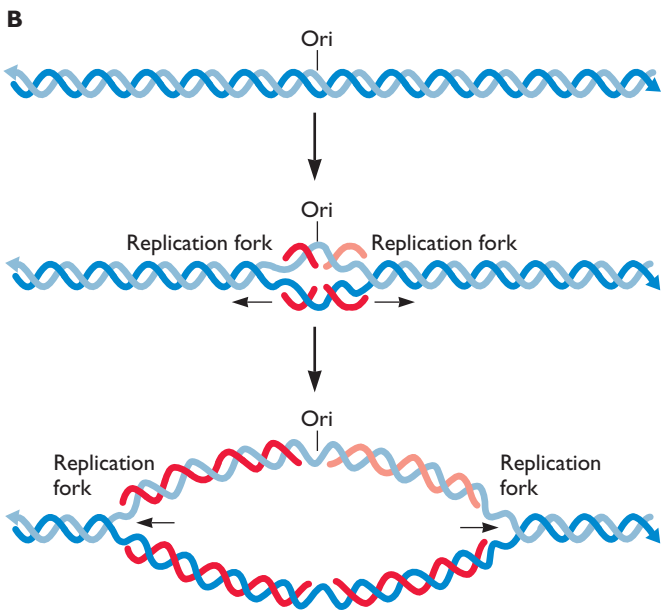


observed *in vivo*, a typical human chromosome could not be replicated as a single unit in less than 10 days! Each chromosome therefore contains many replicons, ranging in length from about 20 to 300 kbp. With some exceptions, no viral DNA genome is larger than the upper limit reported for such cellular replicons. Nevertheless, all but the smallest contain two or three origins (see "Properties of Viral Replication Origins" below).

Each replicon contains a single origin at which replication begins. The sites at which nascent DNA chains are being synthesized, the ends of "bubbles" seen in the electron microscope (Fig. 9.1A), are termed **replication forks**. In bidirectional replication, two replication forks are established at a single origin and move away from it as the new DNA strands are synthesized (Fig. 9.1B). However, as DNA must be synthesized in the 5′ → 3′ direction, only one of the two parental strands can be copied continuously from a primer deposited at the origin. The long-standing conundrum of how the second strand is synthesized was solved with the elucidation of the discontinuous mechanism of synthesis (Fig. 9.2A). Primers for DNA synthesis are synthesized at multiple sites, such that this new DNA strand is made initially as short, discontinuous segments.

The discontinuous mechanism of DNA synthesis creates a special problem at the ends of linear DNAs, where excision of the terminal primer creates a gap at the 5′ end of the daughter DNA molecules (Fig. 9.2B). In the absence of a mechanism for completing synthesis of termini, discontinuous DNA synthesis would lead to an intolerable loss of genetic information. In chromosomal DNA, specialized elements, called **telomeres**, at the ends of each chromosome prevent loss of end sequences. These structures comprise simple, repeated sequences maintained by reverse transcription of an RNA template, which is an essential component of the ribonucleoprotein enzyme **telomerase**. Complete replication of all sequences of linear viral DNA genomes (Table 9.1) is achieved by a variety of elegant mechanisms.

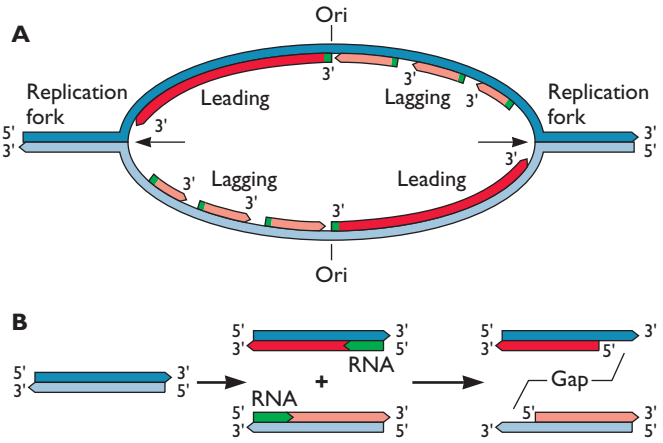


Figure 9.2 Semidiscontinuous DNA synthesis from a bidirectional origin. (A) Semidiscontinuous synthesis of the daughter strands. Synthesis of the RNA primers (green) at the origin allows initiation of continuous copying of one of the two strands on either side of the origin in the replication bubble. The second strand cannot be made in the same way (see the text). The nascent DNA population contains many small molecules termed **Okazaki fragments** in honor of the investigator who first described them. The presence of short segments of RNA at the 5′ ends of Okazaki fragments indicated that the primers necessary for DNA synthesis are molecules of RNA. With increasing time of replication, these small fragments are incorporated into long DNA molecules, indicating that they are precursors. It was therefore deduced that the second nascent DNA strand is synthesized discontinuously, also in the 5′ → 3′ direction. Because synthesis of this strand cannot begin until the replication fork has moved some distance from the origin, it is called the **lagging strand**, while the strand synthesized continuously is termed the **leading strand**. Complete replication of the lagging strand requires enzymes that can remove RNA primers, repair the gaps thus created, and ligate the individual DNA fragments to produce a continuous copy of the template strand. **(B)** Incomplete synthesis of the lagging strand. When a DNA molecule is linear, removal of the terminal RNA primer from the 5′ end of the lagging strand creates a gap that cannot be repaired by any DNA-dependent DNA polymerase.

Origins of Cellular Replication

Identification of replication origins of eukaryotes (other than budding yeasts) has proved to be one of the most frustrating endeavors of modern molecular biology. A large body of evidence indicates that replication initiates at specific sites *in vivo*. With the availability of cloned genomic DNA segments, these origins can be identified and mapped with some precision. The difficulty lies in their functional identification. *Saccharomyces cerevisiae* origins can be assayed readily, because they support replication of small plasmids and hence their maintenance as episomes. All yeast origins behave as such **autonomously replicating sequences**. In contrast, this simple functional assay generally has failed to identify analogous mammalian sequences, even when applied to DNA segments containing origins mapped *in vivo*. Conversely, many long DNA fragments (including prokaryotic DNAs) support replication of plasmids in mammalian cells, but apparently direct random initiation of DNA replication. The paradox of why DNA sequences that support plasmid replication are so difficult to find has yet to be fully resolved. However, there is accumulating evidence that metazoan DNA replication initiates within long DNA segments (several kilobase pairs or more), and that their function can depend on far more distant sequences, or epigenetic changes. Despite the considerable differences in size and complexity of fungal and mammalian origins of replication, the origin recognition machinery is highly conserved among eukaryotes.

The Origin of Simian Virus 40 DNA Replication

Because the identification of functional origins of most eukaryotic genomes was so difficult, progress in elucidation of the mechanisms of origin-dependent DNA synthesis came through study of viral origins, notably that of simian virus 40. This origin, which supports bidirectional replication, was the first viral control sequence to be located on a physical map in which the reference points are restriction endonuclease cleavage sites (Fig. 9.1A; Box 9.2). We now possess a detailed picture of this origin (Fig. 9.3), and of the binding sites for the simian virus 40 origin recognition protein, large T antigen (LT).

A 64-bp sequence, the core origin, which lies between the initiation sites of early and late transcription, is sufficient for initiation of simian virus 40 DNA synthesis in infected cells. This sequence contains four copies of a pentanucleotide-binding site for LT, flanked by an AT-rich element and a 10-bp imperfect palindrome (Fig. 9.3). Additional sequences within this busy control region of the viral genome increase the efficiency of initiation of DNA synthesis from the core origin.

Cellular Replication Proteins and Their Functions during Simian Virus 40 DNA Synthesis

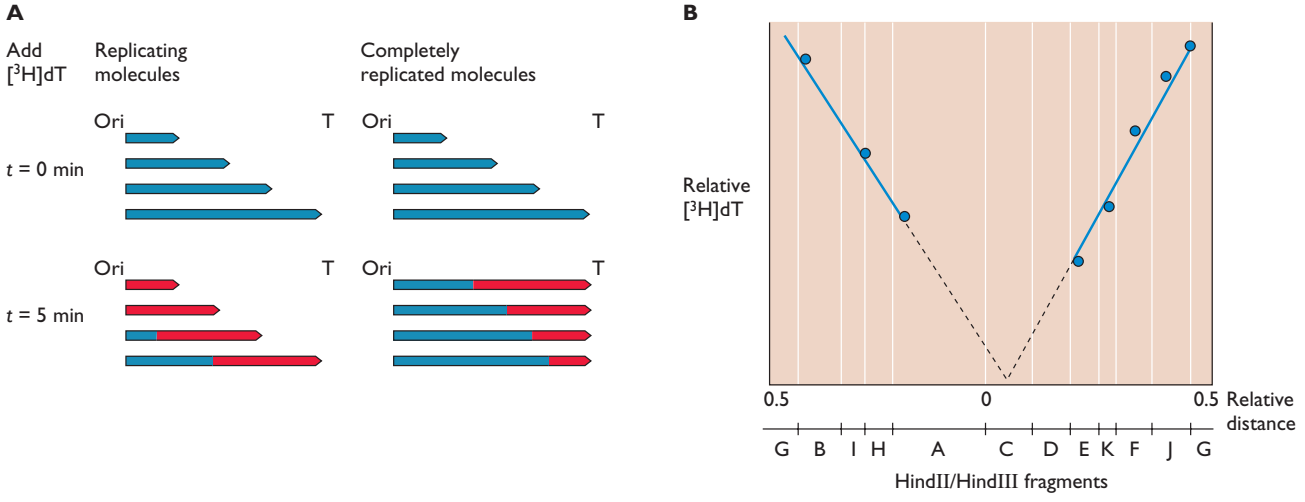
Eukaryotic DNA Polymerases

It has been known for over 50 years that eukaryotic cells contain DNA-dependent DNA polymerases. Mammalian cells contain several such nuclear enzymes, which are distinguished by such properties as sensitivities to various inhibitors and their degree of **processivity**, the number of nucleotides incorporated into a nascent DNA chain per initiation from a primer carrying a 3′-OH group. These characteristics can be readily assayed in *in vitro* reactions with artificial template-primers, such as gapped or

BOX 9.2 EXPERIMENTS

Mapping of the simian virus 40 origin of replication

As illustrated in panel **A** (left), exposure of simian virus 40-infected monkey cells to [^{3}H]thymidine ([^{3}H]dT) for a period less than the time required to complete one round of replication (e.g., 5 min) results in labeling of the growing points of replicating DNA. If replication proceeds from a specific origin (Ori) to a specific termination site (T), the DNA replicated last will be preferentially labeled in the population of completely replicated molecules (panel **A**, right). The distribution of [^{3}H]thymidine among the fragments of completely replicated viral DNA generated by cleavage with HindII and HindIII is shown in panel **B**. The simian virus 40 genome is represented as cleaved within the G fragment, and relative distances are given with respect to the junction of the A and C fragments. The observation of two decreasing gradients of labeling that can be extrapolated (dashed lines) to the same region of the genome confirmed that simian virus 40 replication is bidirectional (Fig. 9.1A) and allowed location of the origin on the physical map of the viral genome. Modified from K. J. Danna and D. Nathans, *Proc. Natl. Acad. Sci. USA* **69:**3097–3100, 1972, with permission.



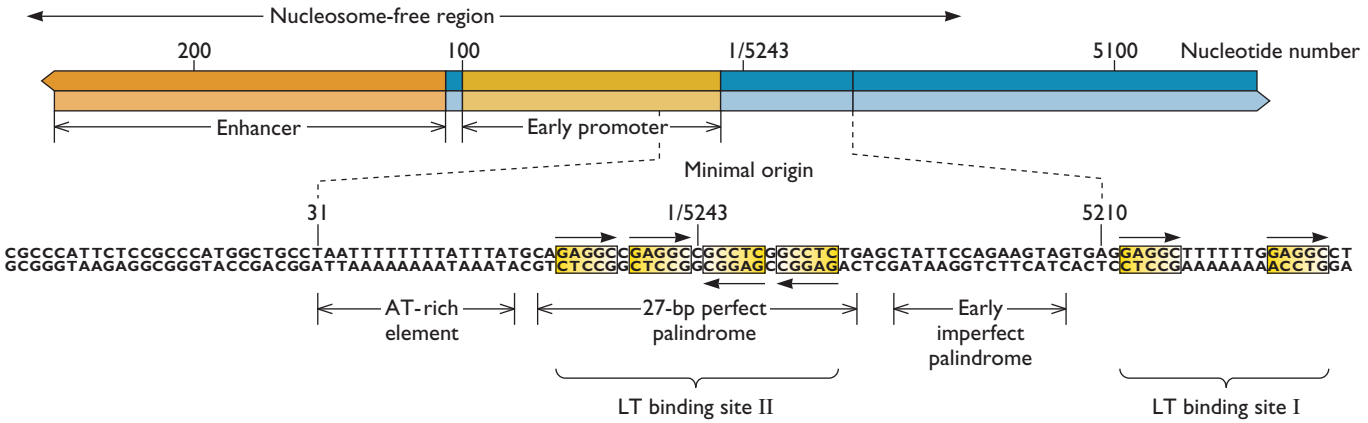


Figure 9.3 The origin of simian virus 40 DNA replication. The positions of the minimal origin necessary for simian virus 40 DNA replication *in vivo* and *in vitro* and of the enhancer and early promoter (see Chapter 8) in the viral genome are indicated. The sequence of the minimal origin is shown below, with the pentameric LT-binding sites ($G^A_C GGC$) in yellow. The AT-rich element and early imperfect palindrome, as well as LT-binding site II, are essential for replication. A second LT-binding site (site I) modestly stimulates replication *in vivo*. Other sequences, including the enhancer and Sp1-binding sites of the early promoter, increase the efficiency of viral DNA replication at least 10-fold. The activation domains (see Chapter 8) of these transcriptional regulators might help recruit essential replication proteins to the origin. Alternatively, the binding of transcriptional activators might induce remodeling of chromatin in the vicinity of the origin. This possibility is consistent with the fact that, as indicated at the top, the region of the genome containing the origin and transcriptional control regions is nucleosome free in a significant fraction (about 25%) of minichromosomes in infected cells.

nicked DNA molecules. However, distinguishing their roles in DNA replication was impossible until simian virus 40 origin-dependent DNA synthesis was reproduced *in vitro* (see the next section). The requirements for viral DNA synthesis *in vitro* and genetic analyses (performed largely with yeasts) identified DNA polymerases α, δ, and ε as replication enzymes. Only DNA polymerase α is associated with priming activity. A heteromeric **primase** is tightly bound to DNA polymerase α, and copurifies with it.

One of the most striking properties of these replicative DNA polymerases is their obvious evolutionary relationships to prokaryotic and viral enzymes. Six regions are highly conserved in both primary amino acid sequence and relative position in a wide variety of these DNA polymerases. As discussed in Chapters 6 and 7, all template-directed nucleic acid polymerases share several sequence motifs and probably a similar core structure, indicating that important features of the catalytic mechanisms are also common to all these enzymes.

Origin-Dependent Viral DNA Replication In Vitro

Studies of eukaryotic DNA replication took a quantum leap forward with the development of a cell-free system for synthesis of adenoviral DNA from an exogenous template. This breakthrough was soon followed by origin-dependent replication of simian virus 40 DNA *in vitro*. Because cellular components are largely responsible for simian virus 40 DNA synthesis, this system proved to be the watershed in the investigation of eukaryotic DNA replication: it allowed the identification of previously unknown cellular replication proteins, and elucidation of the mechanism of DNA synthesis.

Mechanism of Simian Virus 40 DNA Synthesis

Origin recognition and unwinding. The first step in simian virus 40 DNA synthesis is the recognition of the origin by LT, the major early gene product of the virus. This viral protein can bind to the core origin to form a hexamer on any of the pentanucleotide repeats (Fig. 9.3). However, initiation of viral DNA synthesis requires the flanking sequences of the minimal origin and ATP-dependent formation of two LT hexamers that encase the DNA sequence (Fig. 9.4). The double hexamers bind over the palindromic pairs of pentanucleotide repeats in the origin, with additional non-sequence-specific contacts with the flanking DNA. In reactions that require ATP but not its hydrolysis, the LT hexamers undergo conformational change and, in turn, induce structural transitions in the AT-rich and the early imperfect palindrome sequences that flank the LT-binding sites (Fig. 9.4). The next reaction, LT-induced unwinding of the origin, requires cellular replication protein A (Rp-A), which possesses single-stranded-DNA-binding activity and binds specifically to LT

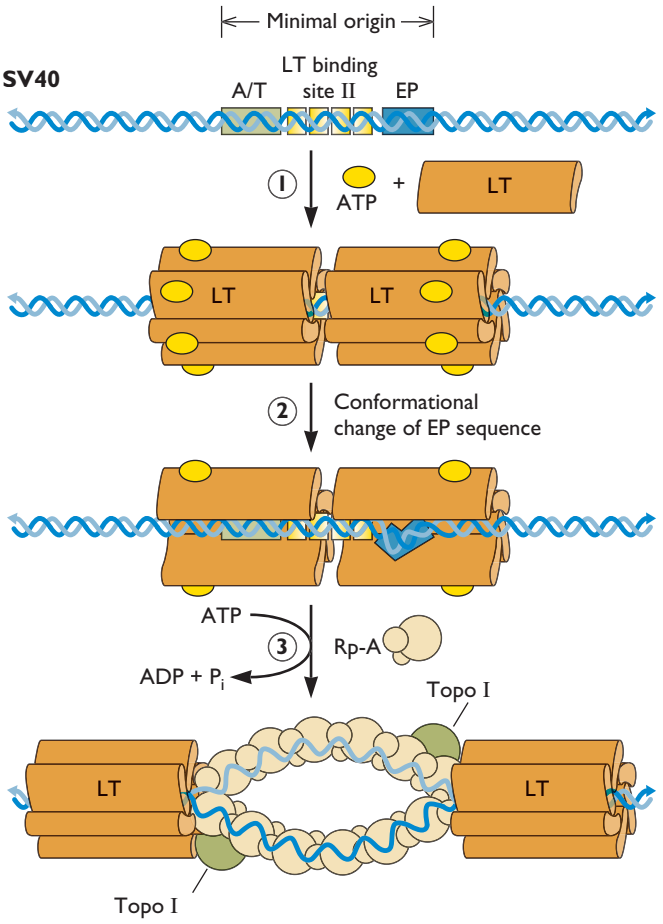


Figure 9.4 Model of the recognition and unwinding of the simian virus 40 (SV40) origin. In the presence of ATP, two hexamers bind to the origin via the pentanucleotide LT-binding sites (step 1). Binding of LT hexamers protects the flanking AT-rich (A/T) and early palindrome (EP) sequences of the minimal origin from DNase I digestion and induces conformational changes, for example distortion of the early palindrome (step 2). Stable unwinding of the origin requires the cellular, single-stranded-DNA-binding protein replication protein A (Rp-A), which binds to LT. LT helicase activity, in concert with Rp-A and topoisomerase I, progressively unwinds the origin (step 3). For simplicity, the two LT hexamers are shown moving apart, but they probably remain in contact (Box 9.3).

(Table 9.2). In concert with Rp-A and cellular topoisomerase I, the intrinsic 3′ → 5′ helicase activity of LT harnesses the energy of ATP hydrolysis to unwind DNA bidirectionally from the core origin (Fig. 9.4). Formation of the LT complex at the simian virus 40 origin resembles assembly reactions at well-characterized bacterial origins, such as *Escherichia coli* OriC, or the origin of phage λ, in which multimeric protein structures form on AT-rich sequences. Furthermore, formation of hexamers around DNA is a property common to several viral and cellular replication proteins.

Leading-strand synthesis. Binding of DNA polymerase α-primase to both LT and Rp-A at the simian virus 40 origin completes assembly of the **presynthesis complex**, and sets the stage for the initiation of leading-strand synthesis (Fig. 9.5). The primase synthesizes the RNA primers of the leading strand at each replication fork, while DNA polymerase α extends them to produce short fragments. The 3′-OH ends of these DNA fragments are then bound by cellular replication factor C (Rf-C), proliferating-cell nuclear antigen (Pcna), and DNA polymerase δ (Fig. 9.5). Pcna, which is highly conserved, is the processivity factor for DNA polymerase δ: it is required for synthesis of long DNA chains from a single primer and essential for simian virus 40 DNA replication *in vitro*. This mammalian protein is the functional analog of the β subunit of *E. coli* DNA polymerase III and phage T4 gene 45 product. These remarkable proteins are **sliding clamps**, which track along the DNA template and serve as movable platforms for DNA polymerases (Fig. 9.6). As sliding clamp proteins are closed rings, Pcna cannot load onto DNA molecules that lack ends, such as the circular simian virus 40 genome or chromosomal domains of genomic DNA. This essential, ATP-dependent step is carried out by the clamp-loading protein Rf-C, which induces transient opening of the Pcna ring. Because Rf-C binds to the 3′-OH ends of DNA fragments, it places the processivity protein at the replication forks (Fig. 9.5). Binding of these two proteins inhibits DNA polymerase α, and induces dissociation of the DNA polymerase α-primase complex. Subsequent binding of DNA polymerase δ completes assembly of a multiprotein structure capable of leading-strand synthesis by continuous copying of the parental template strand.

DNA replication can neither initiate nor proceed without the action of enzymes that unwind the strands of the DNA template. In the simian virus 40 *in vitro* replication system, LT is the helicase responsible for unwinding of the origin, and remains associated with each of the two replication forks, unwinding the template during elongation (Fig. 9.5).

Lagging-strand synthesis. The first Okazaki fragment of the lagging strand is synthesized by the DNA polymerase α-primase complex (Fig. 9.5, step 4). The lagging strand is also synthesized by DNA polymerase δ, and transfer of the 3′ end of the first Okazaki fragment to this enzyme is thought to proceed as on the leading strand. The lagging-strand template is then copied **toward** the origin of replication. Consequently, synthesis of the lagging strand requires initiation by DNA polymerase α-primase at multiple sites progressively further from the origin (Fig. 9.5). The mechanisms by which leading- and lagging-strand synthesis are coordinated are not fully understood. If the replication

Table 9.2 Cellular proteins required for simian virus 40 DNA replication

Protein	Synonym(s)	Contacts	Functions
Rp-A	Rf-A	Primase	Binds to single-stranded DNA; origin unwinding in cooperation with LT
	ssB	LT	
DNA polymerase α-primase	Polα/primase	LT	Synthesis of RNA primers and Okazaki fragments on leading and lagging strands
Rf-C	Activator 1	Pcna	ATP-dependent clamp loading; also required for release of Pcna from DNA
Pcna		Rf-C	Sliding clamp
DNA polymerase δ		Pcna	Processive synthesis of leading and lagging strands when bound to Pcna
Rnase H1			Endonucleotytic degradation of RNA base-paired with DNA; removal of RNA primers
Fen1			5′→3′ exonuclease; removal of RNA primers
DNA ligase I			Sealing of daughter DNA fragments

machinery tracked along an immobile DNA template, the complexes responsible for leading- and lagging-strand synthesis would have to move in opposite directions. Furthermore, lagging-strand synthesis would require repeated assembly of priming complexes at sites that do not contain LT-binding sequences. A more attractive alternative is that the DNA template is spooled through an immobile replication complex that contains all the proteins necessary for synthesis of both daughter strands. This mechanism would allow simultaneous copying of the template strands in opposite directions by a single replication machine present at each fork (Fig. 9.7). Consistent with this idea, replication of chromosomal DNA occurs at fixed sites in the nucleus. Moreover, structures indicative of DNA spooling have been observed in the electron microscope during the initial, LT-dependent unwinding of the simian virus 40 origin (Box 9.3). While a model invoking an immobile replication machine possesses the virtues of elegance and parsimony, it remains to be firmly established.

Termination and resolution. Because the circular simian virus 40 DNA genome possesses no termini, its replication does not lead to gaps in the strands made discontinuously. Nevertheless, additional cellular proteins are needed for the production of two daughter molecules from the circular template. These essential components of the simian virus 40 replication system are cellular enzymes that alter the topology of DNA, topoisomerases I and II. These enzymes, which differ in their catalytic mechanisms, and functions in the cell, reverse the winding of one duplex DNA strand around another (**supercoiling**). Because they remove supercoils, topoisomerases are said to **relax** DNA. In a closed circular DNA molecule the unwinding of duplex DNA at the origin and subsequently at the replication forks is necessarily accompanied by supercoiling of the remainder of the DNA (Fig. 9.8A). If not released, the torsional stress so introduced would act as a brake on movement of the replication forks, eventually bringing them to a complete halt. Both topoisomerases I and II can remove such torsional stress to allow movement of SV40 DNA replication forks. These enzymes play an analogous role during replication of chromosomal DNA *in vivo*. Topoisomerase II is also required specifically for the separation of the viral daughter molecules from late replication intermediates. A cycle of simian virus 40 DNA synthesis produces two interlocked (catenated) circular DNA molecules that can be separated only when one DNA molecule is passed through a double-strand break in the other. The break is then resealed (Fig. 9.8B). Topoisomerase II catalyzes this series of reactions.

Replication of chromatin templates. The simian virus 40 genome is associated with cellular nucleosomes both in the virion and in infected cell nuclei. It is therefore replicated as a minichromosome, in which the DNA is wrapped around nucleosomes. This arrangement raises the question of how the replication machinery rapidly copies a DNA template that is tightly bound to nucleosomal histones. A similar problem is encountered during the replication of many viral RNA genomes, when the template RNA is packaged by viral RNA-binding proteins in a ribonucleoprotein. The mechanisms by which replication complexes circumvent the nucleosomal barriers to movement are not understood in detail. Nevertheless, as discussed in Chapter 8, numerous proteins that couple ATP hydrolysis to remodeling of nucleosomal DNA have been identified. The organization of the simian virus 40 genome into a minichromosome also implies that viral DNA replication must be coordinated with binding of newly synthesized DNA to cellular nucleosomes. In fact, new nucleosomes are deposited at viral replication forks, a reaction that is catalyzed by the essential human protein chromatin assembly factor 1.

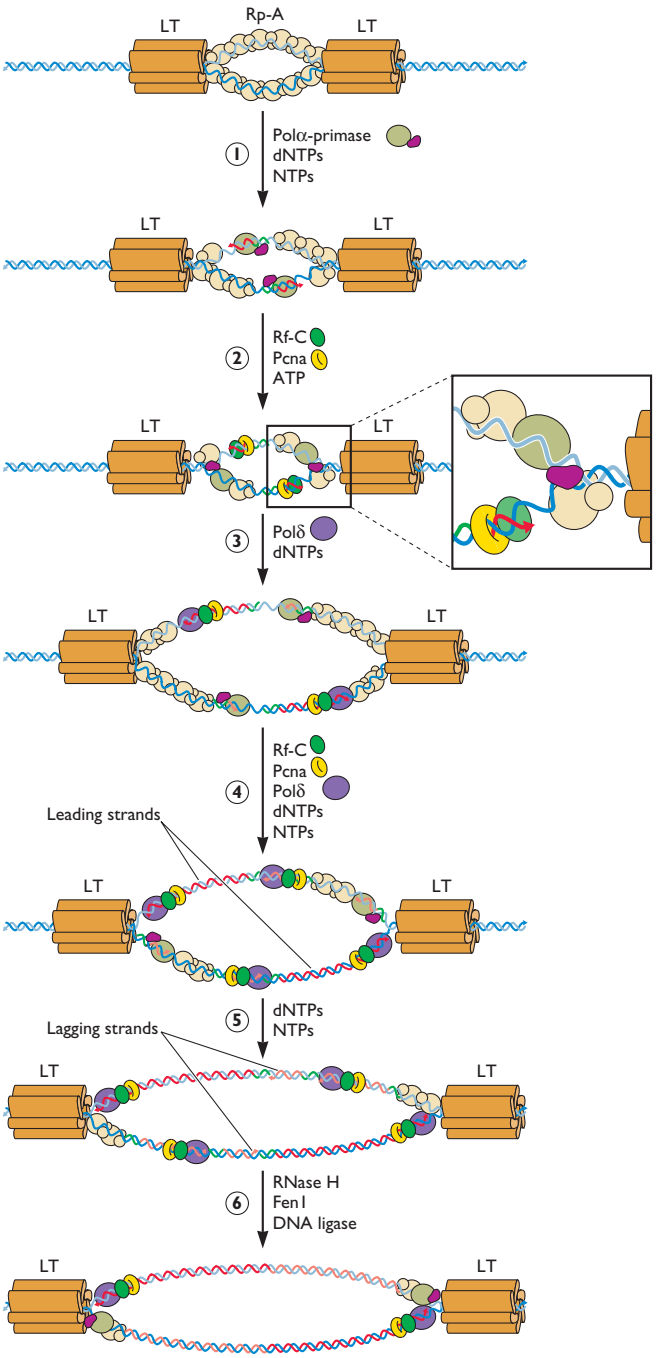


Figure 9.5 Synthesis of leading and lagging strands. The DNA polymerase α-primase responsible for the synthesis of Okazaki fragments binds specifically to both the Rp-A and LT proteins assembled at the origin in the presynthesis complex. Once bound, this enzyme complex synthesizes leading-strand RNA primers that are then extended as DNA (step 1). The 3′-OH group of the nascent RNA-DNA fragment (about 30 nucleotides in total length) is then bound by replication factor C (Rf-C) in a reaction that requires ATP but not its hydrolysis. Rf-C allows ATP-dependent opening of the proliferating-cell nuclear antigen (Pcna) (Fig. 9.6) ring and its loading onto the template (step 2). DNA polymerase δ then binds to the Pcna/Rf-C complex (step 3). This replication complex is competent for continuous and highly processive synthesis of the leading strands (steps 4 and 5). Lagging-strand synthesis begins with synthesis of the first Okazaki fragment by DNA polymerase α-primase (step 3). DNA polymerase δ is recruited as during leading strand synthesis, and produces a lagging-strand segment (step 5). The multiple DNA fragments produced by discontinuous lagging-strand synthesis are sealed by removal of the primers by RNase H (an enzyme that specifically degrades RNA hybridized to DNA) and the 5′ → 3′ exonuclease Fen1, repair of the resulting gaps by DNA polymerase δ, and joining of the DNA fragments by DNA ligase I (step 6).

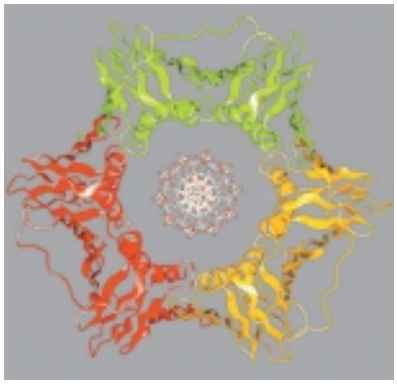


Figure 9.6 Structure of proliferating cell nuclear antigen, Pcna. The protein trimer is represented schematically in ribbon form, with the three subunits colored red, yellow, and orange. Each monomer contains two topologically identical domains. A model of double-stranded B form DNA is shown in the geometric center of the structure to illustrate how the closed ring formed by tight association of the three subunits might encircle DNA. The α-helices from the three subunits line the central channel, forming a surface of positively charged residues favorable for interaction with the negatively charged phosphodiester backbone of DNA. The nonspecificity of such interactions and the orientation of the internal α-helices appear ideal for strong but nonspecific contact with the phosphate groups of the DNA backbone, as modeled in the figure. From T. S. R. Krishna et al., *Cell* **79:**1233–1243, 1994, with permission. Courtesy of J. Kuriyan, Rockefeller University.

Summary. Analysis of simian virus 40 replication *in vitro* has identified essential cellular replication proteins, led to molecular descriptions of crucial reactions in the complex process of DNA synthesis, and provided new insights into chromatin assembly. The detailed understanding of the reactions completed by the cellular DNA replication machinery laid the foundation for elucidation of the mechanisms by which other animal viral DNA genomes are replicated, and of some of the intricate circuits that regulate DNA synthesis and its initiation.

Mechanisms of Viral DNA Synthesis

The replication of all viral DNA genomes within infected cells comprises reactions analogous to those necessary for simian virus 40 DNA synthesis, namely, origin recognition

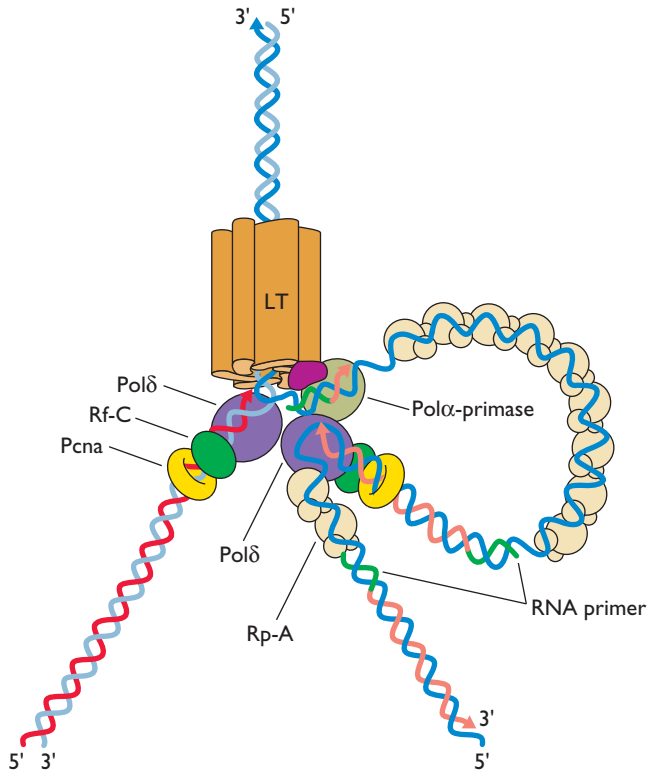


Figure 9.7 A hypothetical simian virus 40 replication machine. A replication machine containing all proteins necessary for both continuous synthesis of the leading strand and discontinuous synthesis of the lagging strand would assemble at each replication fork. Spooling of a loop of the template DNA strand for discontinuous synthesis would allow the single complex to copy the two strands in opposite directions. Pol, polymerase.

and assembly of a presynthesis complex, priming of DNA synthesis, elongation, termination, and often resolution of the replication products. However, the mechanistic problems associated with each of the steps in DNA synthesis are solved by a variety of virus-specific mechanisms.

Priming and Elongation

Synthesis of viral DNA molecules is initiated by a number of unusual mechanisms in which not only RNA, but also DNA and even protein molecules function as primers. Because each of the viral priming strategies has profound consequences for the mechanism of elongation, priming and elongation are considered together.

Synthesis of Viral RNA Primers by Cellular or Viral Enzymes

The standard method of priming is synthesis of a short RNA molecule by a specialized primase. As we have seen, cellular DNA polymerase α-primase synthesizes all RNA primers needed for replication of both template strands of polyomaviral genomes. A similar mechanism operates at certain origins of some herpesviruses, such as that directing replication of the episomal Epstein-Barr viral genome in latently infected cells (see "Regulation of Replication via Different Viral Origins: Epstein-Barr Virus" below). The integrated proviral genomes of retroviruses are also replicated via RNA primers synthesized by the cellular primase at the origin of the cellular replicon into which the provirus is inserted. In actively dividing cells, proviral DNA is therefore replicated once per cell cycle by the cellular replication machinery. Less common among DNA viruses is synthesis of RNA primers by viral proteins. However, this mechanism is characteristic of herpesviral replication in productively infected cells. Such productive replication has been best characterized in cells infected by herpes simplex virus type 1 when a viral primase synthesizes RNA primers. This primase is a heterotrimer of the products of the viral UL5, UL8, and UL25 genes.

An inevitable consequence of DNA synthesis from RNA primers by either cellular or viral DNA polymerases is that one of the two parental strands must be copied discontinuously. In the case of linear templates, a specialized mechanism is also necessary to complete synthesis of the lagging strand. In contrast, other viral DNA genomes are replicated by means of alternative priming mechanisms that eliminate the need for discontinuous synthesis.

Priming via DNA: Specialized Structures in Viral Genomes

Self-priming of viral DNA synthesis via specialized structures in the viral genome is a hallmark of all *Parvoviridae*, among the smallest DNA viruses that replicate in animal cells. This virus family includes the dependoviruses, such as adeno-associated viruses, and the autonomous parvoviruses, such as minute virus of mice. Synthesis of all parvoviral DNAs exhibits a number of unusual features, the most striking being self-priming. This mechanism is illustrated here with adeno-associated virus.

The adeno-associated virus genome is a small molecule (<5 kb) of single-stranded, linear DNA that carries **inverted terminal repetitions** (ITRs). Genomic DNA is of both (+) and (–) polarity, for both strands are encapsidated, but in separate virus particles. As illustrated in Fig. 9.9A, palindromic sequences within the central 125 nucleotides of the ITR base pair to form T-shaped structures. Formation of this structure at the 3′ end of either single strand of viral DNA provides an ideal template-primer for initiation of the first cycle of viral DNA synthesis (Fig. 9.9B). Experimental evidence for such **self-priming** includes the dependence of adeno-associated virus DNA synthesis on self-complementary sequences within the ITR. Following

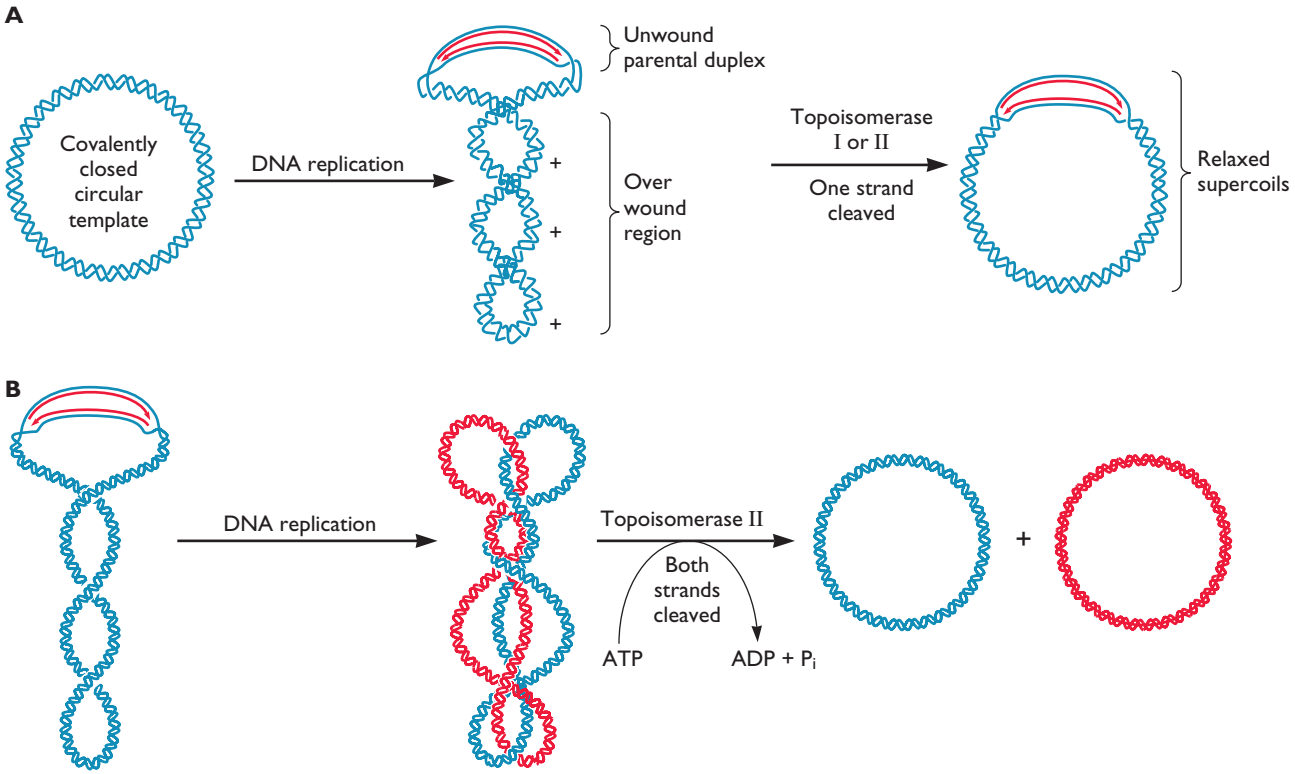


Figure 9.8 Function of topoisomerases during simian virus 40 DNA replication. (A) Relief from overwinding. Unwinding of the template DNA at the origin and two replication forks leads to overwinding (positive supercoiling) of the DNA ahead of the replication forks (middle). Either topoisomerase I or topoisomerase II can remove the supercoils to allow continued movement of the replication fork. **(B)** Decatenation of replication products. Separation of interlocked daughter molecules (middle) requires topoisomerase II, which makes a double-strand break in DNA, passes one double strand over the other to unwind one turn, and reseals the DNA in reactions that require hydrolysis of ATP.

recognition of the viral DNA primer, the single template strand of an infecting genome can be copied by a continuous mechanism, analogous to leading-strand synthesis during replication of double-stranded DNA templates. In subsequent cycles of replication, the same 3′-terminal priming structures form in the duplex replication intermediate produced in the initial round of synthesis (Fig. 9.9B). Adeno-associated virus DNA synthesis is therefore always continuous, and requires cellular DNA polymerase δ, Rf-c and Pcna, but not DNA polymerase α-primase.

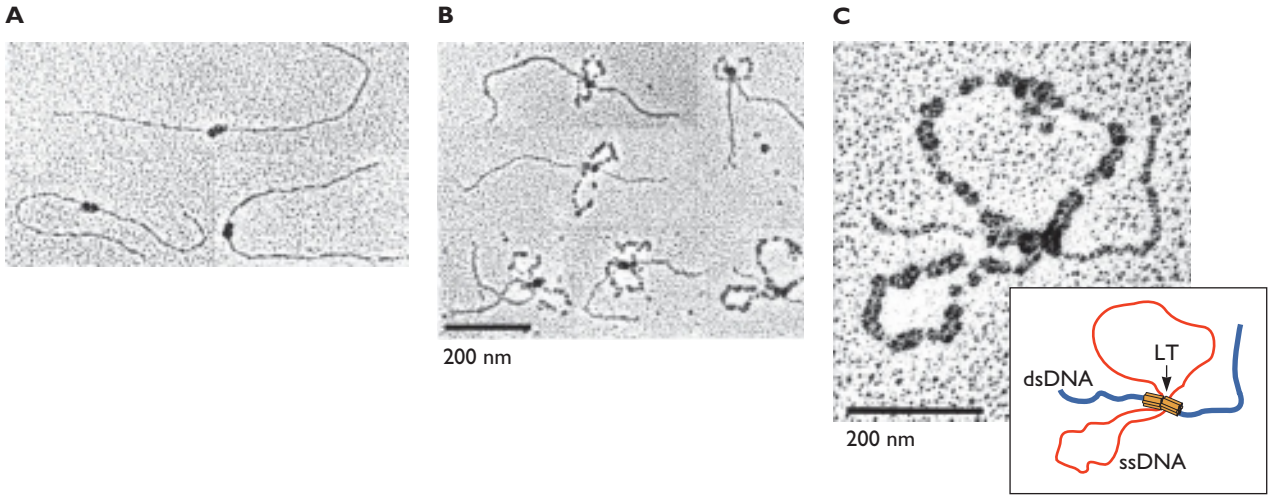
On the other hand, a specialized mechanism **is** necessary to complete replication, by copying of the sequences that form the priming structure: the initial product retains the priming hairpin and is largely duplex DNA in which paren - tal and daughter strands are covalently connected (Fig. 9.9.B, step 2). This step is achieved by nicking of the intermediate within the parental DNA strand at a specific site. The new 3′-OH end liberated in this way primes continuous synthesis to the end of the DNA molecule (Fig. 9.9B, step 4). The nick is introduced by the related viral proteins Rep 78 and Rep 68 (Rep 78/68) (Box 9.4). These proteins are site- and strand-specific endonucleases, which bind to, and cut at, specific sequences within the ITR. During this **terminal resolution** process, Rep 78/68 becomes covalently linked to the cleaved DNA at the site that will become the 5′ terminus of the fully replicated molecule (Fig. 9.9B). Following the synthesis of a duplex of the genomic DNA molecule (the **replication intermediate**), formation of the 3′-terminal priming hairpin allows continuous synthesis of single-stranded genomes by a strand displacement mechanism, with re-formation of the replication intermediate (Fig. 9.9B, steps 6 and 7).

Rep 78 and Rep 68 are similar to simian virus 40 LT in several respects, and can be considered origin recognition proteins (Table 9.3). They are the only viral gene products necessary for parvoviral DNA synthesis. In addition to recognizing and cleaving the terminal resolution site, these proteins provide the ATP-dependent, 3′ → 5′ helicase

BOX 9.3 EXPERIMENTS

Unwinding of the simian virus 40 origin leads to spooling of DNA

Visualization by electron microscopy of structures formed during LT-dependent unwinding from the simian virus 40 origin *in vitro* suggested that the two hexamers remain in contact as DNA is unwound. **(A)** LT bound to the origin, as a characteristic bilobed structure (the double hexamer shown in Fig. 9.4); **(B)** unwinding intermediates; **(C)** the intermediate at the bottom right in panel B at higher magnification. This intermediate contains a bilobed LT complex connecting the two replication forks, so the single-stranded DNA (ssDNA) is looped out as "rabbit ears." The formation of such structures containing a dimer of the LT hexamer, in which each monomer is bound to a replication fork, stimulates the helicase activity of LT. This property supports the view that the DNA template is spooled through an immobile replication machine (see the text). dsDNA, double-stranded DNA. From R. Wessel et al., *J. Virol.* **66:**804–815, 1992, with permission. Courtesy of H. Stahl, Universität des Saarlandes.



activity needed for unwinding of the replicated ITR and reformation of the priming hairpin (Fig. 9.9, step 5). Whether cellular helicases are also required, for example, when double-stranded replication intermediates serve as templates, is not yet clear.

Priming via self-complementary terminal sequences of the viral genome eliminates the need for proteins that make RNA primers, or are themselves primers for DNA synthesis (see the next section). Such a mechanism would therefore seem especially advantageous for viruses with small genomes, such as the parvoviruses. Nevertheless, a similar self-priming mechanism is thought to initiate replication of the large double-stranded DNA genomes of poxviruses such as vaccinia virus.

Protein Priming

Judged by the criterion of simplicity, the most effective mechanism of initiation of DNA synthesis is via a protein primer. Nevertheless, this mechanism is rare, restricted to some bacteriophages (e.g., ϕ29 and PRD1) and to hepadnaviruses and adenoviruses among DNA viruses that infect animal cells. The replication of some viral RNA genomes is also initiated from a protein primer, notably the VPg protein of poliovirus discussed in Chapter 6. Here, we use adenoviral DNA replication to illustrate the mechanism of protein priming.

The primer for human adenoviral DNA synthesis is the precursor to the terminal protein, which is covalently attached to the 5′ ends of the linear, double-stranded DNA genomes. In a template-dependent reaction, the adenoviral DNA polymerase covalently links the α-phosphoryl group of dCMP to the hydroxyl group of a specific serine residue in this preterminal protein (Fig. 9.10). The 3′-OH group of the protein-linked dCMP then primes synthesis of daughter viral DNA strands by the viral DNA polymerase. The nucleotide is added to the preterminal protein **only** when this protein primer is assembled with the DNA polymerase into preinitiation complexes at the origins of replication. As the origins

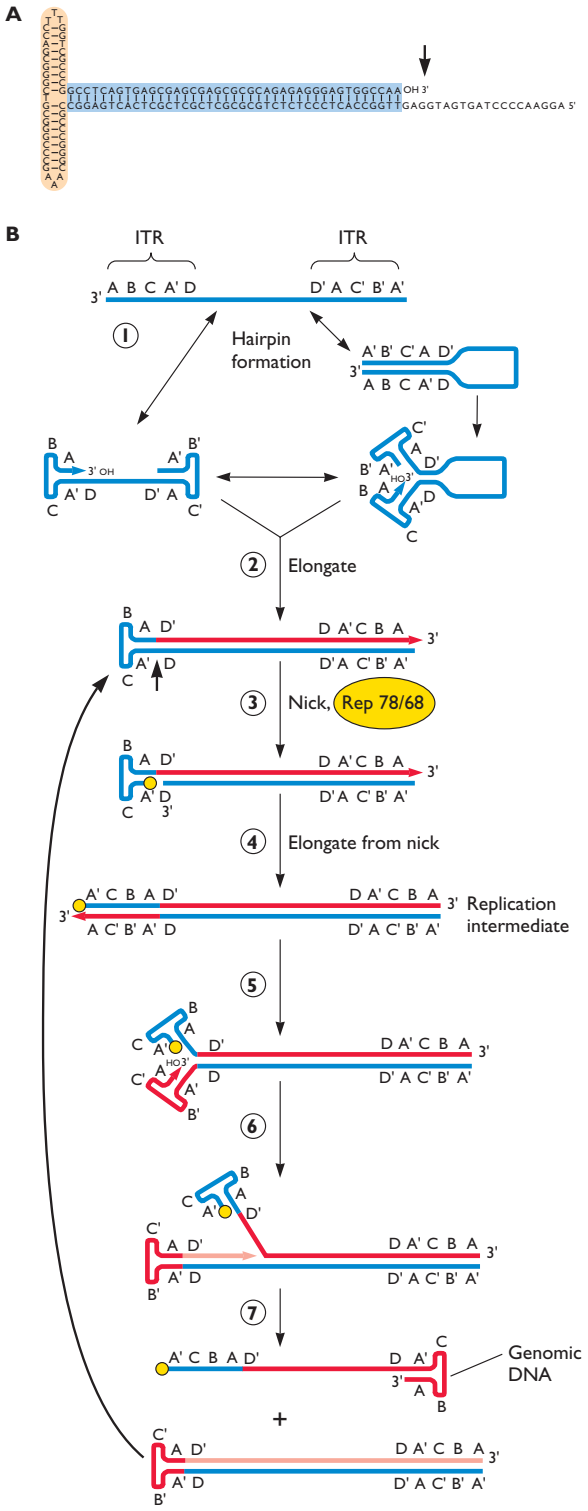


Figure 9.9 Replication of parvoviral DNA. (A) Sequence and secondary structure of the adeno-associated virus type 2 inverted terminal repetition (ITR). A central palindrome (tan background) is flanked by a longer palindrome (light blue background) within the ITR. When these bases pair at the 3′ end of the genome, a T-shaped structure in which the internal duplex stem terminates in a free 3′-OH group (arrow) is formed. **(B)** Model of adeno-associated virus DNA replication. The ITRs are represented by 3′ABCA′D5′ and 5′A′B′C′AD′3′. Formation of the 3′-terminal hairpin provides a primer-template (step 1, left). However, it is possible that the self-complementary terminal repeat sequences first base pair to form a "panhandle," a single-stranded circle stabilized by the duplex terminal sequence (step 1, right). Such a structure could explain the repair of deletions or mutations within one ITR observed when the other is intact. In either case, elongation from the 3′-OH group of the hairpin allows continuous synthesis (red) to the 5′ end of the parental strand (step 2). To complete copying of the parental strand, a nick to generate a new 3′ OH is introduced at the specific **terminal resolution site** (marked by the arrow) by the viral Rep 78/68 proteins. Elongation from the nick results in copying of sequences that initially formed the self-priming hairpin to form the double-stranded replication intermediate (step 4). However, the parental strand then contains newly replicated DNA (red) at its 3′ end. As a result, the ITR of the parental strand is no longer the initial sequence but rather its complement. This palindromic sequence is therefore present in populations of adeno-associated virus DNA molecules in one of two orientations. Indeed, such sequence heterogeneity provided an important clue for elucidation of the mechanism of viral DNA synthesis. The newly replicated 3′ end of the replication intermediate can form the same terminal hairpin structure (step 5) to prime a new cycle of DNA synthesis (step 6) with displacement of a molecule of single-stranded genomic DNA, and the formation of the incompletely replicated molecule initially produced (step 7). The latter molecule can undergo additional cycles of replication as in steps 3 and 4.

lie at the ends of the linear genome, each template strand is copied continuously from one end to the other (Fig. 9.10). The parental template strand initially displaced is copied by the same mechanism, following formation of a duplex stem upon annealing of an ITR sequence. This unusual strand displacement mechanism therefore results in semiconservative replication, even though the two parental strands of viral DNA are not copied at the same replication fork.

Properties of Viral Replication Origins

As we have seen, origins of replication contain the sites at which viral DNA synthesis begins and can be defined experimentally as the minimal DNA segment necessary for initiation of replication in cells or *in vitro* reactions. Viral origins of replication support initiation of DNA synthesis by a variety of mechanisms, including some with no counterpart in cellular DNA synthesis. Nevertheless, they are discrete DNA segments that contain sequences recognized by viral origin recognition proteins to seed assembly of multiprotein complexes. When initiation is by self-priming, DNA sequences essential for replication include those needed to form and maintain a specific secondary structure in the template, as well as the sequence at which replication intermediates are cleaved for complete copying

BOX 9.4 BACKGROUND

Organization of coding sequences in the adeno-associated virus genome

The viral genome is depicted at the top, with the locations of the ITR, the promoters for transcription by RNA polymerase II (p5, p19, and p40), and the single site of polyadenylation (vertical red arrow) indicated. The two coding regions within the viral genome are termed Rep (replication) and Cap (capsid). As shown below, each is expressed as multiple related proteins, which are synthesized from differentially spliced mRNAs transcribed from the different promoters. The two smaller products of the Rep region, Rep 52 and Rep 40, are not required for viral DNA synthesis, but facilitate encapsidation of single-stranded genomic DNA molecules during assembly of virus particles.

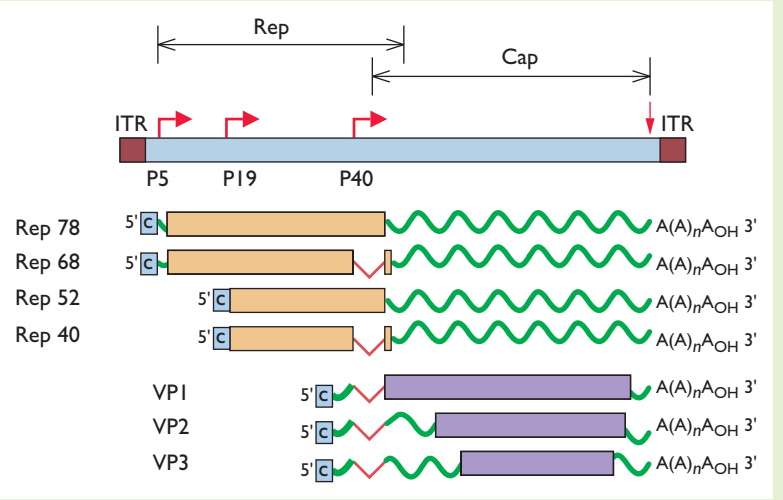


Table 9.3 Viral origin recognition proteins

Virus	Protein(s)	DNA-binding properties	Other activities and functions
Parvovirus			
Adeno-associated virus	Rep 78/68	Binds to specific sequences in ITR; binds as hexamer	Site- and strand-specific endonuclease; ATPase and ATP-dependent helicase; transcriptional regulator
Papovaviruses			
Simian virus 40	LT	Binds cooperatively to origin site II to form double hexamer; distorts origin; DNA binding regulated by phosphorylation at specific sites	DNA-dependent ATPase and 3′→5′ helicase; binds to cellular Rp-A and polymerase α-primase; represses early and activates late transcription; binds cellular Rb protein to induce progression through the cell cycle
Bovine papillomavirus type 5	E1	Binds origin with low affinity; binds strongly and cooperatively in presence of E2 protein	Binding to E2 essential for viral DNA replication; DNA-dependent ATPase and helicase
	E2	Binds as dimer to specific sequences in origin	Regulates transcription by binding to viral enhancers
Adenovirus			
Human adenovirus type 2	Pre-TP-DNA polymerase	Binds to origins; association stimulated by cellular transcriptional activators Nf-1 and Oct-1	Priming of DNA synthesis via addition of dCMP to pre-TP by the DNA polymerase; continuous synthesis of both strands of viral genome
Herpesviruses			
Herpes simplex virus type 1	UL9	Binds cooperatively to specific sites in viral origins; distorts DNA to which it binds	ATPase and 3′→5′ helicase; binds UL29 protein, UL8 subunit of viral primase, and UL42 processivity protein
Epstein-Barr virus	EBNA-1	Binds as dimer to multiple sites in two clusters (FR and DS) in viral OriP	Binding to FR sequences required for maintenance of episomal viral genomes; stimulates transcription from viral promoters

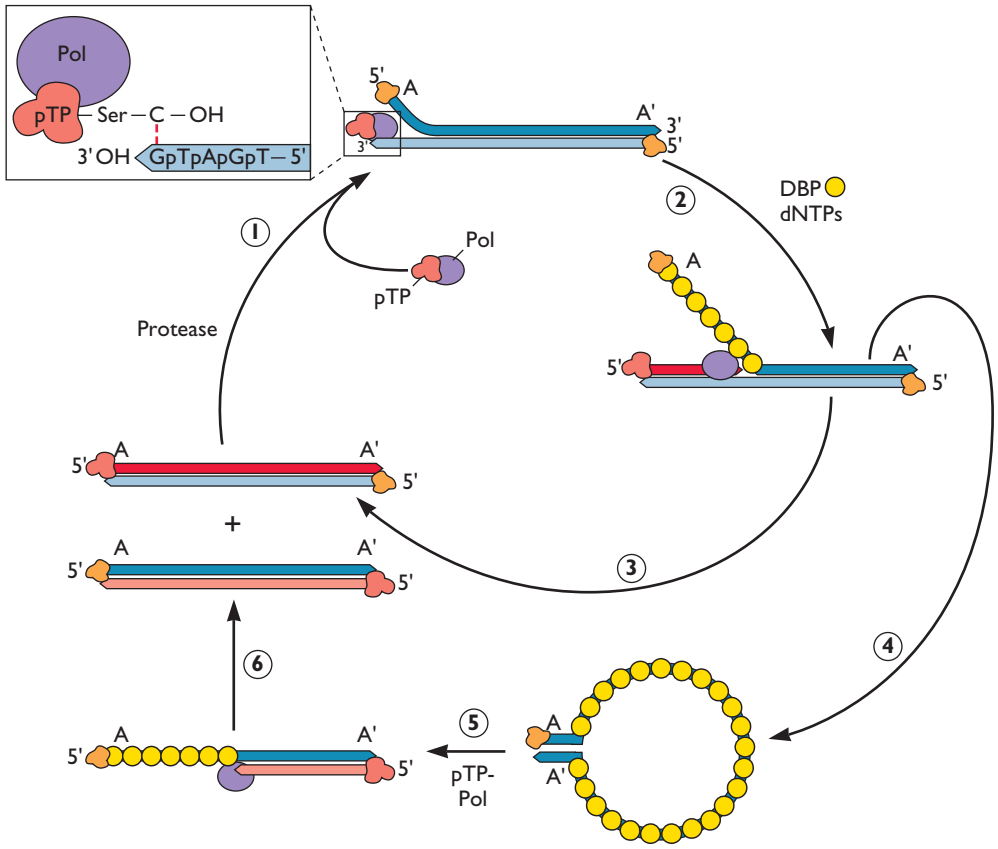


Figure 9.10 Replication of adenoviral DNA. Assembly of the viral preterminal protein (pTP) and DNA polymerase (Pol), into a preinitiation complex at each terminal origin of replication activates covalent linkage of dCMP to a specific serine residue in pTP by the DNA polymerase (step 1). The free 3′-OH group of preterminal protein-dCMP primes continuous synthesis in the 5′ → 3′ direction by Pol (step 2). This reaction also requires the viral E2 single-stranded-DNA-binding protein (DBP), which coats the displaced second strand of the template DNA molecule, and a cellular topoisomerase. As the terminal segments of the viral genome comprise an inverted repeat sequence (A and A′), there is an origin at each end, and both parental strands can be replicated by this displacement mechanism. Reannealing of the complementary terminal sequences of the parental strand initially displaced forms a short duplex stem identical to the terminus of the double-stranded genome (step 3). The origin re-formed in this way directs a new cycle of protein priming and continuous DNA synthesis (steps 4 and 5).

of the parental strand (Fig. 9.9A). Even though viral origins may be unconventional, they exhibit a number of common features, as do the proteins that recognize them.

Number of Origins

In contrast to papillomaviral and polyomaviral DNAs, the genomes of the larger DNA viruses contain not one, but two or three origins. As noted above, the two identical adenoviral origins at the ends of the linear genome are the sites of assembly of complexes containing the viral DNA polymerase and protein primer (Fig. 9.10). The genomes of herpesviruses, such as Epstein-Barr virus and herpes simplex virus type 1, contain three origins of replication. Different functions can be ascribed to the different Epstein-Barr virus origins: a single origin (OriP) allows maintenance of episomal genomes in latently infected cells, while two others (OriLyt) support replication of the viral genome during productive infection. The advantage of three origins, two copies of OriS and one copy of OriL (Fig. 9.11), to herpes simplex viruses is less clear: a full complement of these origins is not necessary for efficient viral DNA synthesis, at least in cells in culture. The two types of origin possess considerable nucleotide sequence similarity, but differ in their organization, and can be distinguished functionally. For example, OriL is activated when differentiated neuronal cells are exposed to a glucocorticoid hormone, but OriS is repressed. As glucocorticoids are produced in response to stress, a condition that reactivates

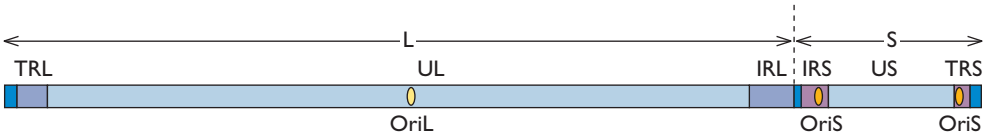
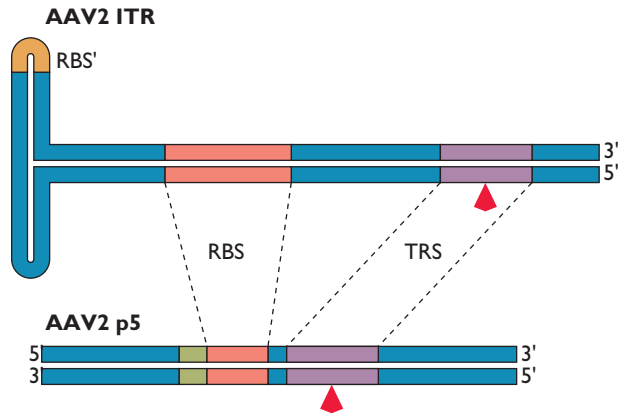


Figure 9.11 Features of the herpes simplex virus type 1 genome. The long (L) and short (S) regions of the viral genome that are inverted with respect to one another in the four genome isomers (Fig. 9.20) are indicated at the top. Each segment comprises a unique sequence (UL or US) flanked by internal and terminal repeated sequences (IR and TR). The locations of the two identical copies of OriS, in repeated sequences, and of the single copy of OriL are indicated.

a latent herpes simplex virus type 1 infection, it has been suggested that replication from OriL may be particularly important during the transition to a productive infection. An unusual feature of these viral origins is that they are required only during the initial stage of replication, which begins early in infection. Subsequent viral DNA synthesis is origin independent.

A recently discovered, and unanticipated, feature of the small adeno-associated virus genome is the presence of a second origin that can support amplification of viral DNA in the absence of the ITR origin described previously. This origin, which includes the p5 promoter (Box 9.4), also contains a binding site for Rep 78/68 and a sequence resembling the terminal resolution site that can be cleaved by the viral protein (Fig. 9.12). The function of this origin during adeno-associated virus infection in the presence of a helper virus is not yet clear. However, it may contribute to viral genome integration in cells infected in the absence of a helper (see "Integrated Parvoviral DNA Can Replicate as Part of the Cellular Genome" below).

Figure 9.12 Sequence features of the adeno-associated virus type 2 ITR and p5 origins. The Rep 78/68-binding sites in the two origins are shown in pink, and the terminal resolution sites (TRS) are shown in purple. The p5 origin TRS, like that of the ITR origin, has been shown to be cleaved by the viral protein (red arrowheads). The TATA box (green) that overlaps the Rep binding site in the p5 origin is required for replication from this origin *in vivo*. Adapted from D. L. Glauser et al., *J. Virol.* 79:12218–12230, 2005, with permission.



Viral Replication Origins Share Common Features

Even though the origins of replication of double-stranded DNA viruses are recognized by different proteins and support different mechanisms of initiation, they exhibit a number of common features (Fig. 9.13). The most prominent of these is the presence of AT-rich sequences. In general, AT base pairs contain only two hydrogen bonds, whereas GC pairs interact via three such noncovalent bonds. The less stable AT-rich sequences are therefore thought to facilitate the unwinding of origins that is necessary for initiation of viral DNA synthesis from either RNA or protein primers. The best-characterized viral origins of DNA replication comprise a minimal essential core origin flanked by sequences that are dispensable, but nonetheless significantly increase replication efficiency. These stimulatory sequences contain binding sites for cellular transcriptional activators (Fig. 9.13). Yet other viral origins, those of papillomaviruses and parvoviruses, and OriLyt of Epstein-Barr virus, include binding sites for viral proteins that are both transcriptional regulators and essential replication proteins. And all three herpes simplex virus type 1 origins lie between sites at which transcription of viral genes is initiated (Fig. 9.13). A close relationship between origin sequences and those that regulate transcription is therefore a second general feature.

Recognition of Viral Replication Origins

The paradigm for viral origin recognition are the polyomaviral LT proteins. We therefore describe the properties of simian virus 40 LT in more detail as a prelude to discussion of other viral proteins with similar functions.

Properties of Simian Virus 40 LT

Functions and organization. The LT proteins of polyomaviruses are remarkable proteins that provide essential replication functions and play several other important roles in the infectious cycle. As we have seen, simian virus 40 LT is both necessary and sufficient for recognition of the viral origin, and also supplies the helicase activity necessary for origin unwinding and perhaps movement of the replication fork. The LT proteins make a major contribution to the species specificity of polyomaviruses. Although the

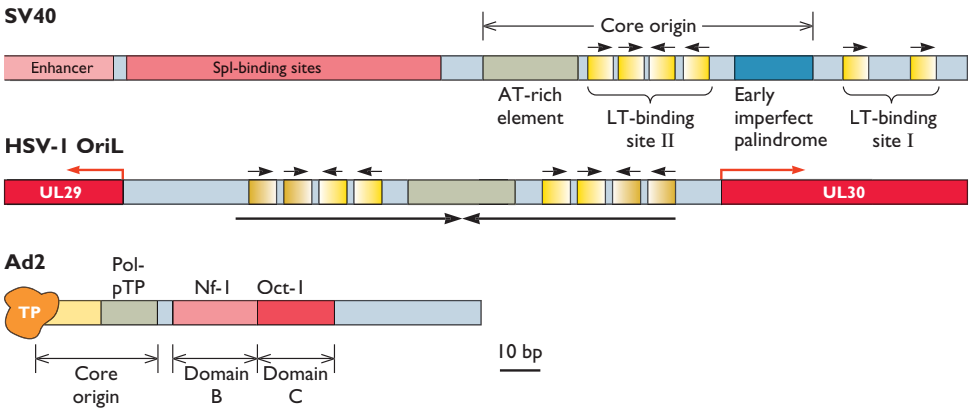
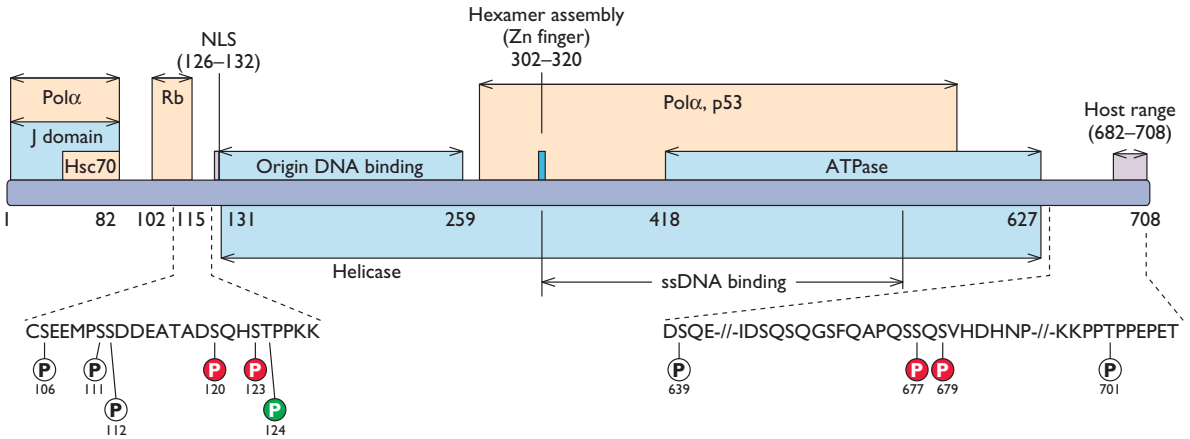


Figure 9.13 Common features of viral origins of DNA replication. The single simian virus 40 (SV40) origin, herpes simplex virus type 1 (HSV-1) OriL (Fig. 9.11), and adenovirus type 2 (Ad2) origin (Fig. 9.10), are illustrated to scale, emphasizing common features. Binding sites for origin recognition proteins and AT-rich sequences are indicated in yellow and green, respectively, and sites of initiation of transcription are indicated by jointed red arrows. Herpes simplex virus OriL, which is a perfectly symmetrical palindrome as indicated by the arrows, is located within the transcriptional control regions of the divergently transcribed UL29 and UL30 genes. The two copies of OriS (Fig. 9.11) are very similar in sequence to OriL. The terminal sequence of the adenoviral origin designated the core origin functions inefficiently in the absence of the adjacent binding site for the transcriptional activator nuclear factor 1 (Nf-1).

genomes of simian virus 40 and mouse polyomavirus are closely related in organization and sequence, they replicate only in simian and murine cells, respectively. Such host specificity is largely the result of species-specific binding of LT to the largest subunit of DNA polymerase α of the host cell in which the virus will replicate (Table 9.3). Although the precise mechanism remains to be determined, assembly of preinitiation complexes competent for unwinding of the origin does not take place when the LT of one polyomavirus binds to the origin of another.

Figure 9.14 Functional organization of simian virus 40 LT. LT is represented to scale by the blue bar. Indicated are the sequences required for binding to the DNA polymerase α-primase complex (Polα), to the cellular chaperone Hsc70, to the cellular retinoblastoma (Rb) and p53 proteins, the origin of replication (origin DNA binding), and single-stranded (ss) DNA. Also shown are segments necessary for the helicase and ATPase activities, hexamer assembly at the origin, the nuclear localization signal (NLS), and a C-terminal sequence necessary for virion production but not viral DNA synthesis (Host range). The region that binds to Hsc70 lies within an N-terminal segment termed the J domain, because it shares sequences and functional properties with the *E. coli* protein DnaJ, a chaperone that assists the folding and assembly of proteins and is required during replication of bacteriophage λ. The chaperone functions of the J domain and Hsc70 are essential for replication, and seem likely to assist assembly or rearrangement of the preinitiation complex. Below are shown the two regions of the protein in which sites of phosphorylation are clustered, indicating modifications that have been shown to inhibit (red) or activate (green) the replication activity of LT.



LT proteins also ensure that the cellular components needed for simian virus 40 DNA synthesis are available in the host cell. By binding and sequestering specific cellular proteins, LT perturbs mechanisms that control cell proliferation and can induce infected cells to enter S phase when they would not normally do so (see below). In addition, LT carries out a number of transcriptional regulatory functions during the infectious cycle, including regulation of its own synthesis and activation of late gene expression.

Sequences of simian virus 40 LT necessary for its numerous activities have been mapped by analysis of the effects of specific alterations in the protein on virus replication in infected cells, DNA synthesis *in vitro*, or the individual biochemical activities of the protein. The properties of such altered proteins indicate that LT contains discrete structural and functional domains, such as the minimal domain for specific binding to sites I and II at the viral origin (Fig. 9.14). However, the activities of such functional regions defined by genetic and biochemical methods may be influenced by distant sites, as discussed in the next section.

High-resolution structures of the LT origin-binding and helicase domains have been determined by X-ray crystallography. The former forms a hexameric spiral that contains a gap (Fig. 9.15A). The central channel of the spiral is more than wide enough, some 30A, to accommodate double-stranded DNA and is lined by residues required for origin binding. In protein crystals, a C-terminal segment of LT that possesses helicase activity also forms a hexameric structure, comprising a two-tiered ring encasing a central channel of varying diameter (Fig. 9.15B). The mechanism by which the helicase domain couples ATP hydrolysis to unwinding of the origin is not fully understood. However, this reaction requires nonspecific binding of LT to double- and single-stranded DNA, and a β-hairpin structure in the helicase domain that is conserved among several viral and cellular proteins with helicase activity (Box 9.5).

Regulation of LT synthesis and activity. As the simian virus 40 early enhancer and promoter are active in many cell types (Chapter 8), the viral early gene encoding LT is transcribed efficiently as soon as the viral chromosome enters the nucleus. The spliced early mRNA encoding LT is the predominant product of processing of these early transcripts. Although production of LT is not regulated during the early phase of infection, its activity is tightly controlled.

Simian virus 40 LT is phosphorylated at multiple Ser and Thr residues, most of which are located within one of two clusters near the N and C termini of the protein (Fig. 9.14). Although the significance of phosphorylation at every site is not known, it is clear that specific modifications regulate the ability of LT to support viral DNA synthesis. For example, phosphorylation of Thr124 is absolutely required. This modification specifically stimulates binding of LT to origin site II and promotes assembly of the double hexamer (Fig. 9.4). It is also essential for unwinding of DNA from the origin. As Thr124 does not lie within the minimal origin-binding domain (Fig. 9.14), such regulation of DNA-binding activity is thought to be the result of conformational change induced by phosphorylation at this site. The best candidate for the protein kinase that phosphorylates Thr124 is cyclin-dependent kinase 2 (Cdk2) associated with cyclin A. The accumulation and activity of this complex are strictly regulated as growing cells traverse the cell cycle, but the mechanisms by which LT ensures that cellular replication proteins are made in simian virus 40-infected cells also result in production of active Cdk2 (see "Viral Proteins Can Induce Synthesis of Cellular Replication Proteins" below).

Viral Origin Recognition Proteins Share Several Properties

Other viral origin recognition proteins share with simian virus 40 LT the ability to bind specifically to DNA sequences within the appropriate origin of replication. They also bind to other replication proteins (although these may be viral or cellular), and several possess the biochemical activities exhibited by LT (Table 9.3). For example, the herpes simplex virus type 1 UL9 protein, which recruits viral rather than cellular replication proteins, binds cooperatively to specific sites that flank the AT-rich sequences of the viral origins (Fig. 9.12), which are then distorted, and it possesses an ATP-dependent helicase activity that unwinds DNA in the 3′ → 5′ direction (Table 9.4). The adeno-associated virus Rep 78/68 protein possesses these same activities (Table 9.4), but is also the site-specific endonuclease that is essential for terminal resolution (Fig. 9.9). The endonuclease active site and the domain that mediates sequence-specific binding adjacent to the terminal resolution site includes a large region very similar in structure to the origin-binding domains of simian virus 40 LT and the papillomavirus E1 protein (Fig. 9.16). Such structural homology is remarkable, as there is no amino acid identity among the three viral proteins. As would be expected, a unique endonuclease active site is present in Rep 78/68 (Fig. 9.16). In addition, this protein contains a second, discrete DNA-binding domain that mediates its binding to a stem-loop structure at the tip of one of the hairpin arms. This interaction greatly stimulates cleavage at the terminal resolution site, by a mechanism that is not yet clear.

In many respects, the herpesviral UL9 protein is a typical origin-binding protein (Table 9.4). However, it is required only during the initial stage of viral DNA synthesis: later in the infectious cycle, replication requires neither the viral origins nor the origin-binding protein. It has been reported recently that the UL9 protein is cleaved by the cellular

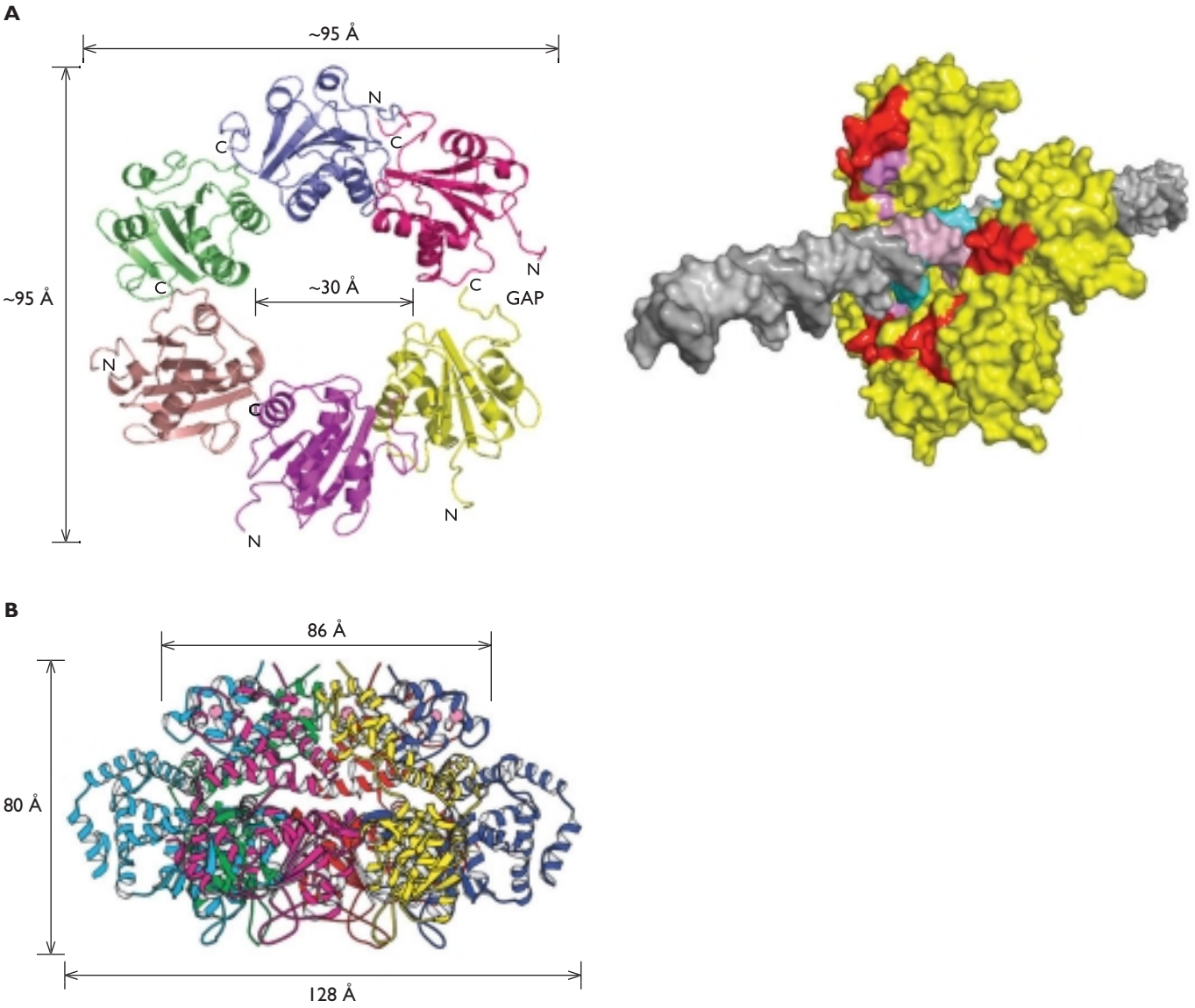


Figure 9.15 Structure of origin-binding and helicase domains of simian virus 40 large T antigen, determined by X-ray crystallography. (A) The structure of the origin-binding domain (amino acids 131 to 260) hexamer is shown in ribbon form on the left, with each subunit a different color. The hexamer forms a left-hand spiral with a gap. In the model of the hexamer shown in surface representation bound to DNA (right), the DNA is gray, with the palindromic LT-binding sequences (Fig. 9.3) in cyan and magenta. The DNA-binding regions of LT are colored red and purple. This model, and the results of mutational analysis, indicate that in the double hexamer of the full-length protein the origin-binding domains in the two hexamers interact with one another. Adapted from G. Meinke et al., *J. Virol.* **80:**4304–4312, 2006, with permission. Courtesy of Andrew Bohm, Tufts University School of Medicine. **(B)** The LT helicase domain (residues 251 to 627) hexamer is shown in ribbon form with each monomer in a different color. The hexameric ring comprises two tiers of different diameters around a central channel. Consistent with the DNA-binding activity of this LT fragment, the central channel is lined with positively charged residues. A large chamber in the middle of the central channel is wide enough (~67 Å in diameter) to accommodate DNA strands separated during DNA unwinding. The diameters of the channel on either side of this chamber are sufficient to accommodate double-stranded DNA in the smaller tier (~25 Å), but only single-stranded DNA (~15 Å) in the larger tier. The sizes of these channels were observed to differ significantly in different crystal forms of the LT hexamer and under different buffer conditions. This property, and structural features of LT monomers and the interfaces that form the two-tier hexamer, have led to the proposal that during DNA unwinding, the central channel might expand and constrict like the diaphragm (iris) of a camera. Adapted from D. Li et al., *Nature* **423:**512–518, 2003, with permission. Courtesy of Xiaojiang Chen, University of Colorado Health Science Center.

BOX 9.5 EXPERIMENTS

A conserved β-hairpin in the simian virus 40 LT helicase domain and unwinding of the origin

The β-hairpin of the LT helicase **(bottom)** is conserved in the polyomavirus and papillomavirus origin-binding proteins, and is present in cellular replicative helicases (Mcm proteins [see Fig 9.23]). Alanine substitution of the lysine and histidine residues shown resulted in complete inhibition of simian virus 40 DNA synthesis *in vitro*. Analysis of effects of the substitutions on interactions of LT established that the β-hairpin residues are required for unwinding of the origin. Furthermore, such unwinding is necessary for oligomerization of the helicase domain. These observations support a model of the action of LT **(top)**, in which initial unwinding of DNA in the EP region of the origin (Fig. 9.3) allows assembly of the hexamer, by coordinated binding of the helicase β-hairpins around a single strand of origin DNA. Adapted from A. Kumar et al., *J. Virol.* **81:**4808–4818, 2007, with permission.

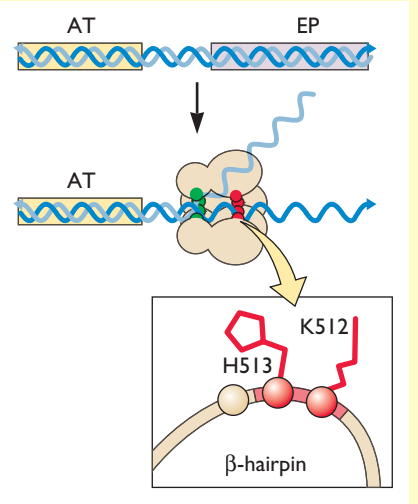


Table 9.4 Viral proteins that participate in genome replication

Virus	Protein	Properties and functions
Adenovirus		
Human adenovirus type 2	DNA polymerase	Initiates replication by covalent linkage of dCMP to the protein primer; completes synthesis of all daughter strands
	Preterminal protein	Protein primer for DNA synthesis
	Terminal protein	Facilitates origin unwinding
	Single-stranded-DNA-binding protein	Binds displaced single-stranded DNA; confers processivity to viral DNA polymerase
Herpesvirus		
Herpes simplex virus type 1	UL9 protein	Origin recognition
	UL30 protein (DNA polymerase)	Synthesis of viral DNA; associated 3′→5′ exonuclease for proofreading; target of several antiherpesviral drugs
	UL5, UL8, and UL52 proteins	Heterotrimer with primase and helicase activities; UL8 protein binds to the viral DNA polymerase
	UL42 protein	Processivity protein for viral DNA polymerase
	UL29 protein (ICP8)	Single-stranded-DNA-binding protein essential for viral DNA synthesis, binds DNA cooperatively; binds to the UL42 protein
Poxvirus		
Vaccinia virus	DNA polymerase	Synthesis of vaccinia virus DNA; associated 3′→5′ exonuclease activity for proofreading
	DNA type I topoisomerase	? essential; present in virions; structurally and functionally similar to cellular counterpart
	A20R protein	Component of DNA polymerase complex

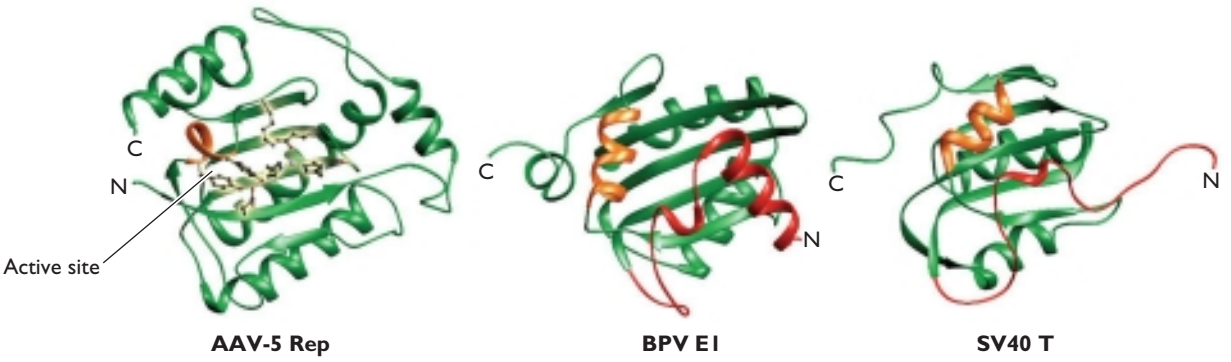


Figure 9.16 Structural homology among DNA-binding domains of viral origin recognition proteins. The X-ray crystal structures of the adenovirus-associated virus type 5 Rep 68 endonuclease domain and the bovine papillomavirus E1 and simian virus 40 LT origin-binding domains are shown in ribbon form. Each protein contains a central antiparallel β-sheet flanked by three α-helices. However, the Rep protein includes a cleft on one surface of the β-sheet that contains the endonuclease active site (residues shown in ball-and-stick). In the other two viral proteins, no cleft is present, as this region is occupied by N-terminal extensions (red). Adapted from A. Hickman et al., *Mol. Cell* **10:**327–337, 2002. Courtesy of Alison Hickman, National Institutes of Health.

protease cathepsin B following the onset of viral DNA synthesis in infected cells. Such cleavage might inactivate the protein, or the stable cleavage product, which retains the origin-binding domain, might act as a dominant negative inhibitor of origin-dependent replication.

Although recognition of viral origins of replication by a single viral protein is common, it is not universal. The human papillomavirus E1 protein possesses the same activities as simian virus 40 LT (Table 9.4), to which it is related in sequence, organization, and structure. The topologies of the origin-binding (Fig. 9.16) and the helicase-ATPase domains of the two viral proteins are strikingly similar. Nevertheless, E1 cannot support papillomaviral DNA replication in infected cells: a second viral protein, the E2 transcriptional regulator, is also necessary. Essential sequences of the minimal origin of replication of papillomaviral genomes include adjacent binding sites for both the E1 and E2 proteins (Fig. 9.17A). The E1 protein binds to DNA, but with only low specificity for origin sequences. In contrast, the E1 and E2 proteins bind cooperatively, and the specificity and the affinity of the E1-DNA interaction are increased significantly. However, the E2 protein is required only transiently: once a specific E1-E2 protein complex has assembled on the origin, the E2 protein dissociates. The results of structural studies of the E1 helicase domain bound to the N-terminal segment of E2 suggest that the latter protein binds to the E1 surface that mediates interaction among subunits in hexameric assemblies (Fig. 9.17B). Binding of ATP to E1 appears to induce conformational change that leads to dissociation of E2. Additional molecules of E1 then associate (Fig. 9.17A). The final product is an E1 hexamer assembled on, and probably closed around, single-stranded DNA at the origin. The E2 protein therefore serves as an initiation protein and loads the E1 helicase.

The adenoviral origins of replication are also recognized by two viral proteins, the preterminal protein and viral DNA polymerase. In this case, the proteins associate as they are synthesized in the cytoplasm of infected cells and, once within the nucleus, bind specifically to a conserved sequence within the minimal origin of replication (Fig. 9.13). Assembly of the viral proteins into a preinitiation complex at the origin is stimulated by their direct interaction with two cellular transcriptional regulators that bind to adjacent sequences. These viral and cellular proteins are sufficient to reconstitute initiation of adenoviral DNA synthesis from templates that carry the mature terminal protein covalently linked to their 5′ ends, like the viral genomes that enter infected cell nuclei (Fig. 9.10). The terminal protein present on such natural adenoviral DNA templates facilitates unwinding of the duplex termini early in the initiation reaction, and may be important for directing viral DNA molecules to the specialized nuclear sites at which replication takes place (see below). Once the first few nucleotides have been incorporated, the DNA polymerase must disassociate from the preterminal protein to allow elongation of the daughter DNA strand (Fig. 9.10). The structure of the ϕ29 DNA polymerase bound to its priming terminal protein suggests that such dissociation is the result of conformational change induced by displacement of the priming domain from the catalytic site in the polymerase (Box 9.6).

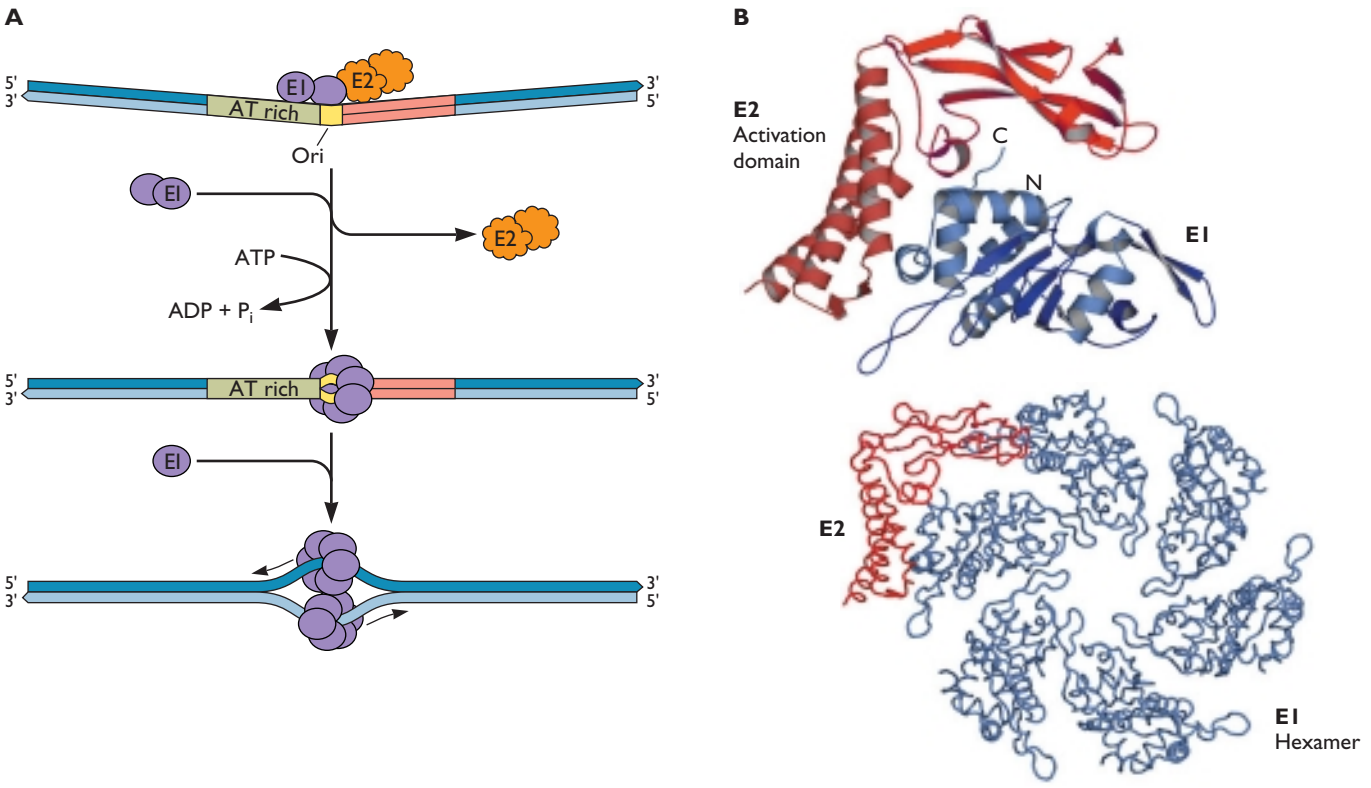


Figure 9.17 Origin loading of the papillomaviral E1 initiation protein by the viral E2 protein. **(A)** Schematic model. The sequence features of the minimal origin of replication of bovine papillomavirus type 1 are depicted as in Fig. 9.13. This origin contains an essential binding site for the viral E2 protein, a sequence-specific transcriptional regulator. The model of the origin loading of the viral E1 by the E2 protein is based on *in vitro* studies of the interactions of these proteins with the origin. The E1 and E2 proteins, which are both homodimers, bind cooperatively to the viral origin, with specificity and affinity far greater than that exhibited by the E1 protein alone. When ATP is hydrolyzed (presumably by the ATPase of the E1 protein), the $(E1)_2(E2)_2$-Ori complex is destabilized, the E2 dimers are displaced, and additional E1 molecules bind. In the resulting E1-Ori complex, the DNA is distorted and becomes partially single stranded. Finally, a larger E1 structure (probably a hexameric ring) is assembled on single-stranded DNA. **(B)** The X-ray crystal structure of the E2 activation domain bound to the E1 ATPase/helicase domain is shown in ribbon form, with E1 and E2 in blue and red, respectively. The overlay of E2 and the E1 hexamer (below) illustrates how association with E2 blocks the E1 surface that mediates hexamer assembly. Hence, E2 must dissociate prior to E1 assembly. Consistent with this model, the E1 and E2 proteins form a 1:1 complex in the absence of ATP, but in the presence of ATP E1 forms of a high-molecular-mass assembly that contains no E2. Adapted from E. Abbate et al., *Genes Dev.* **18:**1981–1986, 2004, with permission. Courtesy of Eric Abbate and Michael Botchan, University of California, Berkeley.

Viral DNA Synthesis Machines

In addition to origin recognition proteins, larger viral DNA genomes encode DNA polymerases and other essential replication proteins (Table 9.4). The simplest viral replication apparatus is that of adenoviruses, which comprises the preterminal protein primer and DNA polymerase and only one other protein, a single-stranded-DNA-binding protein. The latter protein stimulates initiation, and the switch from initiation to elongation. It is essential during elongation, when it coats the displaced strands of the template DNA molecule (Fig. 9.10). Cooperative binding of this protein to single-stranded DNA stimulates the activity of the viral DNA polymerase as much as 100-fold. It also induces highly processive DNA synthesis by this viral enzyme, and progression of replication forks over quite large distances. Remarkably, no ATP hydrolysis is required. The crystal structure of a large segment of the DNA-binding protein indicates that this protein multimerizes via a C-terminal hook (Fig. 9.18A). The formation of long protein chains results in cooperative, high-affinity binding to single-stranded DNA, and is the driving force for ATP-independent unwinding of the duplex template (Fig. 9.18B). Other single-stranded-DNA-binding proteins, such as the herpes simplex virus type 1 UL8 protein and cellular replication

BOX 9.6 DISCUSSION

Model for the transition between initiation and elongation during protein-primed DNA synthesis

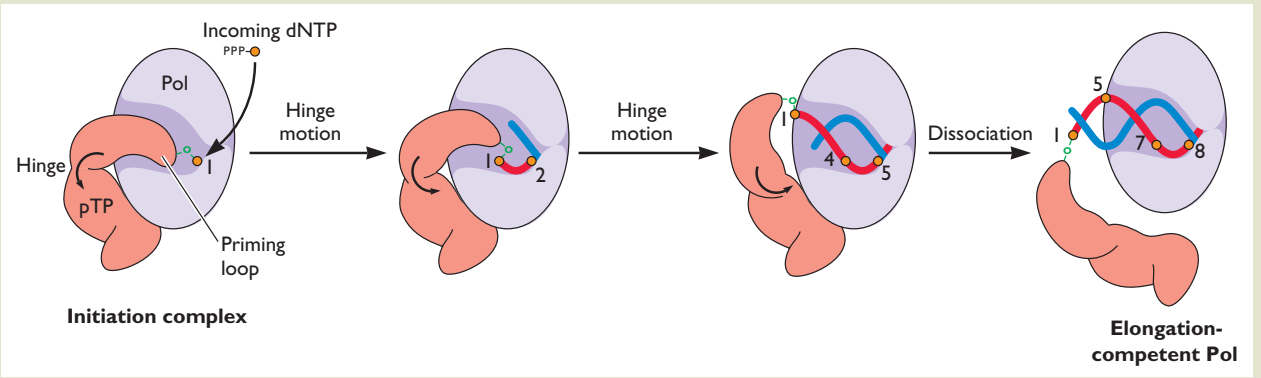
Association of the adenoviral DNA polymerase with the preTP primer is necessary for catalysis of covalent linkage of the priming dCMP to preTP (see the text). However, this interaction must be reversed following initiation to allow processive elongation by the enzyme. Clues about how this transition occurs have come from structural studies of the bacteriophage ϕ29 replication proteins.

Replication of the linear, double-stranded ϕ29 genome is initiated by protein priming from origins at the ends of the genome. The phage DNA polymerase (Pol) and priming terminal protein (TP) form a heterodimer and the enzyme catalyzes linkage of the priming nucleotide to TP, just as in adenoviral DNA synthesis (see Fig. 9.10). The structure of the ϕ29 Pol-TP dimer has been determined by X-ray crystallography. In this complex, the TP priming domain lies in the site occupied by the DNA template-primer in a model of the elongating enzyme. The loop that contains the Ser to which the priming nucleotide is attached lies closest to the Pol active site. The priming domain is connected to a domain that makes extensive contacts with the DNA polymerase via a hinge.

The results of modeling studies indicate that up to 6 or 7 nucleotides can be added to the nascent DNA while TP maintains close contacts with DNA polymerase: motion about the hinge allows displacement of the priming domain while the intermediate domain maintains contact with Pol (see figure). However, this mechanism cannot accommodate further translocation of the priming domain. Rather, the intermediate domain of TP and Pol must dissociate, presumably as a result of addition structural changes. Consequently, the DNA polymerase is released for elongation, as illustrated for incorporation of eight dNMPs in the figure.

Kamtekar, S., A. J. Berman, J. Wang, J. M. Lazaro, M. de Vega, L. Blanco, M. Salas, and T. A. Steitz. 2006. The ϕ29 DNA polymerase: protein primer structure suggests a model for the initiation to elongation transition. *EMBO J.* **25:**1335–1343.

The 3′-OH group of the priming nucleotide attached to TP and nascent DNA are shown in green and red, respectively, with newly incorporated dNMPs indicated by yellow circles. Adapted from S. Kamtekar et al., *EMBO J.* **25:**1335–1343, 2006, with permission.



protein A, may destabilize double-stranded DNA helices by a similar mechanism.

Complete copying of adenoviral DNA templates also requires a cellular topoisomerase, such as topoisomerase I, which relieves overwinding of the template upon extensive replication, as during simian virus 40 DNA synthesis. The simplicity of the adenoviral DNA synthesis system stands in stark contrast to the complexity of the cellular replication machine that is assembled at simian virus 40 replication forks. Such simplicity can be attributed to the protein-priming mechanism, which allows continuous synthesis of full-length daughter DNA strands (Fig. 9.10).

Other viral replication systems include a larger number of accessory replication proteins. Herpes simplex virus type 1 genes encoding essential replication proteins have been identified by both genetic methods and a DNA-mediated transformation assay for the gene products necessary for plasmid replication directed by a viral origin (Fig. 9.19). Replication from a herpes simplex virus type 1 origin requires not only the viral DNA polymerase and origin recognition protein, but also five other viral proteins (Table 9.4). These proteins are functional analogs of essential components of the cellular replication machinery. The products of the UL5, UL8, and UL52 genes form a viral primase, which also functions as a helicase, and the UL42 protein is a processivity factor. Although the herpesviral proteins listed in Table 9.5 provide a large repertoire of replication functions, they do not support viral DNA synthesis *in vitro*. Not all the additional viral and/or cellular proteins needed to reconstitute herpesviral DNA synthesis have

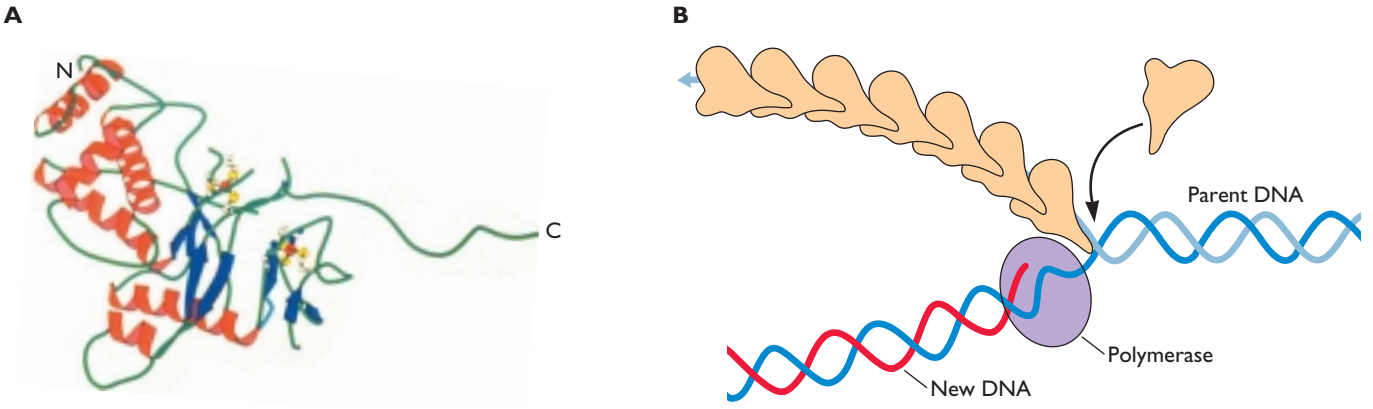


Figure 9.18 Crystal structure of the adenoviral single-stranded-DNA-binding protein. (A) Ribbon diagram of the C-terminal nucleic acid-binding domain (amino acids 176 to 529) of the human adenovirus type 5 protein, showing the two sites of Zn^{2+} (red atom) coordination. The most prominent feature is the long (~40-Å) C-terminal extension. This C-terminal extension of one protein molecule invades a cleft between two α-helices in its neighbor in the protein array formed in the crystal. Deletion of the C-terminal 17 amino acids of the DNA-binding protein fragment eliminates cooperative binding of the protein to DNA, indicating that the interaction of one molecule with another via the C-terminal hook is responsible for cooperativity in DNA binding. From P. A. Tucker et al., *EMBO J.* **13**:2994–3002, 1994, with permission. Courtesy of P. C. van der Vliet, Utrecht University. **(B)** Model of unwinding of double-stranded adenoviral DNA by cooperative interactions among the viral single-stranded-DNA-binding protein.

yet been identified. However, cellular topoisomerase II is essential for replication in infected cells.

Resolution and Processing of Viral Replication Products

Several of the viral DNA replication mechanisms described in preceding sections yield products that do not correspond to the parental viral genome. As we have seen, replication of simian virus 40 DNA yields two interlocked, double-stranded, circular DNA molecules that must be separated by cellular topoisomerase II. Such resolution is required whenever circular templates (e.g., episomal papillomavirus or Epstein-Barr virus DNA) are replicated as monomers. In other cases, replication yields multimeric DNA molecules, from which linear genomes of fixed length and sequence must be processed for packaging into virions. This situation is exemplified by the herpes simplex virus type 1 genome.

The products of herpesviral DNA synthesis are head-to-tail concatemers containing multiple copies of the viral genome. It is well established that the linear viral DNA genomes that enter infected cell nuclei at the start of a productive infection are converted rapidly to "endless" molecules; that is, that the DNA termini are joined together. This reaction requires cellular DNA ligase IV, which normally mediates joining of nonhomologous DNA ends during a cellular repair process. On the other hand, there is no consensus as to whether joining of the herpes simplex viral DNA produces unit-length circles (as found in latently infected cells) or linear concatemers: the large size of the viral genome and the presence of repeated sequences at the termini (Fig. 9.20) has made it difficult to provide an unambiguous experimental demonstration of which type of "endless" structure is formed (Box 9.7). This distinction is of more than esoteric interest, as the configuration of the parental DNA has profound implications about the mechanism of viral DNA synthesis. When a genome is circular, concatemers can be synthesized by the rolling-circle mechanism (Box 9.8), as during replication of the double-stranded DNA genome of bacteriophage lambda. In contrast, recombination is required to produce longer-than-unit-length genomes during replication of a linear template.

However they are made, the concatemeric products of herpesviral DNA synthesis are not simple linear molecules. Rather, they comprise a mixture of structures, including branched (Y- and X-shaped) molecules. A second characteristic feature of herpes simplex virus DNA replication is that it is accompanied by a high degree of recombination. Indeed, conversion of the genome from one of its four isomers to another (Fig. 9.20) occurs by the time that newly replicated DNA can first be detected in infected cells. Such isomerization is the result of recombination between repeated viral DNA sequences (see "Recombination of Viral Genomes" below). These properties suggest that recombination may be an essential reaction during herpes simplex virus type 1 DNA synthesis.

Linear herpes simplex virus type 1 DNA molecules with termini identical to those of the infecting genome are

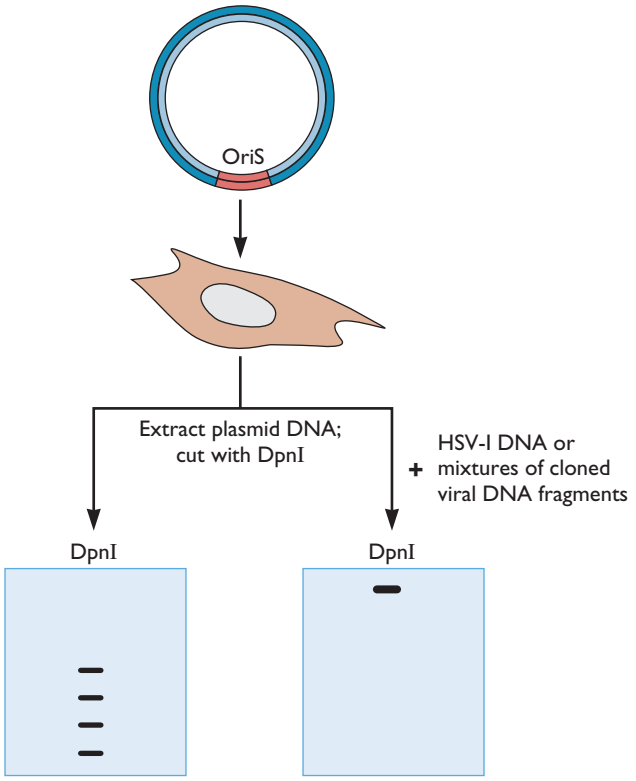


Figure 9.19 DNA-mediated transformation assay for essential herpes simplex virus type 1 replication proteins. A plasmid carrying a viral DNA fragment spanning OriS is introduced into monkey cells permissive for herpesvirus replication. In the absence of viral proteins **(left)**, the plasmid DNA is not replicated and retains the methyl groups added to A residues in a specific sequence by the *E. coli dam* methylation system. As these sequences include the recognition site for the restriction endonuclease DpnI, which cleaves only such methylated DNA, the unreplicated plasmid DNA remains sensitive to DpnI cleavage. When all viral genes encoding proteins required for OriS-dependent replication are also introduced into the cells **(right)**, the plasmid is replicated. Because the newly replicated DNA is not methylated at DpnI sites, it cannot be cleaved by this enzyme. Resistance of the plasmid to DpnI cleavage therefore provides a simple assay for plasmid replication, and hence for the identification of viral proteins required for replication from OriS.

liberated from concatemeric replication products by cleavage at specific sites within the *a* repeats (Fig. 9.11). As described in Chapter 13, such cleavage is coupled with encapsidation of viral DNA molecules during virion assembly.

Mechanisms of Exponential Viral DNA Replication

The details of the mechanisms by which replication of viral DNA genomes is achieved vary considerably from one virus to another. Nevertheless, each of these strategies generally results in efficient replication of viral DNA. Production of 10^4 to 10^5 viral genomes, or more, per infected cell is not uncommon, as the products of one cycle of replication are recruited as templates for the next. Such **exponential** viral DNA synthesis sets the stage for assembly of a large burst of progeny virions. In this section, we discuss regulatory mechanisms that ensure efficient viral DNA synthesis. These regulatory circuits impinge primarily on the expression of cellular or viral genes that encode the proteins that carry out viral DNA synthesis.

Viral Proteins Can Induce Synthesis of Cellular Replication Proteins

With few exceptions, virus reproduction is studied by using established cell lines that are permissive for the virus of interest. Such immortal or transformed cell lines proliferate indefinitely, and differ markedly from the cells in which viruses reproduce in nature. For example, highly differentiated cells, such as neurons or the outer cells of an epithelium, do not divide and are permanently in a specialized resting state, termed the **G_0 state**. Many other cells in an organism divide only rarely, or only in response to specific stimuli, and therefore spend much of their lives in G_0. Such cells do not contain many of the components of the cellular replication machinery, and are characterized by generally low rates of synthesis of RNAs and proteins. Virus reproduction entails the synthesis of large quantities of viral nucleic acids (and proteins), often at a high rate. Consequently, the resting state would not seem to provide a hospitable environment. Nevertheless, viruses often replicate successfully within cells infected when they are in G_0. In some cases, such as replication of several herpesviruses in neurons, the replication machinery is encoded within the viral genome. Infection by other viruses stimulates resting or slowly growing cells to abnormal activity, by disruption of cellular circuits that restrain cell proliferation. This strategy is characteristic of polyomaviruses and adenoviruses. The discovery that infection by these viruses disrupts the same cellular cell growth control circuits was quite unanticipated, and of the greatest importance in elucidating the roles of critical regulators of cell proliferation, such as the cellular retinoblastoma (Rb) protein.

Functional Inactivation of the Rb Protein

Loss or mutation of both copies of the cellular retinoblastoma (*rb*) gene is associated with the development of tumors of the retina in children and young adults. Because it is the **loss** of normal function that leads to tumor formation, *rb* is defined as a **tumor suppressor gene**. As discussed in Volume II, Chapter 7, the Rb protein is an important component of the regulatory program that ensures that cells grow, duplicate their DNA, and divide in an orderly manner. In particular, the Rb protein controls entry into the period of

Table 9.5 Viral enzymes of nucleic acid metabolism

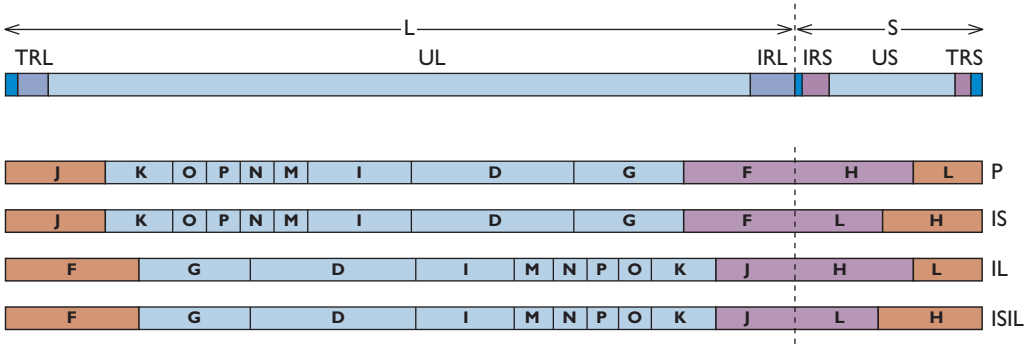
Virus	Protein	Functions
Herpesvirus		
Herpes simplex virus type 1	Thymidine kinase (UL23 protein, ICP36)	Phosphorylates thymidine and other nucleosides; essential for efficient replication in animal hosts
	Ribonucleotide reductase ($\alpha_2\beta_2$ dimer of UL39 and U40 proteins)	Reduces ribose to deoxyribose in ribonucleotides; essential in nondividing cells
	dUTPase (UL50 protein)	Hydrolyzes dUTP to dUMP, preventing incorporation of dUTP into DNA and providing dUMP for conversion to dTMP
	Uracil DNA glycosylase	Corrects insertion of dUTP or deamination of C in viral DNA
	Alkaline nuclease (UL12 protein)	Required for production of infectious DNA
Poxvirus		
Vaccinia virus	Thymidine kinase	Phosphorylates thymidine; required for efficient virus reproduction in animal hosts
	Thymidylate kinase	Phosphorylates TMP
	Ribonucleotide reductase, dimer	Reduces ribose to deoxyribose in ribonucleotides; essential in nondividing cells
	dUTPase	Hydrolyzes dUTP to dUMP (see above)
	DNase	Has nicking-joining activity; present in virion cores
	D4R protein	Uracil DNA glycosylase

the cell cycle in which DNA is synthesized, the **S phase**, from the preceding (G_1) phase. Our current appreciation of the critical participation of this protein in the control of cell cycle progression, and of the mechanism by which it operates, stems from the discovery that Rb binds directly to the two adenoviral E1A proteins (see Chapter 8).

In uninfected cells in the G_1 phase, the Rb protein is bound to cellular transcriptional regulators of the E2f family. These complexes, which bind to specific promoters via the DNA-binding activity of E2f, function as repressors of transcription (Fig. 9.21A). Binding of adenoviral E1A proteins (or of simian virus 40 LT or E7 proteins of highly oncogenic human papillomaviruses) to Rb releases E2f from this association and sequesters Rb. The E2f proteins therefore become available to stimulate transcription of cellular genes that encode proteins that participate directly or indirectly in DNA synthesis, or in control of cell cycle progression (Fig. 9.21A; Volume II, Chapter 7).

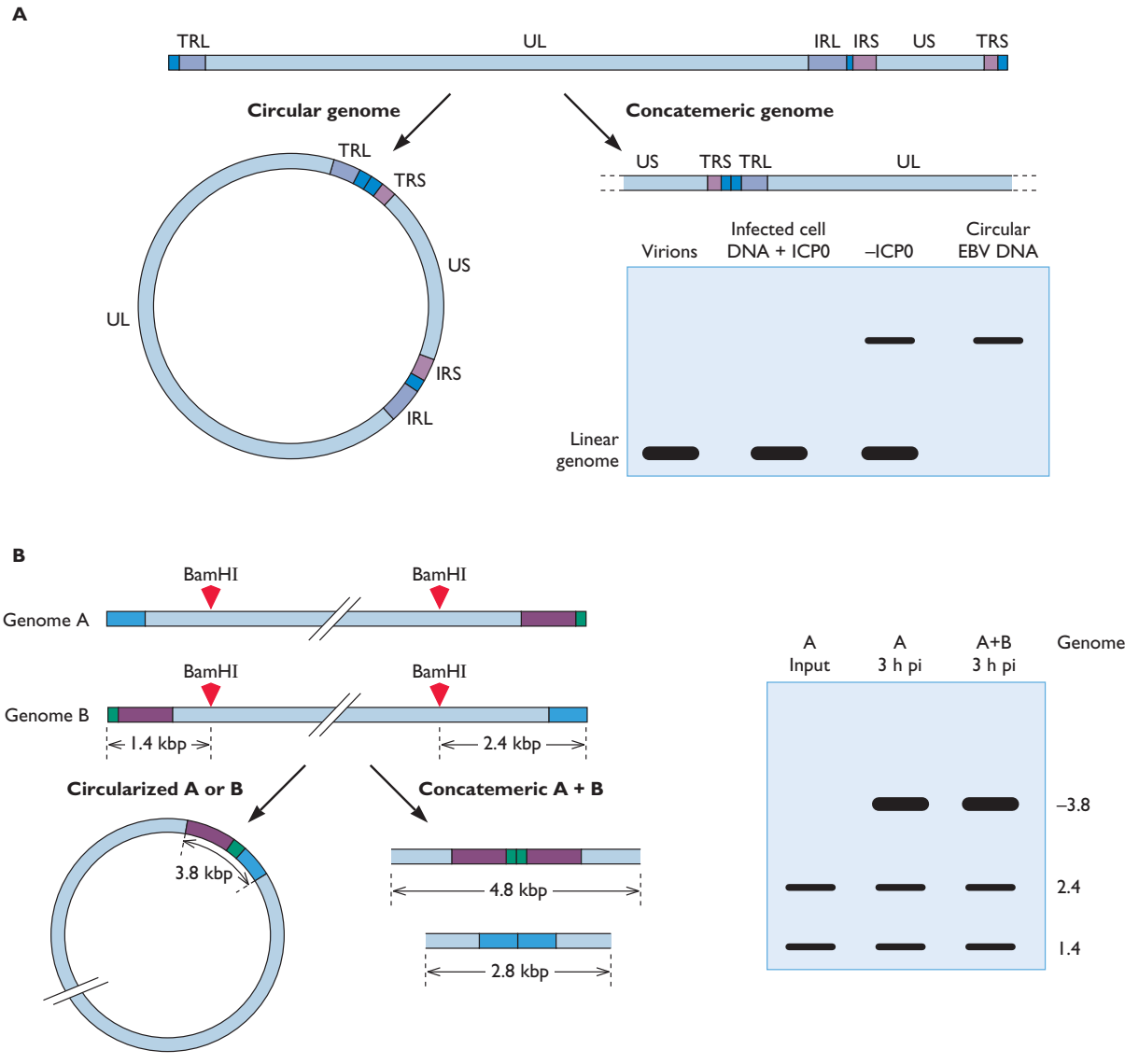
The benefits conferred by activation of E2f when a polyomavirus or an adenovirus infects a cell that is proliferating slowly, or in G_0, are virus specific. While the advantages for viral DNA replication can be deduced, it is important

Figure 9.20 Isomers of the herpes simplex virus type I genome. The organization of the unique and repeated sequences of the viral genome are depicted at the top, as in Fig. 9.11. This orientation is defined as the prototype (P) genome isomer. The other three isomers differ, with respect to the P form, in the orientation of S (IS), in the orientation of L (IL), or in both S and L (ISIL). These differences are illustrated using HindIII fragments. The unusual isomerization of this viral genome was deduced from the presence of fragments that span the terminal or internal inverted repeat sequences at 0.5 and 0.25 molar concentrations, respectively, in such HindIII digests, and examination of partially denatured DNA in the electron microscope.



BOX 9.7 DISCUSSION

Circularization or concatemerization of the herpes simplex virus type 1 genome in productively infected cells?



(A) Evidence for genome concatemerization. These experiments exploited a method of electrophoresis in which infected cells (or nuclei) are lysed in the initial portion of the gel (to minimize damage of the DNA) prior to electrophoresis for a very long period. The viral DNA was then detected by hybridization to labeled viral DNA, following transfer to a membrane. As summarized in the figure, a fraction of infecting viral DNA molecules was detected as a species of low mobility that migrated in similar fashion to circular Epstein-Barr virus DNA. Such molecules were detected only in the absence of the immediate-early gene product ICP0. No such molecules were observed in cells infected by wild-type virus, in the absence or presence of an inhibitor of viral DNA synthesis. These observations led to the conclusion that the herpesviral genome circularized in the absence of ICP0 (as in latently infected cells), but not during productive infection. Consequently, linear viral DNA molecules would serve as the templates for initial viral DNA synthesis. **(B) Evidence for a circular genome.** These experiments exploited mutant viruses that lacked all a repeats (the packaging signal) and contained a minimal packaging signal at an ectopic site. As illustrated, cleavage of the linear mutant genomes designated A and B by BamHI yields terminal fragments of 1.4 and 2.4 kb. Circularization of either genome generates a new, unique junction fragment of 3.8 kb, whereas head-to-tail concatemerization of A with B would produce distinguishable BamHI fragments. The fragment characteristic of circularization was detected within 1 h of infection by either virus in cells that synthesized ICP0. In contrast, neither of the junction fragments diagnostic of concatemers were observed in cells coinfected by the two viruses. These observations led to the conclusion that the genome circularizes very soon after entry into infected cell nuclei, and in this form serves as a template for initial viral DNA synthesis.

(continued)

BOX 9.7 DISCUSSION

Circularization or concatemerization of the herpes simplex virus type 1 genome in productively infected cells? (continued)

It has been known for some time that the linear herpes simplex virus type 1 DNA that enters nuclei of productively infected cells rapidly adopts a new conformation in which the termini are fused: restriction endonuclease cleavage of viral DNA recovered shortly after infection established that cleavage products generated from free ends decrease in concentration as those characteristic of joined ends increase. However, the origin of the latter fragments has been difficult to determine, for several reasons.

- Formation of either unit length circles or concatemers results in loss of free termini.
- The presence of an internal inverted copy of the joined terminal repeats precludes the use of an assay based on detection of joined termini.
- Conventional methods for separation and identification of linear and circular DNA molecules by electrophoresis cannot be applied to the large herpesviral genome.
- Because of its large size, the herpesviral genome may be easily damaged during extraction from infected cells.
- Under conditions that facilitate detection of viral DNA, high multiplicity of infection, the majority of infecting DNA molecules may be neither transcribed nor replicated.

These different conclusions have yet to be reconciled. Complications of approach A in the figure include the difficulty of interpreting electrophoretic mobility in terms of specific DNA structures and the possible trapping of molecules with complicated structure in the wells of the gel. Approach B (see figure) appears straightforward. However, the mutant genomes lack sequences present in wild-type DNA, and the authors could not detect circular genomes by the electrophoretic method.

Jackson, S. A., and N. A. DeLuca. 2003 Relationship of herpes simplex virus genome configuration to productive and persistent infections. *Proc. Natl. Acad. Sci. USA* **100:**7871–7876.

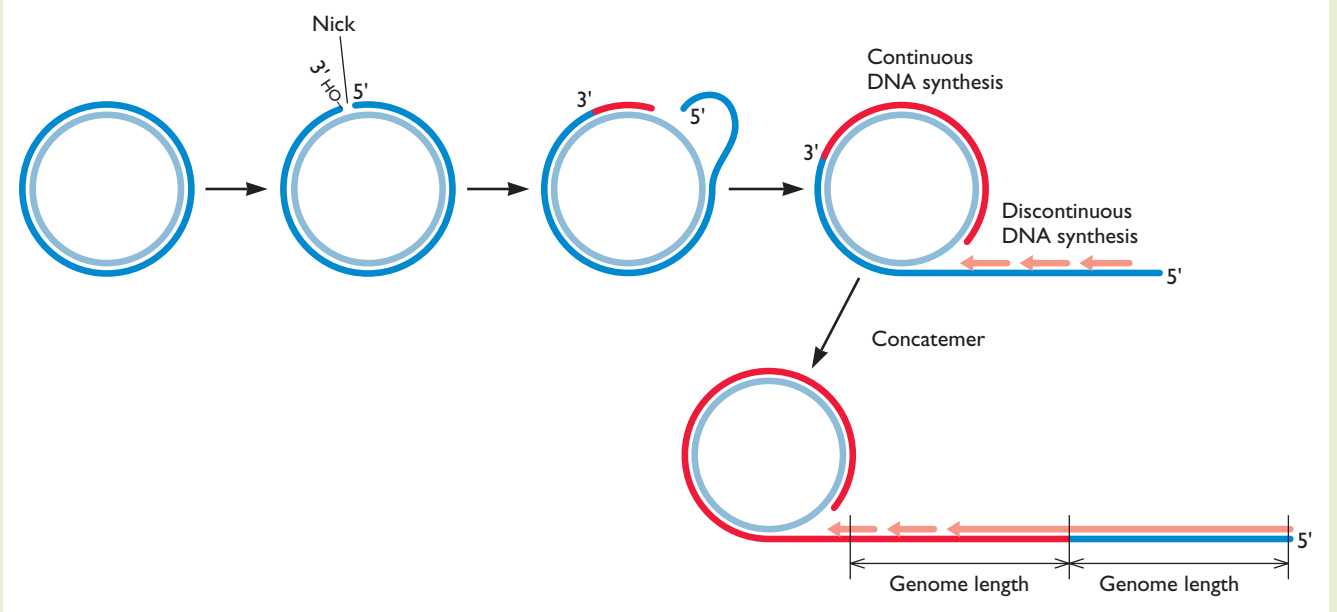
Strang, B. L., and N. D. Stow. 2005 Circularization of the herpes simplex virus type 1 genome upon lytic infection. *J. Virol.* **79:**12487–12494.

BOX 9.8 BACKGROUND

Rolling-circle replication

The rolling-circle replication mechanism of DNA synthesis was discovered during studies of the replication of the single-stranded DNA genome of bacteriophage ϕX174. However, it also operates during replication of double-stranded genomes, such as that of bacteriophage lambda.

Rolling-circle replication is initiated by introduction of a nick that creates a 3′-OH end into one strand of a double-stranded circular DNA. One strand of the template is copied continuously, and multiple times, while the displaced strand is copied discontinuously. As shown, this mechanism produces genome concatemers.



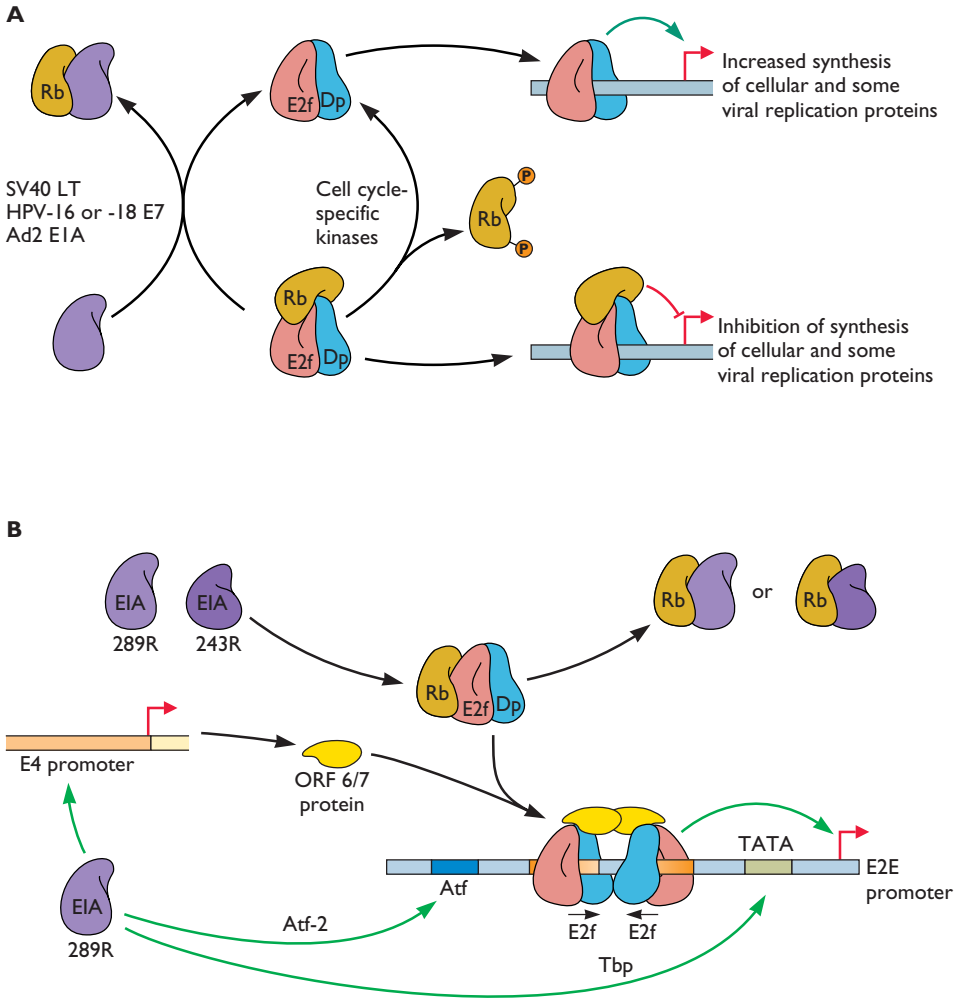


Figure 9.21 Regulation of production of cellular and viral replication proteins. (A) Model for the abrogation of the function of the Rb protein by viral proteins. E2f transcriptional regulators are heterodimeric proteins, each containing one E2f and one Dp subunit. E2f dimers stimulate transcription of cellular genes encoding replication proteins, histones, and proteins that allow passage through the cell cycle (green arrow). Binding of Rb protein does not prevent promoter recognition by E2f. However, Rb protein represses transcription (red bar). Phosphorylation of Rb protein at specific sites induces its dissociation from E2f, and activates transcription of cellular genes expressed in S phase. The adenoviral E1A proteins, simian virus 40 LT, and the E7 proteins of certain human papillomaviruses (types 16 and 18) bind to the region of Rb protein that contacts E2f to disrupt Rb-E2f complexes and activate E2f-dependent transcription. **(B)** Stimulation of transcription from the adenoviral E2 early promoter by E1A proteins. The E2E promoter-binding sites for the cellular Atf, E2f, and TfIId proteins are necessary for E2E transcription in infected cells. The inversion of the two E2f sites (arrows) and their precise spacing are essential for assembly of an E2f-DNA complex unique to adenovirus-infected cells, in which the E4 Orf6/7 protein is bound to each E2f heterodimer. Binding of the E4 protein promotes cooperative binding of E2f, and increases the lifetime of E2f-DNA complexes. The availability of the cellular E2f and viral E4 Orf6/7 proteins is a result of the action of immediate-early E1A proteins: either the 243R or 289R protein can sequester unphosphorylated Rb to release active E2f from Rb-E2f complexes, and the 289R protein stimulates transcription from the E4 promoter. This larger E1A protein can also stimulate transcription from the E2E promoter directly.

to stress that essentially all studies of the cell growth control functions of LT, E7, or E1A proteins have been directed toward elucidation of mechanisms of transformation. For example, it has been shown that the smaller adenoviral E1A protein is necessary for efficient viral DNA synthesis in quiescent human cells. However, it has not been established that the E1A protein plays a critical role in activation of cellular E2f, as assumed in the model described below.

E2f and simian virus 40 DNA synthesis. Expression of the cellular genes encoding DNA polymerases, accessory replication proteins, histones, and enzymes that synthesize substrates for DNA synthesis is tightly controlled during the cell cycle: these proteins are made only just before they are needed in S phase. The transcriptional control regions of many of these genes contain E2f-binding sites, which are required for their efficient transcription. Synthesis of simian virus 40 LT is therefore thought to induce production of the cellular proteins necessary for viral DNA synthesis, and hence to ensure efficient replication of the viral genome regardless of the growth state of the host cell. The ability of simian virus 40 LT both to induce synthesis of components of the cellular replication machinery and to initiate viral DNA synthesis seems likely to coordinate viral replication with the entry of the host cell into S phase. Indeed, as discussed previously, LT replication functions are regulated by phosphorylation, probably by the cyclin A–cyclin-dependent kinase 2 that accumulates as cells enter S phase.

E2f and adenoviral DNA synthesis. Activation of E2f in adenovirus-infected cells is believed to promote viral DNA synthesis in two ways: stimulation of transcription of the cellular genes encoding enzymes that make substrates for DNA synthesis, such as thymidine kinase and dihydrofolate reductase, and activation of production of all three viral replication proteins. The viral DNA polymerase, preterminal protein primer, and DNA-binding protein are encoded within the E2 gene, which is transcribed from an early promoter that contains two binding sites for E2f (Fig. 9.21B). In fact, these critical cellular regulators derive their name from these E2-binding sites, which are necessary for efficient E2 transcription during the early phase of infection. As discussed previously, the viral E1A proteins disrupt Rb-E2f complexes and sequester Rb. They also regulate transcription from the E2 promoter by two other mechanisms (Fig. 9.21B). These E1A-dependent regulatory mechanisms presumably operate synergistically, to allow synthesis of the viral mRNAs encoding replication proteins in quantities sufficient to support numerous cycles of viral DNA synthesis. A posttranscriptional regulatory mechanism may allow production of the appropriate concentrations of the three E2 replication proteins (Box 9.9).

Synthesis of Viral Replication Machines and Accessory Enzymes

The DNA genomes of several viruses, exemplified by that of herpes simplex virus type 1, encode large cohorts of proteins that participate directly or indirectly in viral DNA synthesis. Two classes of such replication proteins are synthesized during productive infection. The first comprises the viral DNA polymerase and other proteins responsible for viral DNA synthesis discussed previously (Table 9.4). The second class contains enzymes, analogous to host cell enzymes, that catalyze reactions by which dNTPs are synthesized (Table 9.5). For example, viral thymidine kinase and ribonucleotide reductase are synthesized in herpesvirus-infected cells. In general, members of this second class of proteins are dispensable for replication in proliferating cells in culture and in animal hosts. However, herpes simplex viruses that lack thymidine kinase or ribonucleotide reductase genes cannot replicate in neurons: such terminally differentiated cells are permanently withdrawn from the cell cycle, and do not make enzymes that produce substrates for DNA synthesis.

Efficient synthesis of all herpes simplex virus type 1 replication proteins is primarily the result of the viral transcriptional cascade described in Chapter 8. Expression of the genes encoding these viral proteins, which are early (β) genes, is regulated by products of immediate-early genes. These immediate-early proteins operate transcriptionally (e.g., ICP0 and ICP4) or posttranscriptionally (e.g., ICP27) to induce synthesis of viral replication proteins at concentrations sufficient to support exponential replication of viral DNA. Whether the multiple origins of herpes simplex virus type 1 and other herpesviruses increase the rate of replication will not be clear until the viral replication mechanism has been better characterized.

Viral DNA Replication Independent of Cellular Proteins

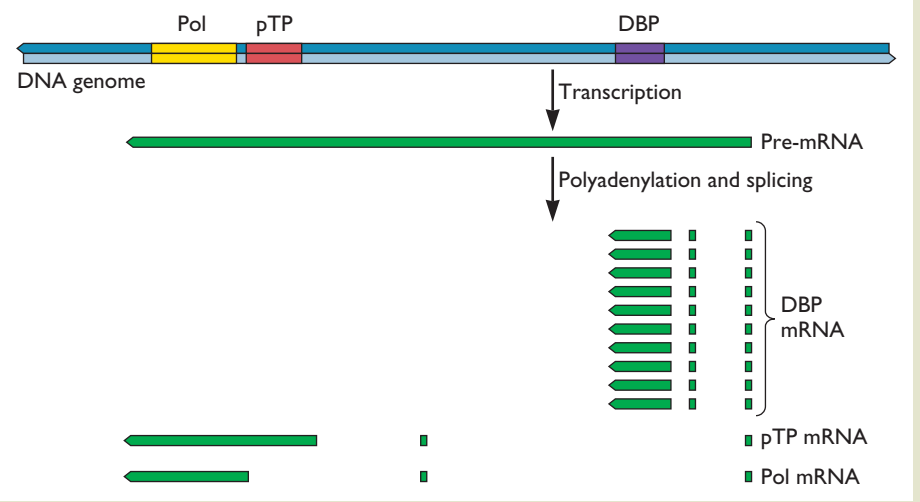
One method guaranteed to ensure replicative success of a DNA virus, regardless of the growth state of the host cell, is encoding of **all** necessary proteins in the viral genome. On the other hand, this mechanism is genetically expensive, which may be the reason why it is restricted to the viruses with the largest DNA genomes, such as the poxvirus vaccinia virus. The genome of this virus, which is replicated in the cytoplasm at specialized foci called **viral factories**, encodes all the proteins needed for viral DNA synthesis. The current catalog of such proteins includes a DNA polymerase and several accessory replication proteins (Table 9.4). The vaccinia virus genome also encodes several other enzymes that would be expected to participate in DNA synthesis or in resolution of replication products, including a type I topoisomerase and a DNase with endonucleolytic activity, as well as several enzymes for synthesis of dNTPs (Table 9.5). None of the latter appear to be essential for virus reproduction in actively growing cells. However, several enzymes, such as the thymidine kinase, are necessary for efficient virus propagation in quiescent cells or animal hosts, where they presumably increase less than optimal substrate pools. Much remains to be learned about the mechanism by which the vaccinia virus genome is replicated and the functions of viral replication proteins.

BOX 9.9 DISCUSSION

Production of appropriate quantities of the adenoviral replication proteins by differential polyadenylation?

The three adenoviral replication proteins, Pol, pTP, and DBP, are all encoded within the E2 transcription unit of the viral genome. However, they are needed in very different quantities during viral DNA synthesis. The replication of one molecule of viral DNA requires four molecules each of Pol and the pTP primer (Fig. 9.10). Once the daughter strands have been synthesized, Pol dissociates and can catalyze additional replication cycles. This protein is therefore required at **catalytic** concentrations. In contrast, the pTP remains covalently attached to the 5′ end of each newly synthesized DNA strand and is incorporated into progeny virus particles. This protein is therefore required at a greater concentration than Pol. The DBP coats the single-stranded DNA displaced during viral DNA synthesis (Fig. 9.10). This protein, which interacts with about 12 nucleotides, binds cooperatively to such DNA to form a long chain (Fig. 9.18). Thousands of molecules of DBP are needed during replication of a single molecule of the viral genome.

As shown in the figure, E2 primary transcripts can be polyadenylated at either a promoter-proximal (DBP mRNA) or a promoter-distal (Pol and pTP mRNAs) site. The former appears to be utilized much more frequently than the latter: DBP mRNA accumulates to 10-to 20-fold-higher concentrations than the Pol and pTP mRNAs. In this way, the replication proteins can be made in the appropriate relative concentrations, even though they are encoded in the same transcription unit.



Delayed Synthesis of Virion Structural Proteins Prevents Premature Packaging of DNA Templates

During productive infection by DNA viruses, each cycle of replication increases the number of DNA molecules that can be copied in the subsequent cycle. This increase in the pool of replication templates, a doubling in each cycle of the simpler viruses like simian virus 40 and adenovirus, undoubtedly makes an important contribution to rapid amplification of genomes. Progeny viral DNA molecules are eventually encapsidated during the assembly of new virus particles, and consequently become unavailable to serve as templates for DNA synthesis. However, assembly and sequestration of the genome are delayed with respect to initiation of viral DNA synthesis, because transcription of late genes encoding virion proteins cannot begin until viral DNA has been replicated (Chapter 8).

Inhibition of Cellular DNA Synthesis

When viral DNA replication is carried out largely by viral proteins, cellular DNA synthesis is often inhibited, presumably to increase the availability of critical substrates. Infection by the larger DNA viruses (herpesviruses and poxviruses) induces severe inhibition of synthesis of cellular DNA. Cellular DNA synthesis is also blocked when adenoviruses infect proliferating cells in culture, despite the elaborate mechanisms by which these viruses induce quiescent host cells to reenter the cell cycle. Inhibition of cellular DNA synthesis in cells infected by these DNA viruses was described in some of the earliest studies of their infectious cycles. However, very little is known about the mechanisms that shut down this cellular process.

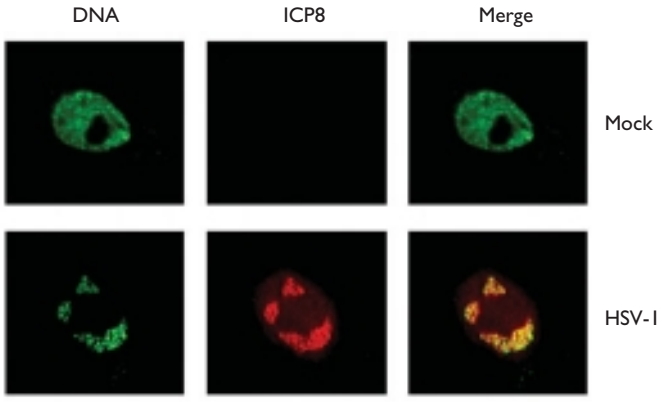
There is accumulating evidence that inhibition of cellular DNA synthesis is an active process, rather than an indirect result of passive competition between viral and

cellular DNA polymerases for the finite pools of dNTP substrates. For example, infection of proliferating cells by adenovirus or the herpesvirus human cytomegalovirus has been reported to induce cell cycle arrest. Synthesis of the Epstein-Barr virus Zta protein, a sequence-specific transcriptional regulator and origin-binding protein, also arrests cells at a point in the cell cycle prior to S phase, such that cellular DNA synthesis is precluded. In this case, arrest is the result of increased concentrations of cellular proteins that negatively regulate progression through the cell cycle, such as the Rb protein.

Viral DNAs Are Synthesized in Specialized Intracellular Compartments

A common, probably universal, feature of cells infected by viruses with DNA genomes is the presence of virus-specific structures that are the sites of viral DNA synthesis. Vaccinia virus DNA is replicated in the cytoplasm, in discrete viral factories that contain both the DNA templates and viral replication proteins. The replication of viral DNA genomes within infected cell nuclei also takes place in specialized compartments, which can be visualized as distinctive, infected cell-specific structures containing viral proteins (Fig. 9.22). Such structures, known as **replication centers** or **replication compartments**, have been best characterized in human cells infected by adenovirus or herpes simplex virus type 1. They contain newly synthesized viral DNA and all the viral proteins necessary for viral DNA synthesis, as well as other viral and cellular proteins.

Figure 9.22 Nuclear replication compartments of herpes simplex virus type 1-infected monkey cells. The locations of the viral ICP8 single-stranded-DNA-binding protein and newly replicated DNA were visualized 6 h after infection by indirect immunofluorescence with antibodies against ICP8 and bromodeoxyuridine incorporated into DNA, respectively. Newly synthesized viral DNA and ICP8 colocalize to a limited number of globular patches, which contain all seven known viral replication proteins. Courtesy of Lindsey Silva and David Knipe, Harvard Medical School.



Among the cellular proteins associated with herpes simplex virus type 1 replication centers are several enzymes, such as DNA polymerases α and γ, and topoisomerase II, and numerous DNA repair and recombination proteins.

The localization of both the templates for viral DNA synthesis and the replication proteins at a limited number of sites undoubtedly facilitates exponential viral DNA replication: this arrangement increases the local concentrations of proteins that must interact with one another, or with viral origin sequences or replication forks, favoring such intermolecular interactions by the law of mass action. In addition, the high local concentrations of replication templates and proteins are likely to allow for efficient recruitment of the products of one replication cycle as templates for the next.

Viral replication centers also serve as foci for viral gene expression, presumably in part by concentrating templates for transcription with the proteins that carry out or regulate this process. For example, the herpes simplex virus type 1 immediate-early ICP4 and ICP27 proteins, as well as the host cell's RNA polymerase II, are recruited to these nuclear sites. Similarly, an adenoviral early protein complex necessary for selective export of viral late mRNAs from the nucleus (Chapter 10) is associated with the periphery of viral replication centers, as is nascent viral RNA.

Viral replication centers do not assemble at random sites, but rather are formed by viral colonization of specialized niches within mammalian cell nuclei. When they enter the nucleus, infecting adenoviral or herpes simplex virus type 1 genomes (as well as those of papillomaviruses and polyomaviruses) localize to preexisting nuclear bodies that contain specific cellular proteins, the promyelocytic leukemia proteins (Pmls). These structures are therefore called **Pml bodies**, or nuclear domain 10 structures, a name derived from the average number present in most cells. Viral proteins then induce reorganization of Pml bodies as viral replication centers are established (Fig. 9.23). For example, the herpes simplex virus type 1 ICP0 protein causes disruption of these cellular structures. This viral protein is an E3 ubiquitin ligase. Such enzymes catalyze addition of polyubiquitin chains to proteins, a modification that targets them for degradation by the proteasome. ICP0 induces the degradation of several components of Pml bodies, including some Pml proteins. Others, including specific isoforms of the Pml protein, Daxx, and many proteins that participate in repair, recombination, and chromatin remodeling, become associated with viral replication centers. Recruitment of specific proteins present in Pml bodies to viral replication centers also occurs in adenovirus-infected cells, and requires the E4 Orf 3 protein.

The association of replication centers of different DNA viruses with constituents of the same intranuclear

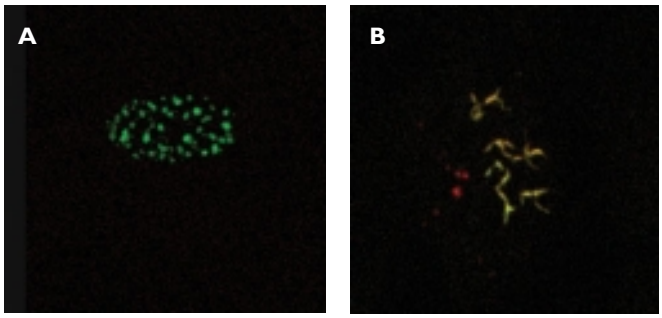


Figure 9.23 Reorganization of Pml bodies by the adenoviral E4 Orf3 protein. Plasmids for expression of human Pml isoforms I to VI linked to a FLAG tag were introduced in *Pml*-null mouse embryo fibroblasts in the absence **(A)** or presence **(B)** of an expression vector for the viral E4 Orf3 protein. The Pml (green) and E4Orf3 (red) proteins were visualized by using indirect immunofluorescence. All Pml isoforms formed Pml-like bodies alone, but only structures containing PmlI were disrupted and reorganized by the E4 Orf 3 protein. Adapted from A. Hoppe et al., *J. Virol.* **80:**3042–3049, 2006, with permission. Courtesy of K. Leppard, University of Warwick, Coventry, United Kingdom.

structures suggests that reorganization of host cell nuclei facilitates viral DNA synthesis. The discovery that viral DNA genomes home to Pml bodies has stimulated characterization of their constituents, but much remains to be learned about the molecular functions of these nuclear structures. There is accumulating evidence that Pml bodies represent a form of intrinsic antiviral defense (Volume II, Chapter 3). However, other advantages conferred by the degradation or dispersal of Pml body proteins are likely to be virus specific. For example, the human papillomavirus type 18 E6 protein induces proteasomal degradation of a Pml isoform (Pml-IV) that causes primary human cells to become senescent (a state in which cellular proteins required for replication of the viral genome are not made). In cells infected by adenovirus, alterations in specific Pml components block disadvantageous concatemerization of the viral genome (see below). In contrast, herpesviral DNA synthesis may require cellular repair and recombination proteins that become relocalized to viral replication centers. Furthermore, it has been reported recently that formation of herpesviral replication compartments represents but an early step in the massive alterations in nuclear architecture necessary for egress of newly formed nucleocapsids (see Chapter 13).

Limited Replication of Viral DNA

Exponential replication of viral DNA is the typical pattern when the majority of DNA viruses infect cells in culture. Nevertheless, several can establish long-term relationships with their hosts and host cells, in which the number of genomes produced is limited. Various mechanisms that effect such copy number control are described in this section.

Integrated Parvoviral DNA Can Replicate as Part of the Cellular Genome

The adeno-associated viruses reproduce only in cells coinfected with a helper adenovirus or herpesvirus. Although the latter viruses are widespread in hosts infected by adeno-associated viruses, the chances that a particular host cell will be infected simultaneously by two viruses are very low. The strategy of exploiting other viruses to provide functions for efficient expression of the genetic information of adeno-associated viruses would therefore appear to be potentially lethal for individual virus particles. In fact, this is not the case, for adeno-associated virus can survive in the absence of a helper virus by an alternative mechanism: its genome becomes integrated into that of the host cell, and is replicated as part of a cellular replicon.

This program for long-term survival of the adeno-associated virus genome depends on expression of its regulatory region (Rep) (Box 9.4). The two larger proteins encoded by this region, Rep 78/68, are multifunctional and control all phases of the viral life cycle (Table 9.3). When helper virus proteins, such as adenoviral E1A, E1B, and E4 proteins, allow synthesis of large quantities of Rep 78/68, adeno-associated virus DNA is replicated by the mechanism described previously. In the absence of helper functions, only very small quantities of Rep 78/68 are made, even in healthy, dividing cells. Consequently, there is little viral DNA synthesis, and the genome is integrated into that of the host cell. The latter reaction is mediated by Rep 78/68.

One of the most unusual features of this integration reaction is that it occurs preferentially near one end of human chromosome 19. It was believed for many years that integration required the recognition of the viral ITR origin (Fig. 9.12) by Rep 78/68. However, the observation that integration of DNA molecules containing only the ITR was exceedingly inefficient led to the identification of a viral sequence that increased the frequency of site-specific integration by up to 100-fold. This sequence corresponds to the viral origin that overlaps the p5 promoter (Fig. 9.12) and is, in fact, sufficient for integration. The Rep 78/68 protein can bind simultaneously to both viral and human chromosomal 19 DNA sequences required for integration, at least *in vitro*. The current model of integration therefore proposes that its specificity is the result of such simultaneous binding to the two DNA molecules by Rep 78/68. Rep mediates nonhomologous recombination reactions that result in integration of the viral genome, with concomitant, large deletions or duplications of cellular DNA.

Following high-multiplicity infection of cells in culture, as many as 40% of the cells contain the integrated adeno-associated virus genome. However, the results of more recent studies indicate that site-specific integration is very rare when the virus infects humans or laboratory animals. Rather, the viral genome can persist in various tissues, for example tonsil and lung in humans, as a double-stranded, circular episome. The mechanisms by which such episomes are produced from the linear, single-stranded DNA genome are not yet clear.

Regulation of Replication via Different Viral Origins: Epstein-Barr Virus

During herpesviral latent infections, the viral genome is stably maintained at low concentrations, often for long periods (Volume II, Chapter 5). Furthermore, replication of viral and cellular genomes can be coordinated. This pattern is characteristic of human B cells latently infected by Epstein-Barr virus. Many such cell lines have been established from patients with Burkitt's lymphoma, and this state is the usual outcome of infection of B cells in culture. Characteristic features of latent Epstein-Barr virus infection include expression of only a small number of viral genes, the presence of a finite number of viral genomes, and replication from a specialized origin. Because replication from this origin, which is not active in lytically infected cells, is responsible for maintenance of episomal viral genomes, it is termed the **origin for plasmid maintenance** (OriP).

The Epstein-Barr virus genome is maintained in nuclei of latently infected cells as a stable circular **episome**, present at 10 to 50 copies per cell. For example, one Burkitt's lymphoma cell line (Raji) has carried about 50 copies per cell of episomal viral DNA for over 40 years. When Epstein-Barr virus infects a B cell, the linear viral genome circularizes by a mechanism that is not well understood. The circular viral DNA is then amplified during S phase of the host cell to the final concentration listed above. Such replication is by the cellular DNA polymerases and accessory proteins that synthesize simian virus 40 DNA. However, it also requires OriP (Fig. 9.24A), and the viral protein that binds specifically to it, EBNA-1 (Table 9.3), which is invariably synthesized in latently infected cells. In contrast to exponential replication, such amplification of the episomal viral genome is limited to a few cycles. Following such limited amplification, the viral DNA is duplicated once per cell cycle, in S phase, such that its concentration is maintained as the host lymphocyte divides. The EBNA-1 protein and OriP are sufficient for both such once-per-cell cycle replication and orderly segregation of viral genomes to daughter cells.

The availability of cellular replication proteins only in late G_1 and S can account for the timing of Epstein-Barr virus replication in latently infected cells. However, this property **cannot** explain why each genome is replicated only once in each cell cycle. Such controlled initiation of replication is analogous to the tight control of initiation of replication from cellular origins, each of which also fires **once and only once** in each S phase. The mechanisms that control such once-per-cycle firing of eukaryotic origins, a process termed **replication licensing**, were initially elucidated in budding yeasts, which contain compact origins of replication. Mammalian homologs of the yeast origin recognition complex (Orc) and proteins that regulate initiation of DNA synthesis, such as Mcm and Cdc6, have been identified in all other eukaryotes examined. The human Orc proteins are associated with OriP and can bind to EBNA-1. Experimental manipulations that reduce the concentrations of specific Orc proteins severely inhibit OriP-dependent replication. Human Mcm proteins are also associated with OriP during G_1 phase. However, they are not present at OriP during G_2, as would be expected if replication from OriP were licensed by the mechanism shown in Fig. 9.24B. Overproduction of a protein that prevents recruitment of Mcm inhibits the OriP-dependent replication complex (Fig. 9.24B). This observation provides strong support for the conclusion that synthesis of viral DNA genomes in latently infected cells is governed by the mechanisms that ensure once-per-cell-cycle firing of cellular origins.

In addition to EBNA-1-binding sites, OriP contains three copies of a nonameric sequence that resemble repeated sequences present in telomeres. In the presence of EBNA-1, several cellular proteins bind to the repeats. Such cellular proteins include telomerase-associated poly(ADP-ribose) polymerases and telomere repeat-binding protein 2. These protein regulate OriP-dependent replication negatively and positively, respectively, but how they do so is not yet clear.

Orderly segregation of episomal viral DNA molecules during mitosis requires binding of EBNA-1 to its high affinity sites in the family of repeat (FR) sequences of OriP (Fig. 9.24A). Direct observation of episomal viral genomes by *in situ* hybridization has established that these DNA molecules become tethered to the cellular sister chromatids that are separated during mitosis. Tethering of viral DNA chromosomes, and their partitioning during mitosis, is mediated by an N-terminal sequence of EBNA-1 that contains two domains that can bind directly to AT-rich DNA. In metaphase chromosomes, regions of uncondensed (that is, accessible) AT-rich DNA are found between segments of the genome that are highly condensed. Any derivative of EBNA-1 that contains two such AT-hook domains (even if they are derived from cellular proteins) binds to chromosomes and supports maintenance of OriP-containing episomes in a host cell population.

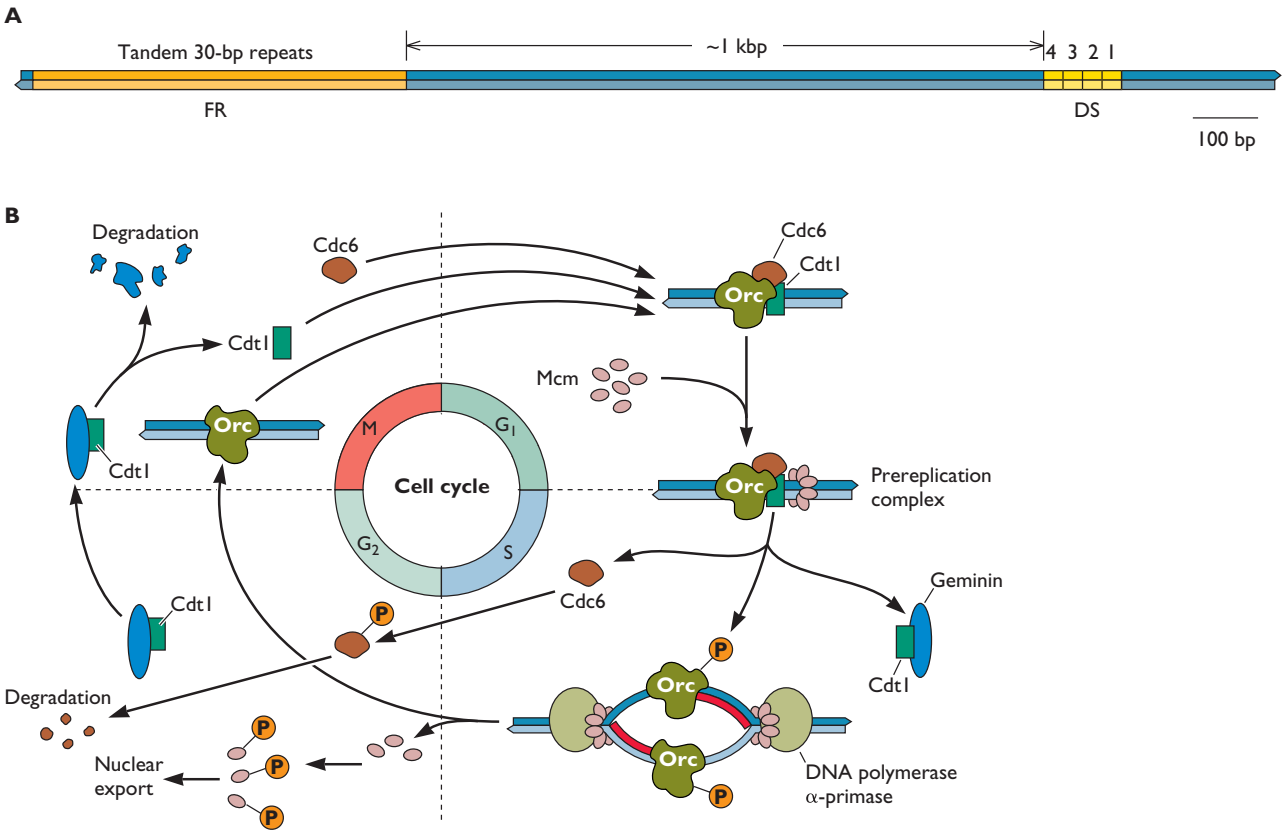


Figure 9.24 Licensing of replication from Epstein-Barr virus OriP. (A) Organization of EBNA-binding sites, shown to scale. The dyad symmetry (DS) sequence, which comprises four binding sites (1 to 4) for EBNA-1 dimers, is the site of initiation of DNA synthesis. The activity of the DS origin is regulated by adjacent sequences recognized by cellular telomere-binding proteins (see the text) and stimulated by the family of repeat (FR) sequence. The mechanism of such stimulation is not clear. However, binding of EBNA-1 to both DS and FR sequences, with the intervening 1 kbp of DNA looped out, appears to be important. Binding of EBNA-1 to FR sequences is also necessary for maintenance of episomal viral DNA in latently infected B cells. **(B)** The multiprotein origin recognition complex (Orc) is present throughout the cell cycle and is associated with replication origins. However, initiation of DNA synthesis requires loading of the hexameric minichromosome maintenance complex (Mcm), which provides helicase activity. It is the recruitment of Mcm that is controlled during the cell cycle to set the timing of the initiation of DNA synthesis in S phase. This reaction requires two proteins, Cdc6 and Cdt1. The concentrations and activities of both are tightly controlled during the cell cycle. As cells complete mitosis and enter G_1, Cdc6 and Cdt1 accumulate in the nucleus, where they associate with DNA-bound Orc. These interactions permit loading of Mcm at the G_1-to-S-phase transition, and subsequently of components of the DNA synthesis machinery, such as Rpa and DNA polymerase α-primase. The latter step requires phosphorylation of specific components of the prereplication complex by cyclin-dependent kinases that accumulate during the G_1-to-S-phase transition (Volume II, Chapter 7). Reinitiation of DNA synthesis is prevented by several mechanisms. A cyclin-dependent kinase that accumulates during the G_2 and M phases phosphorylates both Mcm proteins and Cdc6. This modification induces nuclear export of the former and degradation of the latter. In addition, the protein called geminin is present in the nucleus from S until M phase (when it is degraded). This protein binds to Cdt1, sequestering it from interaction with Cdc6 and Orc. As a consequence of such regulatory mechanisms, the prereplication complex can form **only** in the G_1 phase, ensuring firing of the origin once per cell cycle.

As a latent infection is established, the Epstein-Barr virus genome becomes increasingly methylated at C residues present in CG dinucleotides, although sequences that must function in latently infected cells, such as OriP, generally escape this modification. Such DNA methylation is associated with repression of transcription, and contributes to inhibition of expression of viral genes. The viral genome also becomes packaged by cellular nucleosomes and is therefore replicated as a circular minichromosome, much like that of simian virus 40. Replication of the Epstein-Barr

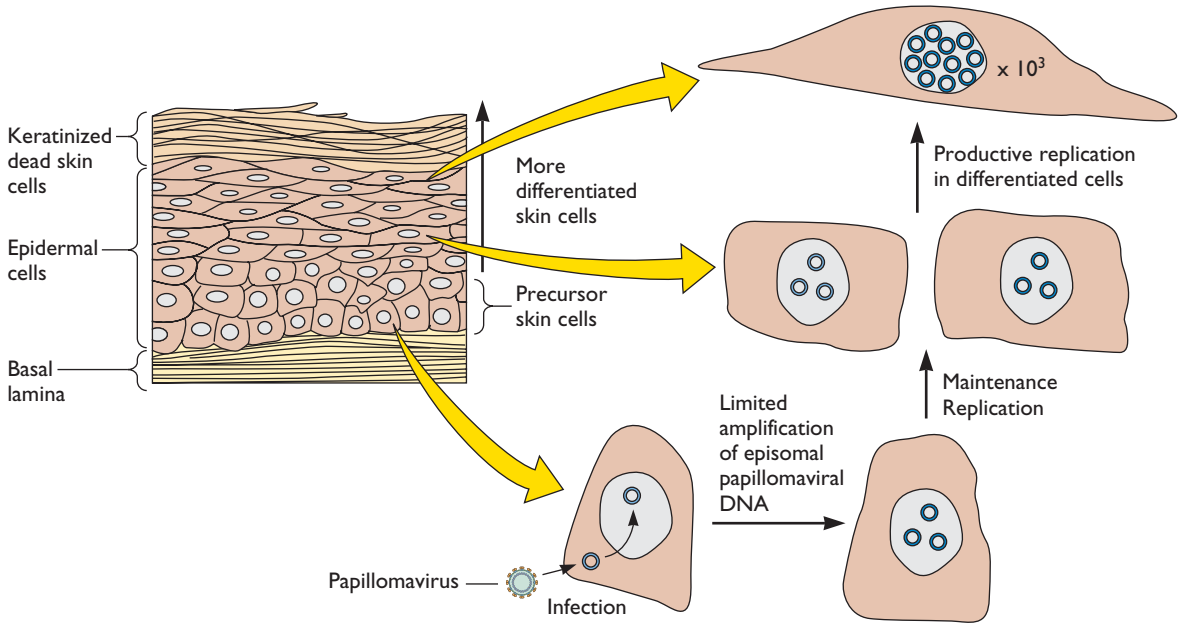
virus genome once per cell cycle persists unless conditions that induce entry into the viral productive cycle are encountered. The critical step for this transition is activation of transcription of the viral genes encoding the transcriptional activators Zta and Rta (Chapter 8). These proteins induce expression of the viral early genes that encode the viral DNA polymerase and other proteins necessary for replication from OriLyt. In addition, Zta appears to be the viral OriLyt recognition protein. Consequently, once this protein is made in an Epstein-Barr virus-infected cell, its indirect and direct effects on viral DNA synthesis ensure a switch from OriP-dependent to OriLyt-dependent replication, and progression through the infectious cycle.

Controlled and Exponential Replication from a Single Origin: the Papillomaviruses

Three different modes of viral DNA replication are associated with papillomavirus infection (Fig. 9.25). Entry of a papillomaviral genome into the nucleus of a host cell initiates a period of amplification of the circular genome, just as during the early stages of latent infection by Epstein-Barr virus. Replication continues until a moderate number of viral genomes (~50 to 100) accumulates in the cell. A maintenance replication pattern, in which the complement of viral episomes is duplicated on average once per cell cycle, is then established. The mechanism that governs the switch from amplification to maintenance replication is not known. In natural human papillomavirus infections, these two types of replication take place in the proliferating basal cells of an epithelium. They can also be reproduced in cells in culture transformed by these viruses.

The single viral origin and the viral E1 and E2 proteins that bind to specific origin sequences (Fig. 9.17; Table 9.3) are necessary for both the initial amplification of the papillomavirus genome and its maintenance for long periods at a more-or-less constant concentration. Initial studies of bovine papillomavirus indicated that such maintenance replication is not the result of strict, once-per-cell-cycle replication of viral DNA. Rather, replication of individual viral episomes occurs at random, taking place on average once per cell cycle. Subsequent studies of human papillomavirus DNA replication in different epithelial cell lines, including those derived from naturally infected cervical epithelia, have established that the viral genome can be replicated by both random and strict, once-per-cell-cycle mechanisms (Box 9.10). Which mode of replication prevails is determined by both the host cell and the concentration of the viral E1 protein: at high concentrations, this

Figure 9.25 Regulation of papillomaviral DNA replication in epithelial cells. The outer layers of the skin are shown as depicted in Fig. 2.5. The virus infects proliferating basal epithelial cells, to which it probably gains access after wounding. The double-stranded, circular viral DNA genome is imported into the infected cell nucleus and initially amplified to a concentration of 50 to 100 copies per cell. This concentration of viral DNA episomes is maintained by further limited replication as the basal and parabasal cells of the epithelium divide **(maintenance replication)**. As cells move to the outer layers of the epidermis and differentiate, productive replication of the viral genome to thousands of copies per cell takes place.



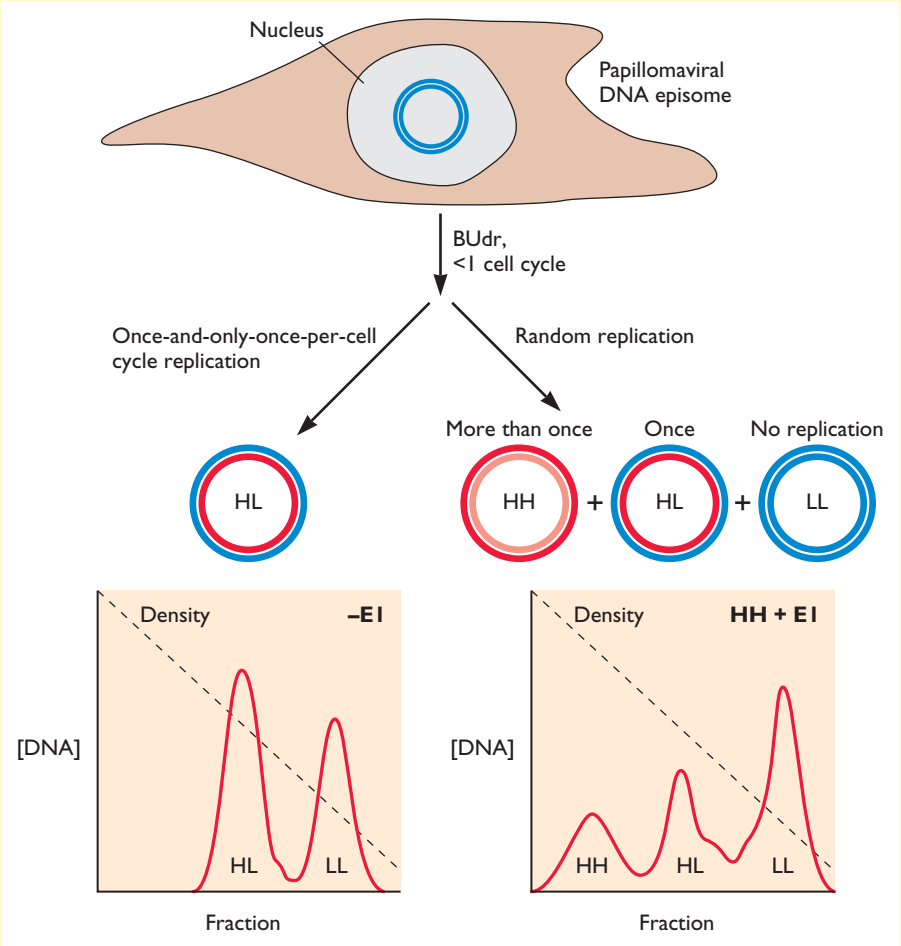
BOX 9.10 EXPERIMENTS

Distinguishing one-per-cell cycle from random replication of human papillomavirus DNA

In once-per-cell cycle replication, each molecule of episomal viral DNA is replicated just once per cell cycle during S phase. In random replication, some DNA molecules are replicated several times in a single cell cycle, some are replicated once, and some do not replicate. As illustrated in the figure, these mechanisms can be distinguished by the densities of the DNA molecules synthesized when cells are incubated with the dense analog of thymidine, bromodeoxyuridine (BUdR), for a period less than the time required to complete one cell cycle.

Results obtained when this method was applied to W12 cervical keratinocytes that contain human papillomavirus type 16 DNA are shown schematically in the figure. In these cells, viral DNA replication is by the once-per-cell cycle mechanism: no HH DNA could be detected **(left)**. When a vector for expression of the viral E1 protein was introduced, random replication of the viral DNA ensued **(right)**.

Hoffman, R., B. Hirt, V. Bechtold, P. Beard, and K. Raj. 2006. Different modes of human papillomavirus DNA replication during maintenance. *J. Virol.* **80:**4431–4439.



protein converts once-per-cell cycle viral DNA synthesis to random-choice replication.

Stable maintenance of the viral genome requires an additional sequence, called the **minichromosome maintenance element**, which is composed of multiple binding sites for the E2 protein. When bound by the viral protein, the minichromosome maintenance element is attached to mitotic chromosomes and remains associated with them during all stages of mitosis. This association is mediated by binding of E2 to the C-terminal domain of the cellular bromodomain-containing protein 4 (Brd 4), an acetylated histone H4-binding protein that interacts with mitotic chromosomes. The C-terminal domain of Brd4 acts as a dominant negative inhibitor of E2 binding: when

overproduced, it induces release of viral DNA from mitotic chromosomes and loss of the viral genome from cells transformed by bovine papillomavirus type 1. This protein also appears to mediate binding to mitotic chromosomes of episomal DNA of human herpesvirus type 8, a gammaherpesvirus that is associated with various human tumors (Volume II, Chapter 7).

Remarkably, the final stage of papillomaviral DNA replication, production of high concentrations of the viral genome for assembly into progeny virions, is restricted to nondividing, differentiated epithelial cells, such as terminally differentiated keratinocytes formed as basal cells move toward the surface of an epithelium (Fig. 9.25). Cell culture systems that support such so-called productive replication have now been developed, and should allow elucidation of the mechanism of this poorly understood process. The viral E7 protein is necessary for productive replication and induction of synthesis of the cellular replication proteins needed for viral DNA synthesis, such as DNA polymerase α and Pcna, by the mechanism shown in Fig. 9.21A. This protein perturbs the program of keratinocyte differentiation. The viral E5 and E1^E4 proteins, which are produced at high concentrations following differentiation, also contribute to maintenance of proliferative competence in infected keratinocytes. The function of these viral proteins therefore appears to be to establish a cellular environment favorable for productive replication of the papillomaviral genome.

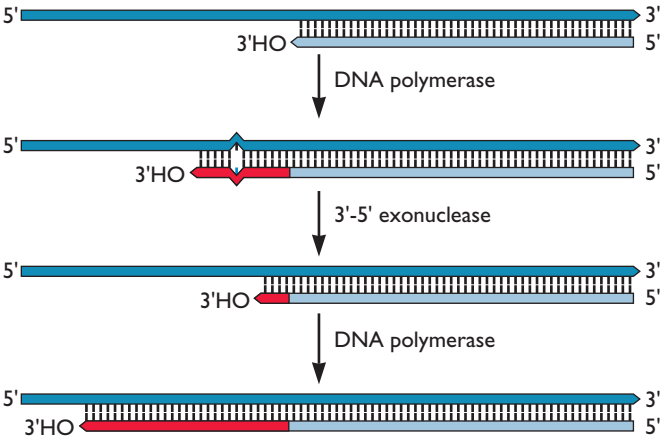


Figure 9.26 Proofreading during DNA synthesis. If permanently fixed into the genome, mispaired bases would result in mutation. However, the majority are removed by the proofreading activity of replicative DNA polymerases. A mismatch at the 3′-OH terminus of the primer-template during DNA synthesis activates the 3′ →5′ exonuclease of all replicative DNA polymerases, which excises the mismatched region to create a perfect duplex for further extension. In the best-characterized case, DNA polymerase I of *E. coli*, the rate of extension from a mismatched nucleotide is much lower than when a correct base pair is formed at the 3′ terminus of the nascent strand. This low rate of extension allows time for spontaneous unwinding (breathing) of the new duplex region of the DNA and transfer of the 3′ end to the 3′ → 5′ exonuclease site for removal of the mismatched nucleotide. Because preferential excision of mismatched nucleotides is the result of differences in the **rate** at which the polymerase can add the next nucleotide, this mechanism is called **kinetic proofreading**.

Origins of Genetic Diversity in DNA Viruses

Fidelity of Replication by Viral DNA Polymerases

Proofreading Mechanisms

Cellular DNA replication is a high-fidelity process with an error rate of only about one mistake in every 10^9 nucleotides incorporated. Such fidelity, which is essential to maintain the integrity of the genome, is based on the accurate pairing of substrate and template deoxyribonucleotide bases prior to synthesis of each phosphodiester bond. Nonstandard base pairs can form quite readily. However, DNA synthesis requires perfect base pairing between the nascent and template strands, and DNA synthesis does not begin if the terminal nucleotide, or the preceding region of the primer-template, is mismatched. In such circumstances, the mismatched base in the primer strand is excised by a 3′ → 5′ exonuclease present in all replicative DNA polymerases until a perfectly base-paired primer-template is created (Fig. 9.26). Replicative DNA polymerases are therefore self-correcting enzymes, removing errors made in newly synthesized DNA as replication continues.

The cellular DNA polymerases that replicate small viral DNA genomes possess such proofreading exonucleases. Infection by these viruses (e.g., papillomavirus and polyomavirus) does not result in inhibition of cellular protein synthesis, and indeed may induce expression of cellular replication proteins. The cellular mechanisms of mismatch repair (Fig. 9.27), which correct errors in the daughter strand of newly replicated DNA missed during proofreading, are therefore available to operate on progeny viral genomes. The replication of the genomes of these small DNA viruses is therefore likely to be as accurate as that of the genome of their host cells.

Proofreading by Viral DNA Polymerases

The question of how accurately viral DNA is replicated by viral DNA polymerases, such as those of adenoviruses, herpesviruses, and poxviruses, has received relatively little attention. However, each of these viral enzymes possesses an intrinsic 3′ → 5′ exonuclease that preferentially excises mismatched nucleotides from duplex DNAs *in vitro*. These viral enzymes contain short, conserved sequences related

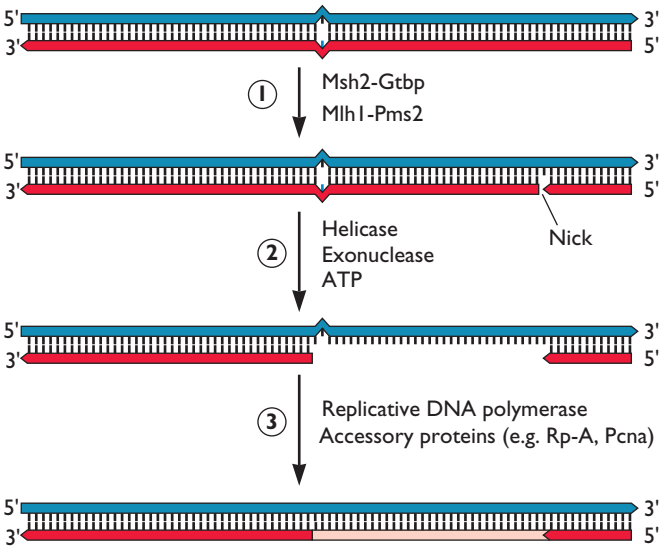


Figure 9.27 Mismatch repair in newly synthesized DNA. This activity requires recognition of the newly synthesized strand containing a misincorporated nucleotide and introduction of a nick (step 1), exonucleolytic degradation of the new strand from the nick as it is unwound (step 2), and resynthesis of DNA to repair the gap (step 3). The long-patch repair system is responsible for removal of mismatches introduced during DNA synthesis in eukaryotic cells. The mammalian Msh2-Gtbp and Mlh1-Pms2 heterodimers are composed of homologs of the well-characterized *E. coli* mismatch repair proteins MutS and MutL, respectively. This relationship suggests that they participate in steps 1 and 2. The importance of this repair system is illustrated by the predisposition to tumor development induced by mutations in the human genes encoding MutS or MutL homologs.

to those that flank the exonuclease active site residues of well-characterized cellular DNA polymerases. Indeed, mutations that impair the exonuclease activity of the herpes simplex virus type 1 DNA polymerase greatly increase the mutation rate.

At this juncture, relatively little is known about the effects of infection by the larger DNA viruses on the production or function of cellular mismatch repair proteins that normally back up proofreading by replicative DNA polymerases. As expression of cellular genes and cellular DNA synthesis are generally inhibited in cells infected by these viruses, it is possible that such mismatch repair proteins are not present in the concentrations necessary for effective surveillance and repair of newly synthesized viral DNA. Indeed, infection of primary human fibroblasts by human cytomegalovirus (a betaherpesvirus) reduces the activity of an enzyme important for excision of alkylated bases. On the other hand, it has been reported that the herpes simplex virus type 1 UL8 and UL42 replication proteins interact with two proteins that participate in mismatch repair, Pcna and Msh6. More detailed information about the rates at which viral DNA polymerases introduce errors during DNA synthesis *in vitro*, and the rates of mutation of viral DNA genomes during productive infection, would help establish the role of cellular repair systems in maintaining the integrity of these viral genomes. Similarly, the contributions of viral enzymes that could prevent or repair DNA damage, such as the dUTPase and uracil DNA glycosylases of herpesviruses and poxviruses (Table 9.5), remain to be established.

Inhibition of Repair of Double-Stranded Breaks in DNA

Exposure of mammalian cells to ultraviolet (UV) or infrared light, as well as stalling or collapse of replication forks, can produce double-stranded breaks in the DNA genome. Such lesions are potentially lethal, so it is not surprising that they elicit powerful and sensitive damage-sensing and response systems. Proteins that recognize double-stranded DNA ends initiate signaling to effector proteins that halt progression through the cell cycle (to allow time for repair) and that repair the broken ends (Fig. 9.28). The DNA ends are sealed either by nonhomologous end joining or homologous recombination repair (Fig. 9.28). In the former process, which is prone to errors, double-stranded ends of DNA are simply joined together. This important repair pathway is blocked in cells infected by several DNA viruses.

The products of adenoviral DNA synthesis are unit-length copies of the linear viral genome that require no processing (Fig. 9.10). However, accumulation of these viral DNA molecules requires inactivation of nonhomologous end joining. In the absence of the viral E4 Orf 3 and Orf 6 proteins, newly synthesized viral DNA forms concatemers far too large to be packaged into progeny virions. Accumulation of such multimeric DNA molecules depends on the cellular MreII-Rad50-Nsb1 repair complex, which normally accumulates at sites of DNA damage (Fig. 9.28), and is necessary for repair of double-stranded breaks. In adenovirus-infected cells, the protein components of this complex become redistributed within nuclei, and are then degraded by the proteasome. These alterations are induced by the Orf 3 and Orf 6 proteins, respectively. When the E4 proteins cannot be made, the cellular repair proteins accumulate in viral replication centers. These E4 gene products therefore appear to prevent triggering of a double-strand-break repair mechanism by the accumulation of linear viral DNA genomes in infected cell nuclei.

Infection by alpha-, beta-, or gammaherpesviruses induces phosphorylation of the Atm and Chk2 proteins (Fig. 9.28). These modifications are signatures of an active

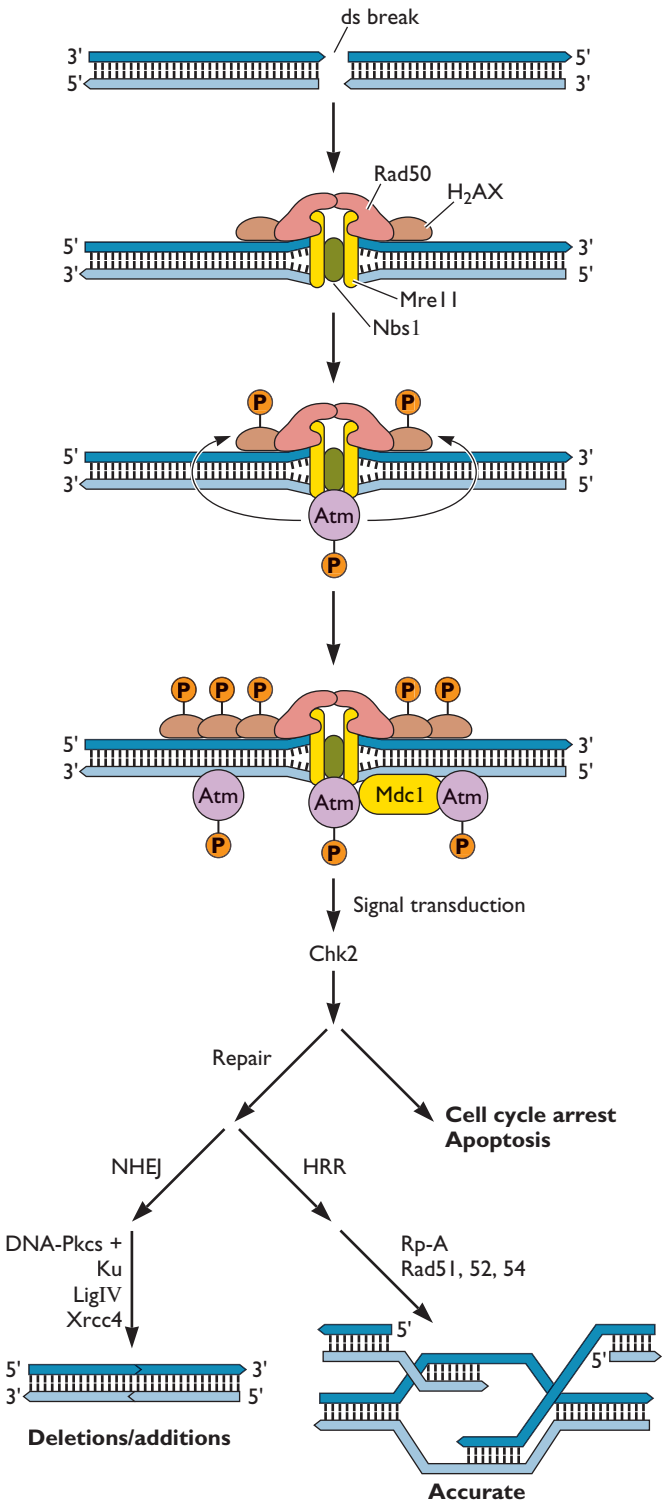


Figure 9.28 Detection of double-stranded breaks in DNA. Induction of a double-stranded break in the DNA genome triggers rapid accumulation of the MRN complex at the break. This complex contains two copies each of the Mre11 and Rad50 proteins, which move from the cytoplasm into the nucleus, and

DNA damage response which is thought to be triggered by free DNA ends present in the viral genome and replication intermediates. The mechanisms and consequences of activation of this cellular response for viral replication are not understood. However, in the case of cells infected by human cytomegalovirus, the signaling pathway is blocked upstream of effectors.

Recombination of Viral Genomes

General Mechanisms of Recombination

Genetic recombination is an important source of genetic variation in populations. It also makes a major contribution to repair of breaks in a DNA genome (Fig. 9.28) and can rescue replication when this process has stalled at unfavorable sequences (or chromosomal sites) in the template. Much of our understanding of the mechanisms of recombination is based on studies of bacterial viruses, such as bacteriophage lambda. Similar principles apply to recombination of DNA genomes of animal viruses.

Two types of recombination are generally recognized: site specific and homologous. In **site-specific recombination**, exchange of DNA takes place at short DNA sequences that are specifically recognized by proteins that catalyze recombination. These sequences may be present in only one or both of the DNA sequences that are recombined in this way. Much more common during reproduction of DNA viruses is **homologous recombination**, the exchange of genetic information between **any** pair of related DNA sequences.

Viral Genome Recombination

The integration of adeno-associated virus DNA into the cellular genome, and its excision when conditions are appropriate, are the result of **site-specific** recombination reactions mediated by the Rep 78/68 viral proteins, which bind to specific sequences in both DNA molecules.

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one of the Nbs1 protein. Mre11 possesses 3′ → 5′ exonuclease, single-stranded DNA endonuclease, and helicase activities. It is thought that these activities unwind the DNA ends at the site of the break, allowing recruitment of the large protein kinase ataxia telangiectasia mutated, Atm. This kinase then becomes activated, perhaps by conformational change and autophosphorylation, and phosphorylates substrates such as the variant histone H2AX. This modification allows amplification of the signal via binding of additional MRN complexes and of mediator of DNA damage checkpoint protein 1, Mdc1. Both this protein and Nsb1 bind phosphorylated H2AX. The Mdc1 protein transduces the signal via additional protein kinases (e.g., Chk2) and other proteins to induce such responses as cell cycle arrest and DNA repair. The two major repair pathways are nonhomologous end joining (NHEJ) and homologous recombination repair (HRR).

In contrast, integration of retroviral DNA (Chapter 7) is site specific only for the viral DNA.

All viral DNA genomes undergo homologous recombination. Indeed, viral recombination is favored by the large numbers of viral DNA molecules present in productively infected cells, and their concentration within specialized replication compartments: the initial step in recombination, pairing of homologous sequences with one another, depends on random collision and is therefore concentration dependent. Furthermore, the structures of replication intermediates, or the nicking of viral DNA during replication or packaging that yields DNA ends, can facilitate recombination among viral DNA molecules. The formation of replication compartments can also result in the concentrations of cellular proteins that participate in recombination (and repair) with viral genomes, as, for example, observed in cells infected by herpes simplex virus type 1.

The ease with which viral DNA sequences can recombine is an important factor in the evolution of these viruses. It is also of great benefit to the experimenter, facilitating introduction of specific mutations into the viral genome or construction of viral vectors (see Chapter 3). Conversely, recombination may be necessary for productive replication of some viral genomes (those of herpesviruses [see below]) or stimulation of viral DNA synthesis, for example, from the nicks that initiate recombination.

As viral genomes do not generally encode homologous recombination proteins, it is thought that this process is catalyzed by host cell enzymes. One exception is the herpes simplex virus type 1 alkaline nuclease (Table 9.5). This enzyme is a 5′ → 3′ exonuclease with homology to the Red α component of the bacteriophage lambda recombinase (Box 9.11). In conjunction with the viral single-stranded DNA-binding protein (ICP8), the alkaline nuclease can mediate the exchange of strands between two DNA molecules *in vitro*. The precise role of recombination mediated by these viral proteins during infection is not known. However, this process is important for production of normal, infectious genomes: the viral DNA synthesized and packaged in cells infected by mutants that do not direct synthesis of the active nuclease contains structural abnormalities and is poorly infectious.

Although recombination among animal viral DNA sequences has been widely exploited in the laboratory, the mechanisms have not received much attention. One exception is adenovirus recombination. Recombination between markers in two adenovirus genomes exhibits many properties typical of this process, such as the dependence of recombination frequency on the distance between the markers. However, several features suggest that recombination is coupled with viral DNA synthesis, because the initial invasion is by the single strands of viral DNA displaced during replication of the adenoviral genome (Fig. 9.10). In particular, this mechanism accounts for the **polarity** of adenovirus recombination, the decreasing gradient of recombination frequency with distance of the recombining sequences from the ends of the viral genome. Another important exception is the homologous recombination of DNA sequences of some herpesviruses, including herpes simplex virus type 1, which is responsible for isomerization of the genome.

Populations of viral DNA molecules purified from herpes simplex virus type 1 virions contain four isomers of the genome, defined by the relative orientations of the two unique sequence segments (L and S) with respect to one another (Fig. 9.20). These unique sequences are flanked by several inverted repeats, including the conserved *a* sequence. The viral DNA population isolated from a single plaque contains all four isomers at equimolar concentrations, suggesting that a single virus particle containing just one genome isomer gives rise to all four by recombination between repeated DNA sequences. It was initially thought that the *a* sequences mediate these recombination reactions by a site-specific mechanism, for example, because insertion of an *a* sequence at an ectopic site induces additional inversions. However, it is now clear that an *a* sequence can act as a hot spot for recombination simply because it contains the viral packaging signal: double-stranded DNA breaks that promote recombination are made within this sequence during cleavage of replication products for packaging of the viral genome. In fact, the *a* sequences are dispensable for production of all four genome isomers at the normal frequency, and recombination between **any** of the inverted repeat sequences in the viral genome promotes inversion of the L and S segments. Such homologous recombination takes place during viral DNA synthesis and requires the viral replication machinery.

Despite some 30 years of study, the function of the unusual isomerization of the genome of herpes simplex virus type 1 and certain other herpesviruses remains enigmatic. Isomerization is not absolutely essential for virus replication in cells in culture, because viruses "frozen" as a single isomer by deletion of internal inverted repeats are viable. On the other hand, the reduced yield of such viruses, and the presence of the inverted repeat sequences in **all** strains of herpes simplex virus type 1 examined, emphasizes the importance of the repeated sequences. It may be that these sequences themselves fulfill some beneficial function (as yet unknown). Recombinational isomerization would then be a secondary result of the presence of multiple, inverted copies of these sequences in the viral genome. Alternatively, isomerization might be a consequence of an important role

BOX 9.11 DISCUSSION

Replication and recombination/repair are two sides of the same coin: earliest insights from bacteriophage λ

In the early 1970s, studies of the replication of bacteriophage λ showed that mutants defective in viral recombination genes (*redα*$^-$ or *redβ*$^-$, *gam*$^-$) synthesize DNA at only half to one-third the wild-type rate, the concatemers typical of late DNA synthesis were on average shorter than usual, and viral bursts were only 30 to 40% of wild-type levels. The role of Gam was explained by its inhibition of the cellular RecBCD nuclease, which would be expected to destroy free concatemer ends. However, the role of Red proteins was not so readily apparent. Furthermore, the fact that viral *red*$^-$ mutants failed to plate at all on certain cells, for example those that were deficient in host DNA polymerase A or ligase, suggested a critical role for recombination and repair functions in λ DNA replication.

An elegant series of genetic and biochemical experiments led to a model (shown here) for the transition from circle to rolling-circle replication, which proposed a mechanism by which viral recombination or host DNA repair functions might produce new replication forks when encountering damage induced by a single-strand break.

It was suggested at the time that the principles illustrated in this model might very well be applicable to cellular DNA metabolism. The idea that recombination could generate a replication origin was novel at the time, but current schemes for the repair of stalled replication forks in both prokaryotic and eukaryotic cells incorporate the very same ideas elaborated from studies of λ over 30 years ago.

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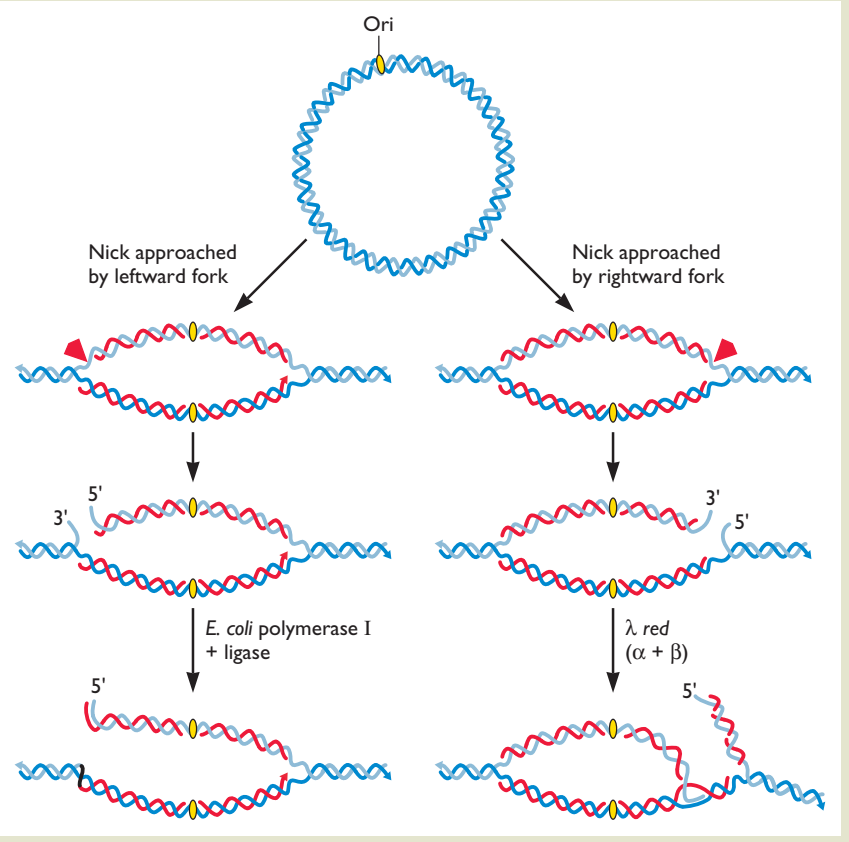

for recombination in replication of the viral genome, as during replication of bacteriophage lambda (Box 9.11). The switch to origin-independent replication later in the infectious cycle is consistent with this idea, but a definitive answer is not yet available. The value of the unusual isomerization of the herpes simplex virus type 1 genome may become clearer as understanding of the mechanism of viral DNA synthesis improves.

Perspectives

Our understanding of mammalian replication proteins and the intricate reactions they carry out during DNA synthesis would still be rudimentary were it not for the simian virus 40 origin of replication. This relatively simple viral DNA sequence, which was initially well characterized by genetic methods, supports origin-dependent replication *in vitro* when cellular proteins are supplemented with a single viral protein, LT.

The mechanism of synthesis of this small viral DNA genome has also provided the conceptual framework within which to appraise the considerable diversity in replication of viral DNA genomes. One parameter that varies considerably is the degree of dependence on the host cell's replication machinery. In contrast to those of papillomaviruses, parvoviruses, and polyomaviruses, the genomes of the larger DNA viruses (herpesviruses and poxviruses) encode the components of a complete DNA synthesis system as well as accessory enzymes responsible for the production of dNTP substrates. Nevertheless, replication of **all** viral DNA genomes requires proteins that carry out the reactions first described for simian virus 40 DNA synthesis, notably an origin recognition protein(s), one or more DNA polymerases, proteins that confer processive DNA synthesis, origin-unwinding and helicase proteins, and, usually, proteins that synthesize, or serve as, primers.

Replication of viral DNA genomes ranges from simple, continuous synthesis of both strands of a linear, double-stranded DNA template (adenovirus) to baroque (and not well understood) mechanisms that produce DNA concatemers (herpesviruses). These various replication strategies represent alternative mechanisms for circumventing the inability of all known DNA-dependent DNA polymerases to initiate DNA synthesis *de novo*. In some cases, initiation of viral DNA synthesis requires RNA primers and the lagging strand is synthesized discontinuously, but in others, the priming mechanism leads to continuous synthesis of all daughter DNA strands from protein or DNA sequence primers.

Efficient reproduction of DNA viruses requires the rapid production of very large numbers of progeny viral DNA molecules for assembly of viral particles. One factor contributing to such exponential replication is the efficient production of the proteins that mediate or support DNA synthesis, be they viral or cellular in origin. However, it is also likely that viral DNA replication at specialized intracellular sites, a common feature of cells infected by these viruses, contributes to efficient viral DNA synthesis. Further exploration of this incompletely understood phenomenon should shed new light on host cell biology, in particular the structural and functional compartmentalization of the nucleus. Also far from well understood are the cues that set the stage for the alternative mode of limited replication characteristic of some DNA viruses, when the number of replication cycles and their timing with respect to the host cell cycle are governed by the nature (e.g., alphaherpesviruses) or differentiation state (papillomaviruses) of the host cell. Elucidation of the mechanisms that result in such close integration of viral DNA synthesis with the physiological state of the host cell seems certain to provide important insights into both host cell control mechanisms and the long-term relationships these viruses can establish with their hosts.

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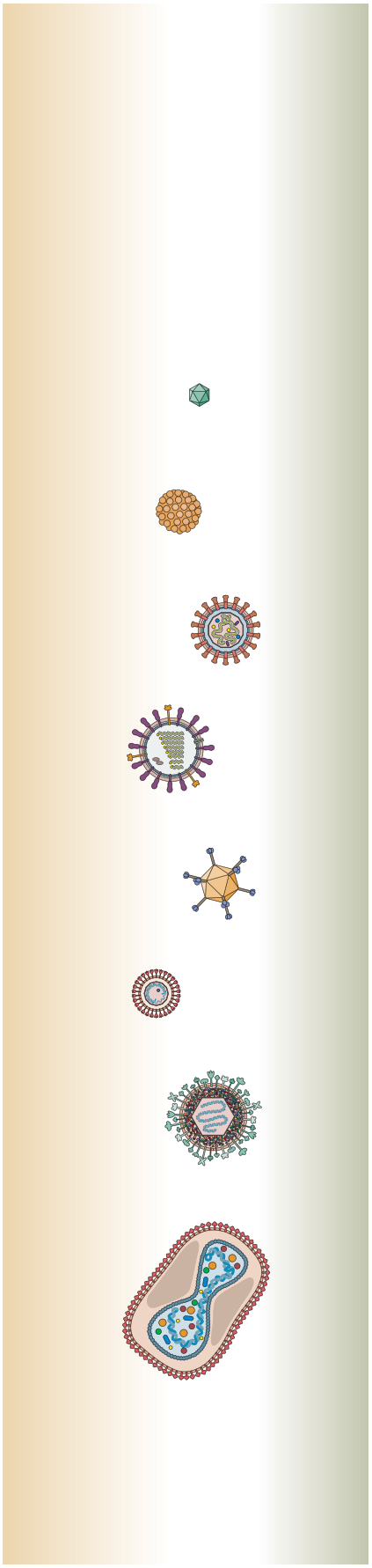

10

Processing of Viral Pre-mRNA

All is flux, nothing stays still.
HERACLITUS

Introduction

Viral messenger RNAs (mRNAs) are synthesized by either viral or cellular enzymes and may be made in the nucleus or the cytoplasm of an infected cell. Regardless of how and where they are made, all must be translated by the protein-synthesizing machinery of the host cell. Consequently, they must conform to the requirements of the cell's translational system. A series of covalent modifications, collectively known as **RNA processing** (Fig. 10.1), endow mRNAs with the molecular features needed for recognition by the protein synthesis machinery, and translation of the protein-coding sequences by cellular ribosomes. Most RNA-processing reactions were discovered in viral systems, primarily because virus-infected cells provide large quantities of specific mRNAs for analysis.

Two modifications important for efficient translation are the addition of m^7GpppN to the 5′ end (**capping**) and the addition of multiple A residues to the 3′ end (**polyadenylation**) (Fig. 10.1). The enzymes that perform these chemical additions (Box 10.1) may be encoded by viral or cellular genes. When an RNA is produced in the nucleus, another chemical rearrangement, called **splicing**, is possible. During splicing, short blocks of noncontiguous coding sequences (**exons**) are joined precisely to create a complete protein-coding sequence for translation, while the intervening sequences (**introns**) are discarded (Fig. 10.1). Splicing therefore dramatically alters the precursor mRNA (pre-mRNA) initially synthesized. As no viral genome encodes even part of the complex machinery needed to catalyze splicing reactions, splicing of viral pre-mRNAs is accomplished entirely by cellular gene products. Some viral pre-mRNAs undergo a different type of internal chemical change, in which a single nucleotide is replaced by another, or one or more nucleotides are inserted at specific positions (Box 10.1). Such **RNA-editing** reactions introduce nucleotides that are not encoded in the genome, and therefore may change the sequence of the encoded protein.

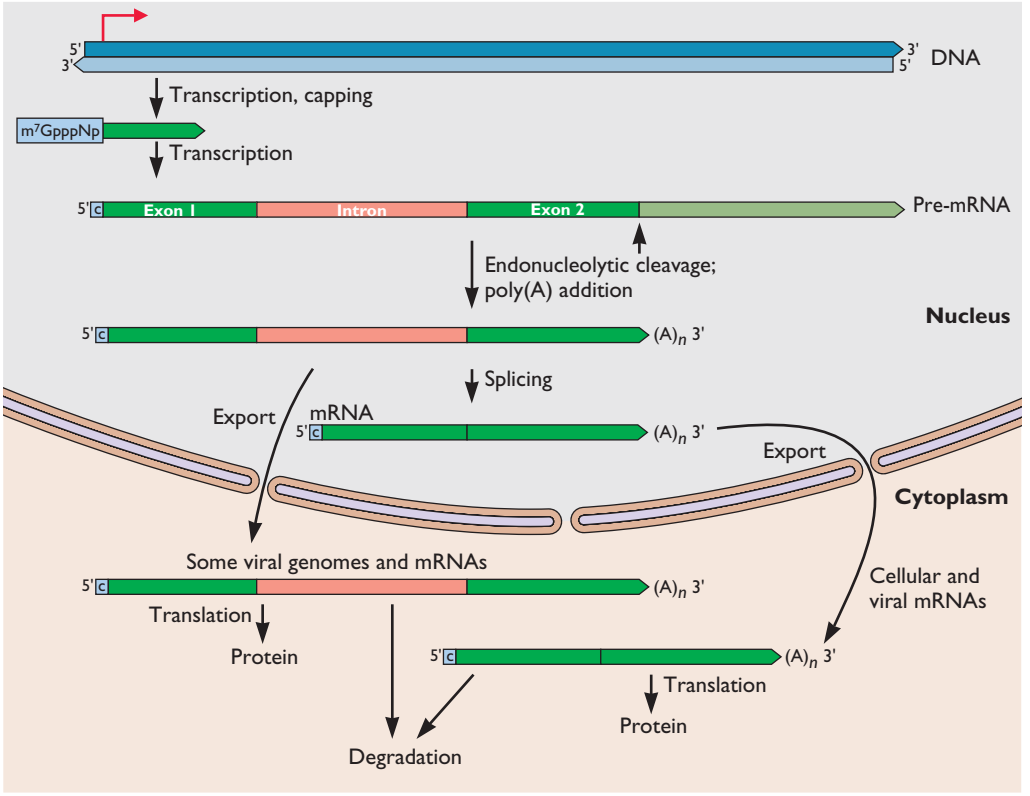

Figure 10.1 Processing of a viral or cellular mRNA synthesized by RNA polymerase II. The reactions by which mature mRNA is made from a typical RNA polymerase II transcript are shown. The first such reaction, capping, takes place cotranscriptionally. For clarity, the exons of a hypothetical, partially processed (i.e., polyadenylated but unspliced) pre-mRNA are depicted, even though polyadenylation and splicing are often coupled, and many splicing reactions are cotranscriptional. Most cellular and viral pre-mRNAs synthesized by RNA polymerase II are processed by this pathway. However, some viral mRNAs that are polyadenylated but not spliced, or are incompletely spliced, are exported to the cytoplasm.

When a viral RNA is produced in the nucleus, it must be exported to the cytoplasm for translation (Fig. 10.1). Such export of mature viral and cellular mRNAs is considered to be part of mRNA processing, even though the RNA is not known to undergo any chemical change during transport. Viral mRNAs invariably leave the nucleus by cellular pathways, but nuclear export mechanisms may be altered in activity or specificity in cells infected by some viruses. Once within the cytoplasm, an mRNA has a finite lifetime before it is recognized and degraded by ribonucleases (RNases). The susceptibilities of individual mRNA species to attack by these destructive enzymes vary greatly, and can be modified in virus-infected cells.

The RNA-processing reactions summarized in Fig. 10.1 not only produce functional mRNAs, but also provide numerous opportunities for posttranscriptional control of gene expression. Regulation of RNA processing can increase the coding capacity of the viral genome, determine when specific viral proteins are made during the infectious cycle, and facilitate selective expression of viral genetic information.

An additional component of the varied repertoire of posttranscriptional mechanisms that regulate viral and cellular gene expression has been recognized much more recently. Cellular and viral genomes encode small RNAs that induce mRNA degradation or inhibition of translation by base pairing to the mRNA. This phenomenon is known as RNA silencing or **RNA interference**. It is mediated by small RNAs 21 to 28 nucleotides in length that are produced by endonucleotytic cleavage of larger precursors in the nucleus and/or the cytoplasm. RNA interference mediated by cellular components is an important component of anti-viral defense. Furthermore, virally encoded small RNAs can alter cellular processes.

In this chapter, we focus on these RNA processing reactions to illustrate both crucial viral regulatory mechanisms, and the seminal contributions of viral systems to the elucidation of essential cellular processes.

BOX 10.1 BACKGROUND

Types of change during pre-mRNA processing

Addition of sequences
- 5′ caps
- 3′ poly(A) tails
- One or a few internal nucleotides by editing

Removal of sequences
- Introns during splicing

Substitution of sequences
- Editing

Relocation
- Export from nucleus to cytoplasm

Covalent Modification during Viral Pre-mRNA Processing

Capping the 5′ Ends of Viral mRNA

The first mRNAs shown to carry the 5′-terminal structure termed the **cap** were those of reovirus and vaccinia virus (Box 10.2). These viral mRNAs are made and processed by virus-encoded enzymes (see "Synthesis of Viral 5′ Cap Structures by Viral Enzymes" below), but subsequent research established that virtually all viral and cellular mRNAs possess the same cap structure, m^7GpppN, where N is any nucleotide (Fig. 10.2A). This structure protects mRNAs from 5′ exonucleolytic attack and allows recognition during splicing of the 5′-terminal exons of pre-mRNAs made by cellular RNA polymerase II. In addition, the cap structure is essential for the efficient translation of most viral and cellular mRNAs, as it is recognized by translation initiation proteins. The principal exceptions are the uncapped mRNAs of certain (+) strand viruses, notably picornaviruses and the flavivirus hepatitis C virus, which are translated by the cap-independent mechanism described in Chapter 11. The cap also blocks recognition of viral RNAs by an antiviral defense mechanism that detects cytoplasmic RNA molecules carrying uncapped 5′ triphosphate groups (see Volume II, Chapter 3).

Although most viral mRNAs carry a 5′-terminal cap structure, there is considerable variation in how this modification is made. Three mechanisms can be distinguished: *de novo* synthesis by cellular enzymes, synthesis by viral enzymes, and acquisition of preformed 5′ cap structures from cellular pre-mRNAs or mRNAs.

Synthesis of Viral 5′ Cap Structures by Cellular Enzymes

Viral pre-mRNA substrates for the cellular capping enzyme are invariably made in the infected cell nucleus by cellular RNA polymerase II (Table 10.1). The formation of cap structures on the 5′ ends of viral and cellular RNA polymerase II transcripts, the first step in their processing, is a cotranscriptional reaction that takes place when the nascent RNA is only 20 to 30 nucleotides in length (Fig. 10.3). Phosphorylation of paired RNA polymerase II at specific serines in the C-terminal domain of the largest subunit is the signal for binding of capping enzyme (see below) and capping of the nascent RNA. The intimate relationship between the cellular capping enzyme and RNA polymerase II ensures that all transcripts made by this enzyme are capped at their 5′ ends. It also explains why RNA species synthesized by other cellular RNA polymerases are not modified in this way: these enzymes do not carry an equivalent of the C-terminal domain of RNA polymerase II.

The 5′ cap structure is assembled by the action of several enzymes as described in Fig. 10.2B. In mammalian cells, a single protein, commonly called **capping enzyme**, contains both the 5′ triphosphatase and the guanylyltransferase required for synthesis of a 5′ cap. Following the action of capping enzyme, the terminal residues are modified by methylation at specific positions (Fig. 10.2B). The cap 1 structure, m^7GpppNm, is common in viral and mammalian mRNAs. However, the sugar of the second nucleotide can also be methylated by a cytoplasmic enzyme, to form the cap 2 structure (Fig. 10.2B).

Synthesis of Viral 5′ Cap Structures by Viral Enzymes

When viral mRNAs are made in the cytoplasm of infected cells, their 5′ cap structures are, of necessity, synthesized by viral enzymes. These enzymes form cap structures typical of those present on cellular mRNA, although with some variations in the mechanism of cap construction. For example, during synthesis of the caps of vesicular stomatitis virus mRNAs, only the α-phosphate group of the initiating nucleotide is retained and guanosine 5′-diphosphate (GDP) is added, and alphaviral mRNAs carry the cap 0 structure (Fig. 10.2B).

Like their cellular counterparts, viral capping enzymes are intimately associated with the viral RNA polymerases responsible for mRNA synthesis. Indeed, in the simplest case, the several enzymatic activities required for synthesis of a 5′ cap structure are supplied by the viral RNA polymerase. This mechanism is exemplified by the vesicular stomatitis virus L protein. This large (>2,000-amino-acid) protein contains discrete domains that catalyze RNA synthesis and cap methylation, and possesses the triphosphatase and guanosine triphosphatase (GTPase) activities necessary for synthesis of the cap. This arrangement presumably facilitates coordination of capping with RNA synthesis. More complex viruses encode dedicated capping enzymes, such as the λ-2 protein of reovirus particles and the VP4 protein of the rotavirus bluetongue

BOX 10.2 EXPERIMENTS

Identification of 5′ cap structures on viral mRNAs

The first clues that the termini of mRNAs made in eukaryotic cells possessed special structures came when viral mRNAs did not behave as predicted from the known structure of bacterial mRNAs. The figure summarizes the identification of 5′ cap structures. The 5′ end of reoviral (or vaccinia virus) mRNA, in contrast to that of a prokaryotic mRNA, could not be labeled by polynucleotide kinase and [γ-^{32}P]ATP (yellow) after alkaline phosphatase treatment. This property established that the 5′ end did not carry a simple phosphate group, but rather was blocked. As indicated below, the structure of the 5′ blocking group (termed the **cap**) was elucidated by differential labeling of specific groups of the viral mRNA, as indicated by colors in the figure, followed by digestion of the mRNA with nucleases with different specificities. In addition to the expected product, 5′pN_{OH}3′, cleavage with P1 nuclease liberated a structure that corresponded to the 5′ terminus of the mRNA (the only position at which β or γ phosphates from nucleoside triphosphates are retained). This structure also contained all the *methyl*-^{3}H label, indicating that it included methyl groups. Its digestion with nucleotide pyrophosphatase produced free phosphate with all the ^{32}P label and two *methyl*-^{3}H-labeled products. Because the terminal structure was known to contain G (from incorporation of ^{32}P from [β,γ-^{32}P]GTP), these nucleotides were identified as m^{7}Gp and 2′-*O*-methylguanosine monophosphate (i.e., methylated at the 2 position of the sugar ring) by comigration with these compounds.

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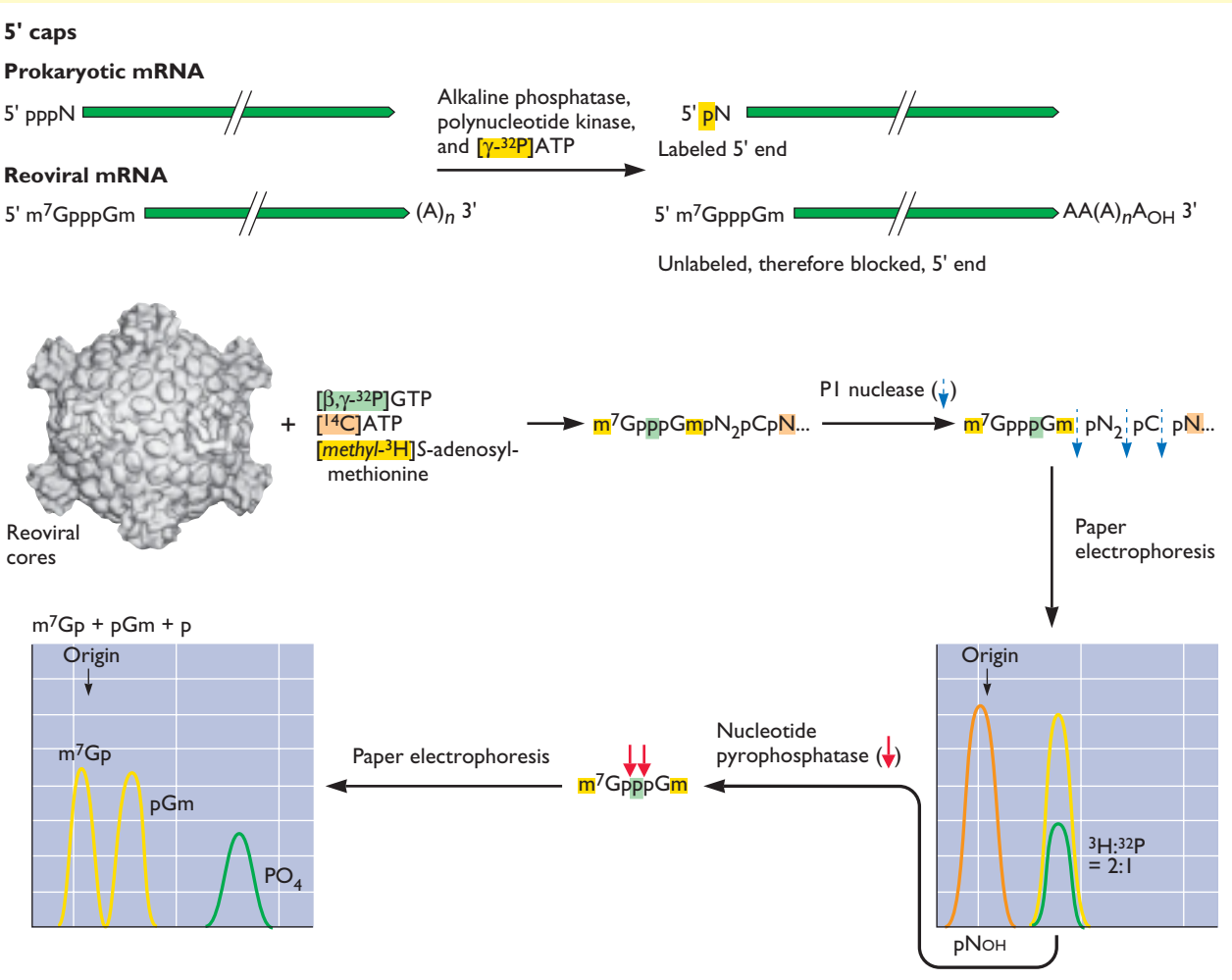


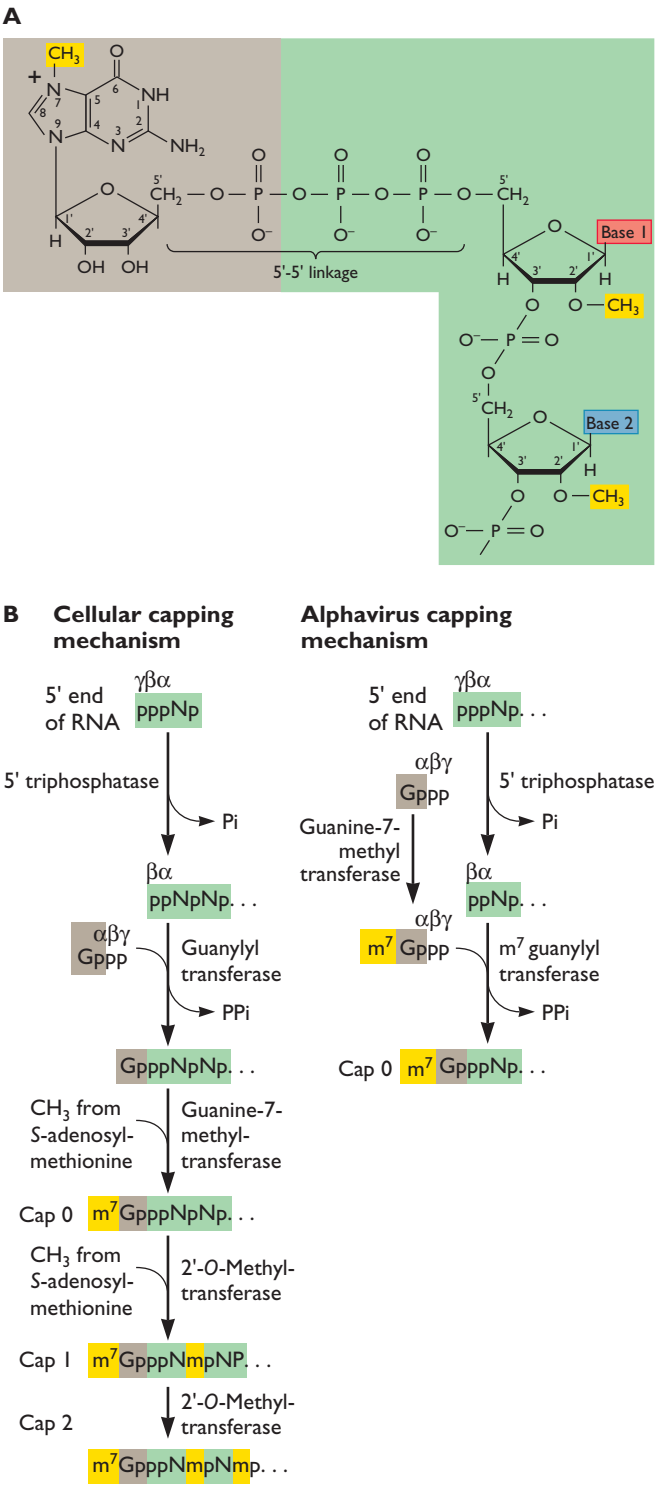


Figure 10.2 The 5′ cap structure and its synthesis by cellular or viral enzymes. (A) In the cap structure shown, Cap 2, the sugars of the two transcribed nucleotides (green) adjacent to the terminal m^{7}G (gray) contain 2′-*O*-methyl groups (yellow). The first and second nucleotides synthesized are methylated in the nucleus and in the cytoplasm, respectively. **(B)** The enzymes and reactions by which this cap is synthesized by cellular enzymes are listed (left) and compared to the synthesis of the caps of Semliki Forest virus (a togavirus) mRNAs by viral enzymes in the cytoplasm of infected cells (right).

virus (both members of the *Reoviridae*). The latter protein catalyses all of the four reactions required for synthesis of the cap 1 structure, and its active sites are organized as a capping "assembly line" (Fig. 10.4). It is closely associated with the viral RNA-dependent RNA polymerase in the core of the virion. One of the first capping enzymes to be analyzed in detail was the vaccinia virus enzyme. This protein binds directly to the viral RNA polymerase and adds 5′ cap structures cotranscriptionally to nascent viral transcripts that are approximately 30 nucleotides in length. The capping enzyme of vaccinia virus therefore displays striking functional similarities to its host cell counterpart.

Most viral capping enzymes cooperate with viral RNA-dependent RNA polymerases that can synthesize both (−) and (+) strand RNAs, but only (+) strand RNAs become capped. The activities of these enzymes must therefore be regulated. The mechanisms that coordinate capping activity with viral mRNA synthesis are not fully understood. In some cases, sequence or structural features of the (+) strand RNA may be recognized by capping enzymes. For example, the methyltransferase of the flavivirus West Nile virus binds specifically to a stem-loop structure at the 5′ end of (+) strand RNA. Substitutions of specific residues within this region inhibit cap methylation and viral replication. In other cases, such as the alphaviruses Sindbis virus and Semliki Forest virus, activation of capping enzymes may be the result of proteolytic processing. As discussed in Chapter 6, the viral P1234 polyprotein is responsible for the initial synthesis of (−) strand RNA from the (+) strand viral genome. This polyprotein includes the sequences of the RNA polymerase and the capping enzyme (Nsp2 and Nsp1, respectively), but the latter is inactive. Cleavage of the polyprotein is necessary for synthesis of viral mRNAs (see Fig. 6.21), and this processing reaction may also activate the capping enzyme.

Acquisition of Viral 5′ Cap Structures from Cellular RNAs

The 5′ cap structures of orthomyxoviral and bunyaviral mRNAs are produced by cellular capping enzymes, but in a unique manner. The 5′ caps of these viral mRNAs are acquired when viral cap-dependent endonucleases cleave cellular transcripts to produce the primers needed for viral mRNA synthesis, a process called **cap snatching** (see Fig. 6.14). The 5′-terminal segments and caps of influenza virus mRNAs are obtained from cellular pre-mRNA in the nucleus. On the other hand, bunyaviral mRNA synthesis

Table 10.1 Mechanisms of synthesis of 5′-terminal cap structures of viral mRNAs

Mechanism	Virus family	Enzyme synthesizing pre-mRNA
Synthesis by host cell enzymes	*Adenoviridae, Hepadnaviridae, Herpesviridae, Papillomaviridae, Parvoviridae, Polyomaviridae, Retroviridae*	Cellular DNA-dependent RNA polymerase II
Synthesis by viral enzymes	*Reoviridae, Rhabdoviridae, Togaviridae*	Viral RNA-dependent RNA polymerase
	Poxviridae	Viral DNA-dependent RNA polymerase
Acquisition from cellular pre-mRNA or mRNA	*Bunyaviridae, Orthomyxoviridae*	Viral RNA-dependent RNA polymerase

is primed with 5′-terminal fragments cleaved from mature cellular mRNAs in the cytoplasm.

Synthesis of 3′ Poly(A) Segments of Viral mRNA

Like the 5′ cap structure, a 3′ poly(A) segment was first identified in a viral mRNA (Box 10.3). This 3′-end modification was soon found to be a common feature of mRNAs made in eukaryotic cells. Like the 5′ cap, the 3′ poly(A) sequence stabilizes mRNA, and it also increases the efficiency of translation. Therefore, it is not surprising that viral mRNAs generally carry a 3′ poly(A) tail. Those that do not, such as reoviral and arenaviral mRNAs, may survive by virtue of a 3′-terminal stem-loop structure that blocks nucleolytic attack. Such structures are also present at the 3′ ends of cellular, poly(A)-lacking mRNAs that encode histones.

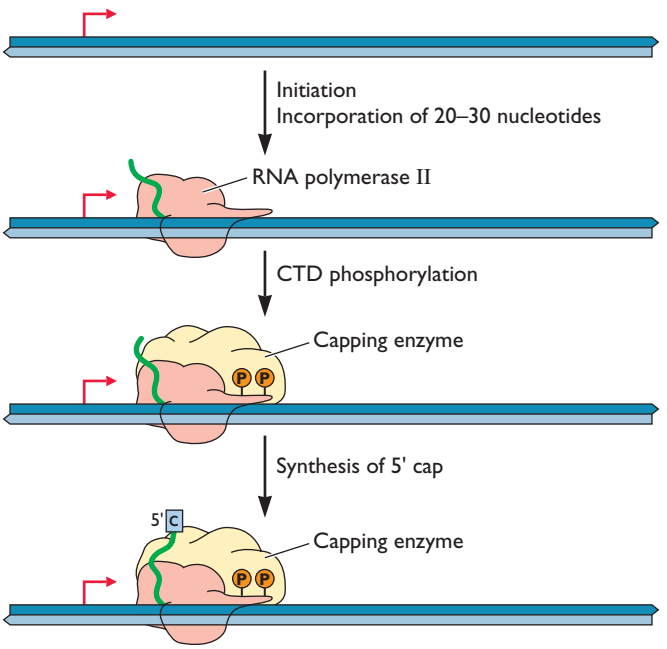
The addition of 3′ poly(A) segments to viral pre-mRNAs, like capping of their 5′ ends, can be carried out by either cellular or viral enzymes (Table 10.2). However, cellular and viral polyadenylation mechanisms can differ markedly.

Polyadenylation of Viral Pre-mRNA by Cellular Enzymes

Viral pre-mRNAs synthesized in infected cell nuclei by RNA polymerase II are invariably polyadenylated by cellular enzymes (Table 10.2). Transcription of a viral or cellular gene by RNA polymerase II proceeds beyond the site at which poly(A) will be added. The 3′ end of the mRNA is determined by endonucleolytic cleavage of its pre-mRNA at a specific position. Such cleavage is also required for termination of transcription. Poly(A) is then added to the new 3′ terminus, while the RNA downstream of the cleavage site is degraded (Fig. 10.5). Cleavage and polyadenylation sites are identified by specific sequences, first characterized in simian virus 40 and adenovirus pre-mRNAs. The 3′ end of mature mRNA is formed 10 to 30 nucleotides downstream of a highly conserved and essential polyadenylation signal, the sequence 5′AAUAAA3′. However, this sequence is **not** sufficient to specify poly(A) addition. For example, it is found within mRNAs at internal positions that are never used as polyadenylation sites. Sequences at the 3′ side of the cleavage site, notably a U- or GU-rich sequence located 5 to 20 nucleotides downstream, are also required. In many mRNAs (particularly viral mRNAs), additional sequences 5′ to the cleavage site are also important.

The first reaction in polyadenylation is recognition of the 5′AAUAAA3′ sequence by the protein termed Cpsf (cleavage and polyadenylation specificity protein) (Fig. 10.5). This interaction is stabilized by other proteins (Fig. 10.5). Poly(A) polymerase is then recruited to the complex, prior to cleavage of the pre-mRNA by a subunit of Cpsf. The polymerase then synthesizes a poly(A) segment typically of 200 to 250 nucleotides in a two-stage process (Fig. 10.5).

Figure 10.3 Model of cotranscriptional capping of RNA polymerase II transcripts. The elongating RNA polymerase II pauses after 20 to 30 nucleotides have been incorporated into a nascent transcript. The C-terminal domain (CTD) of the largest subunit is then phosphorylated (P) at specific serine residues. Such modification is the signal for binding of the capping enzyme to the CTD and capping (blue) of the nascent RNA.



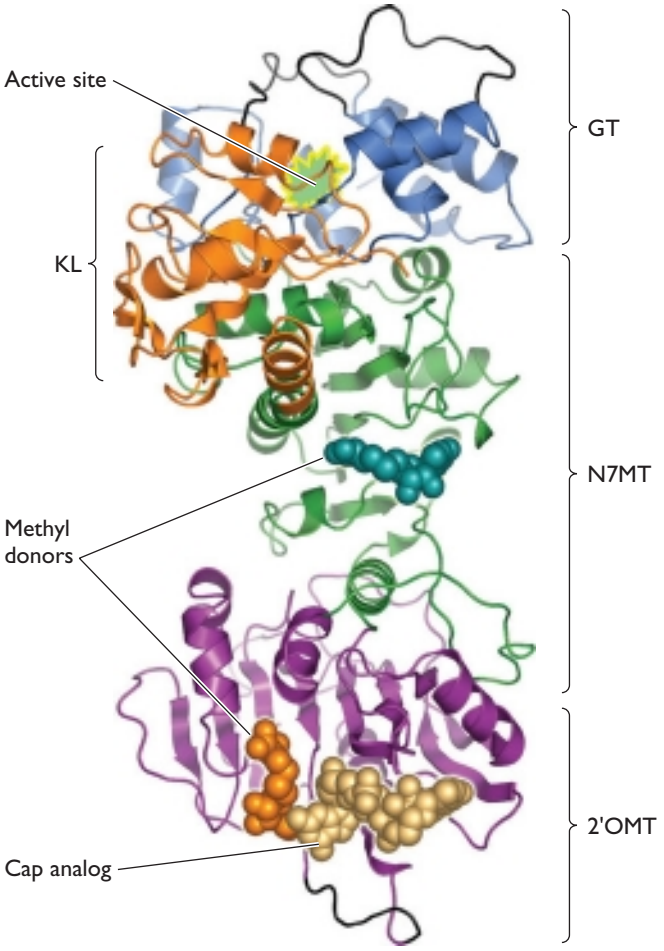


Figure 10.4 A unimolecular assembly line for capping. The structure of the bluetongue virus VP4 protein determined by X-ray crystallography is shown in ribbon form, with each of the four domains in a different color. Localization of the binding sites for substrates and products (e.g., a cap analog) identified the 2′-*O*-methyltransferase (2 O′MT, purple), guanine-7-methyl transferase (N7MT, green), and guanylyltransferase (GT, blue) domains. The latter may also contain the RNA 5′-triphosphatase active site. The linear layout of the active sites in the sequence in which capping reactions take plate (Fig 10.2B) allows efficient coordination of these reactions. The KL domain (orange), which is located on one side of the otherwise linear protein, contains no active sites and is thought to mediate interactions with other proteins, such as the viral RNA-dependent RNA polymerase. Adapted from G. Sutton et al., *Nat. Struct. Mol. Biol.* **14:**449–451, 2007, with permission. Courtesy of Polly Roy, London School of Hygiene and Tropical Medicine.

Components of the polyadenylation machinery, like capping enzymes, are associated with RNA polymerase II, and the polymerase is required for efficient polyadenylation in *in vitro* reactions. Both Cpsf and Csf (Fig. 10.5) bind to the C-terminal domain of the largest subunit of RNA polymerase II. Such interactions are essential for polyadenylation *in vivo*, and are thought to coordinate synthesis of pre-mRNAs with maturation of the 3′ ends.

Polyadenylation of Viral Pre-mRNAs by Viral Enzymes

Formation of 3′ ends by termination of transcription. The 3′ ends of vaccinia virus early mRNAs are formed by termination of transcription by the viral DNA-dependent RNA polymerase at specific sites (Fig. 10.6), a mechanism with no counterpart in cellular or other viral mRNA synthesis systems. Unexpectedly, the vaccinia virus capping enzyme was identified as one protein that is required for termination of transcription. As discussed previously, the capping enzyme binds to the viral RNA polymerase. It is believed to remain with the transcriptional complex until the termination signal 5′UUUUUNU3′ is encountered in the nascent RNA. Termination of transcription, which also requires a specific subunit of the RNA polymerase and a single-stranded DNA-dependent adenosine triphosphatase (ATPase), takes place 30 to 50 nucleotides downstream of this signal. The viral poly(A) polymerase adds poly(A) to the 3′ end thus formed, by a two-step process that is remarkably similar to the mechanism of synthesis of poly(A) by the cellular machinery (compare Fig. 10.6 with Fig. 10.5). The regulatory subunit of the viral poly(A) polymerase that induces processive synthesis of full-length poly(A) after the initial production of short chains is the viral 2′-*O*-methyltransferase. All components of the vaccinia virus capping machinery therefore also participate in formation of the 3′ ends of viral mRNAs. This property seems likely to facilitate coordination of the reactions by which viral mRNAs are synthesized.

Polyadenylation during viral mRNA synthesis. The synthesis of the poly(A) segments of vaccinia virus early mRNAs resembles the cellular mechanism in several respects. In contrast, the poly(A) sequences of mRNAs made by other viral RNA polymerases are produced, during synthesis of the mRNA (Table 10.2). In the simplest case, exemplified by (+) strand picornaviruses, a poly(U) sequence present at the 5′ end of the (–) strand RNA template is copied directly into a poly(A) sequence of equivalent length. The mRNAs of (–) strand RNA viruses like vesicular stomatitis virus and influenza virus are polyadenylated by reiterative copying of short stretches of U residues in the (–) strand RNA template, a mechanism described in Chapter 6.

Splicing of Viral Pre-mRNA

Discovery of Splicing

Between 1960 and the mid-1970s, the study of putative nuclear precursors of mammalian mRNAs established that these RNAs are larger than the mature mRNAs translated in the cytoplasm and are heterogeneous in size.

BOX 10.3 EXPERIMENTS

Identification of poly(A) sequences on viral mRNAs

Polyadenylation of viral mRNAs was first indicated by the observation that an RNA chain resistant to digestion by RNase A, which cleaves after U and C, was produced when vaccinia virus mRNA, but not bacterial mRNA, was treated with this enzyme following labeling with [^{3}H]ATP. The presence of a tract of poly(A) was confirmed by the specific binding of vaccinia virus mRNA, but not of bacterial mRNA, to poly(U)-Sepharose under conditions that allowed annealing of complementary nucleic acids. The position of the poly(A) sequence in viral mRNA was determined by analysis of the products of alkaline hydrolysis, when phosphodiester bonds are broken to produce nucleotides with 5′ hydroxyl and 3′ phosphate (Ap) groups. The liberation of A_{OH3}′ by this treatment indicated that the poly(A) was located at the 3′ end of the mRNA.

Kates, J. 1970. Transcription of the vaccinia virus genome and occurrence of polyriboadenylic acid sequences in messenger RNA. *Cold Spring Harbor Symp. Quant. Biol.* **35:**743–752.

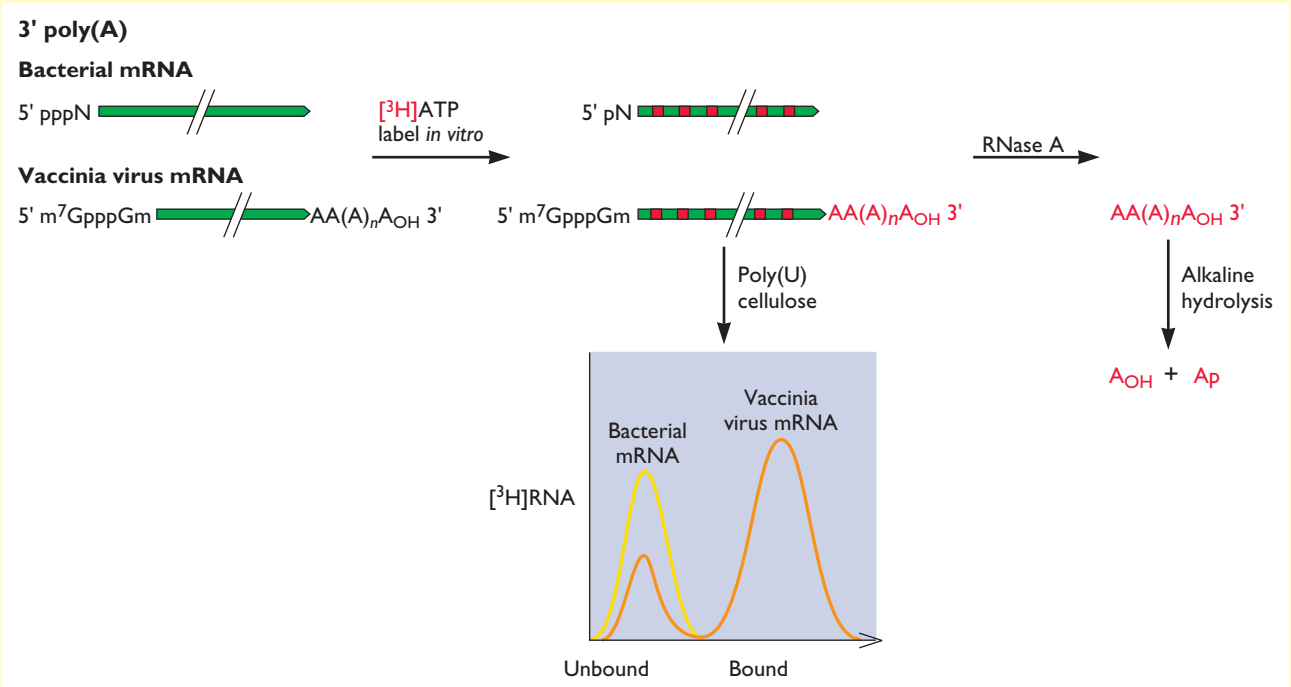


Table 10.2 Mechanisms of addition of poly(A) to viral RNAs

Mechanism	Enzyme	Viruses
During mRNA synthesis		
Reiterative copying of short U stretches in the template RNA	Viral	Orthomyxoviruses, paramyxoviruses, rhabdoviruses
Copying of a long 5′-terminal U stretch in the template RNA	Viral	Picornaviruses, togaviruses
Posttranscriptional		
Cleavage of pre-mRNA followed by polyadenylation	Cellular	Adenovirus, hepadnaviruses, hepatitis delta satellite virus, herpesviruses, papovaviruses, retroviruses
Transcription termination followed by polyadenylation	Viral	Poxviruses

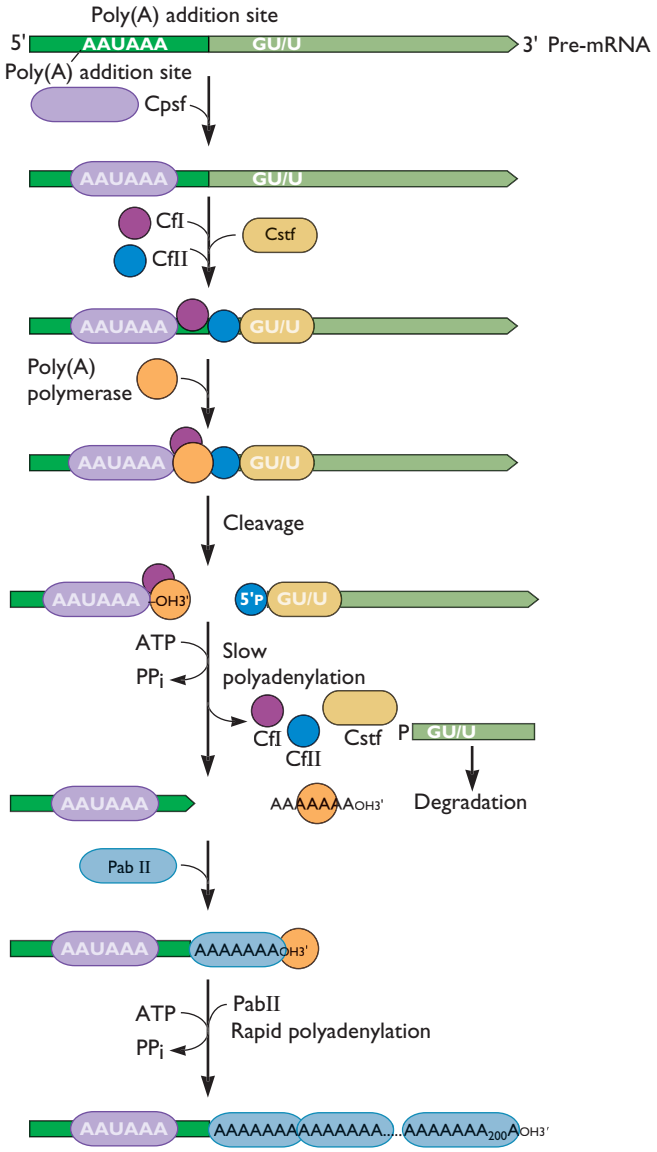


Figure 10.5 Cleavage and polyadenylation of vertebrate pre-mRNAs. The cleavage and polyadenylation specificity protein (Cpsf), which contains four subunits, binds to the 5′AAUAAA3′ poly(A) addition signal that lies upstream of the site at which poly(A) will be added. Cleavage stimulatory protein (Cstf) then interacts with the downstream U/GU-rich sequence to stabilize a complex that also contains the two cleavage proteins, CfI and CfII. Binding of poly(A) polymerase is followed by cleavage at the poly(A) addition site by a subunit of Cpsf, and CfI, CfII, Cstf, and the downstream RNA cleavage product are then released. The polymerase slowly adds 10 to 15 A residues to the 3′-OH terminus produced by the cleavage reaction. Poly(A)-binding protein II (Pab II) then binds to this short poly(A) sequence and, in conjunction with Cpsf, tethers poly(A) polymerase to the poly(A) sequence. This association facilitates rapid and processive addition of A residues until a poly(A) chain of about 200 residues has been synthesized.

They were therefore named **heterogeneous nuclear RNAs** (hnRNAs). Such hnRNAs were shown to carry both 5′-terminal cap structures and 3′ poly(A) sequences, leading to the conclusion that both ends of the hnRNA were preserved in the smaller, mature mRNA. Investigators were faced with the dilemma of deducing how smaller mRNAs could be produced from larger hnRNAs while both ends of the hnRNA were retained.

The puzzle was solved by two groups of investigators, led by Phillip Sharp and Richard Roberts, who shared the 1993 Nobel Prize in physiology or medicine for this work. These investigators showed that adenoviral major late mRNAs are encoded by four **separate** genomic sequences (Box 10.4). The distribution of the mRNA-coding sequences into four separate blocks in the genome, in conjunction with the large size of major late mRNA precursors, implied that these mRNAs were synthesized by excision of noncoding sequences from primary transcripts, with precise joining of coding sequences. The demonstration that nuclear major late transcripts contain the noncoding sequences (introns) confirmed that the mature mRNAs are formed by **splicing** of noncontiguous coding sequences in the pre-mRNA.

This mechanism of mRNA synthesis had great appeal, because it could account for the puzzling properties of hnRNA. Indeed, it was shown within a matter of months that splicing of pre-mRNA is not an obscure, virus-specific device: splicing occurs in all eukaryotic cells. The great majority of mammalian pre-mRNAs, like the adenoviral major late mRNAs, comprise exons interspersed among introns. In all cases, the capped 5′ end and sequences immediately adjacent to the 3′ poly(A) tail are conserved in mature mRNA.

The organization of protein-coding sequences into exons separated by introns has profound implications for the evolution of the genes of eukaryotes and their viruses. Introns are generally much longer than exons, and only short sequences at their ends are necessary for accurate splicing (see "Mechanism of Splicing" below). Consequently, introns provide numerous sites at which DNA sequences can be broken and rejoined without loss of coding information, and greatly increase the frequency with which random recombination reactions can create new functional genes by rearrangement of exons. Evidence of such "exon shuffling" can be seen in the modular organization of many modern proteins. Such proteins comprise combinations of a finite set of structural and functional domains or motifs, or multiple repeats of a single protein domain, each often encoded by a single exon. In similar fashion, the presence of introns is thought to have facilitated the transfer (by recombination) of cellular genetic information into the genomes of DNA viruses and retroviruses.

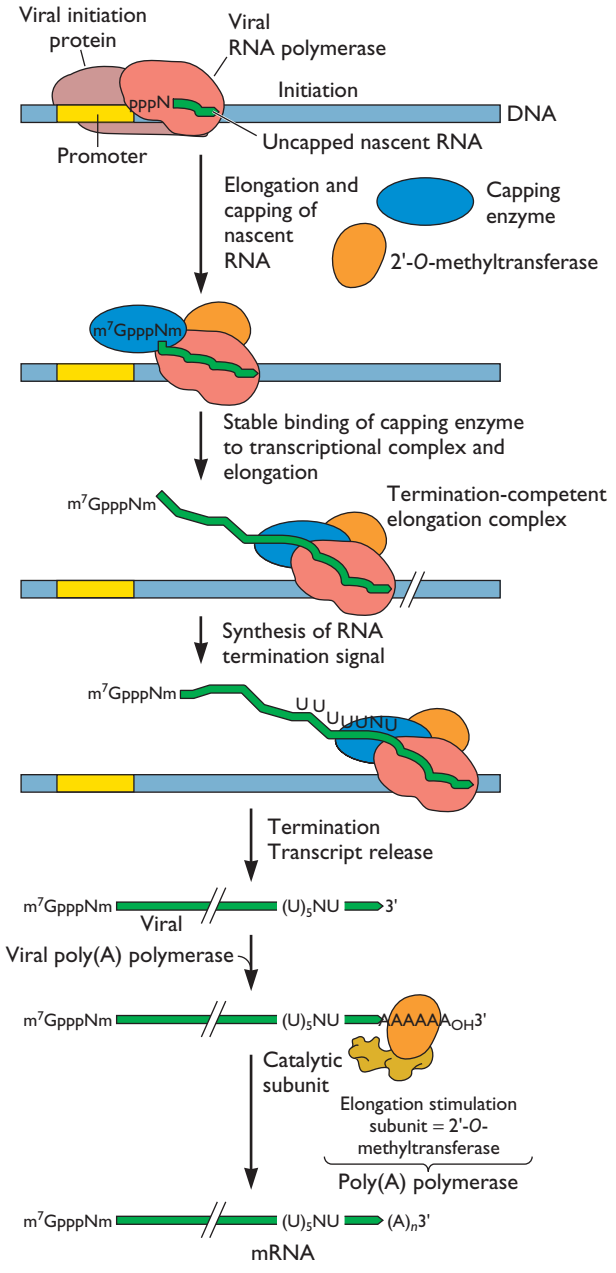


Figure 10.6 The vaccinia virus capping enzyme and 2′-*O*-methyltransferase process both the 5′ and 3′ ends of vaccinia virus mRNAs. After capping the 5′ ends of nascent viral mRNA chains about 30 nucleotides in length (step 1), the capping enzyme remains bound to the nascent RNA chain and to the RNA polymerase as the latter enzyme transcribes the template DNA. The viral 2′-*O*-methytransferase, which produces a cap 1 structure, also binds to the viral RNA polymerase and stimulates elongation during transcription of viral intermediate and late genes (step 2). This protein is also a subunit of the viral poly(A) polymerase. Termination of transcription (step 3), which takes place 30 to 50 nucleotides downstream of the RNA sequence 5′UUUUNU3′, is mediated by the termination protein/capping enzyme and the viral nucleoside triphosphate phosphohydrolase I, which is a single-stranded DNA-dependent ATPase. A fraction of the 2′-*O*-methyltransferase molecules act as an elongation stimulation protein for the viral poly(A) polymerase, analogous to cellular poly(A)-binding protein II. This viral enzyme, like its cellular counterpart (Fig. 10.4), adds poly(A) to the 3′ ends of the mRNA in a two-step process (steps 4 and 5).

Mechanism of Splicing

Sequencing of DNA copies of a large number of cellular and viral mRNAs and of the genes that encode them identified short consensus sequences at the 5′ and 3′ **splice sites**, which are joined to each other in mature mRNA (Fig. 10.7A). The conserved sequences lie largely within the introns. The dinucleotides GU and AG are found at the 5′ and 3′ ends, respectively, of almost all introns. Mutation of any one of these four nucleotides eliminates splicing, indicating that all are essential. Elucidation of the mechanism of splicing came with the development of *in vitro* systems in which model pre-mRNAs (initially of viral origin) are accurately spliced. Numerous components needed for splicing have now been identified by both biochemical studies based on such systems and genetic analyses in organisms such as yeast and the fruit fly *Drosophila melanogaster*.

Pre-mRNA splicing occurs by two *trans*-esterification reactions, in which one phosphodiester bond is exchanged for another without the need for an external supply of energy (Fig. 10.7B). The first reaction yields two products, the 5′ exon and the intron-3′ exon **lariat**. In the second *trans*-esterification reaction, the newly formed hydroxyl group at the end of the 5′ exon attacks the phosphodiester bond at the 3′ splice site, yielding the spliced exon product and the intron lariat.

From a chemical point of view, the splicing of pre-mRNA is a simple process. However, each splicing reaction must be completed with a high degree of accuracy to ensure that coding information is not lost or altered. The chemically active hydroxyl groups (Fig. 10.7B) must also be brought into close proximity to the phosphodiester bonds they will attack. Furthermore, some genes contain 50 or more exons separated by **much** larger introns, which must be spliced in the correct order. It is presumably for such reasons that pre-mRNA splicing requires both many proteins and several RNAs, which associate with the pre-mRNA to form the large structure called the **spliceosome**.

Five small nuclear RNAs (snRNAs) participate in splicing: the U1, U2, U4, U5, and U6 snRNAs. In vertebrate cells, these RNAs vary in length from 100 to 200 nucleotides and are associated with proteins to form **small nuclear ribonucleoproteins** (snRNPs). The RNA components of the snRNPs recognize splice sites and other sequences in cellular and viral pre mRNAs. Indeed, they participate in multiple interactions with the pre-mRNA and with each

BOX 10.4 EXPERIMENTS

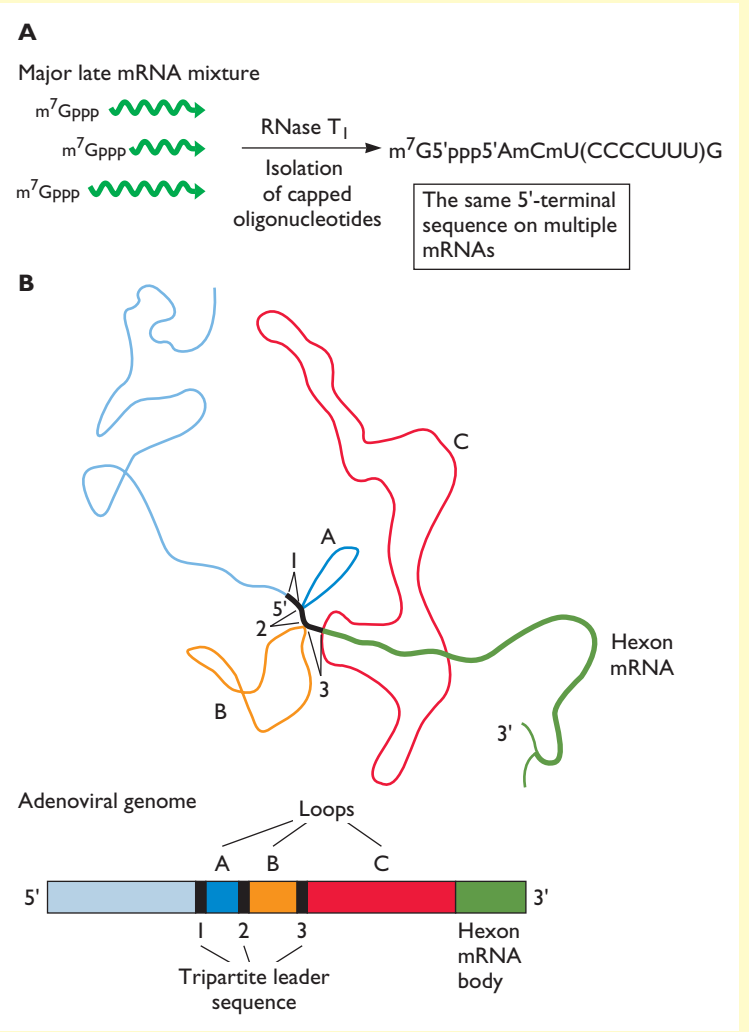
Discovery of the spliced structure of adenoviral major late mRNAs

(A) Digestion of adenoviral major late mRNAs with RNase T_1, which cleaves after G, and isolation of the capped 5′ oligonucleotides indicated the **same** 11-nucleotide sequence was present at the 5′ ends of several different mRNAs. This observation was surprising, and puzzling. Hybridization studies indicated that these 5′ ends were not encoded adjacent to the main segments of major late mRNAs. Direct visualization of such mRNAs hybridized to viral DNA provided convincing proof that their coding sequences are dispersed in the viral genome. **(B)** Schematic diagram of one major late mRNA (hexon mRNA) hybridized to a complementary adenoviral DNA fragment extending from the left end of the genome to a point within the hexon coding sequence. Three loops of unhybridized DNA (thin lines), designated A, B, and C, bounded or separated by three short segments (1, 2, and 3) and one long segment (hexon mRNA) of DNA-RNA hybrid (thick lines) were observed. Other adenoviral late mRNAs examined yielded the same sets of hybridized and unhybridized viral DNA sequences at their 5′ ends, but differed in the length of loop C, and the length and location of the 3′-terminal RNA-DNA hybrid. It was therefore concluded that the major late mRNAs contain a common 5′-terminal segment (segments 1, 2, and 3) built from sequences encoded at three different sites in the viral genome, and termed the tripartite leader sequence. This sequence is joined to the mRNA body, a long sequence complementary to part of the hexon coding sequence in the example shown. (B) Adapted from S. M. Berget et al., *Proc. Natl. Acad. Sci. USA* **74:**3171–3175, 1977, with permission.

Berget, S. M., C. Moore, and P. A. Sharp. 1977. Spliced segments at the 5′ terminus of adenovirus 2 late mRNA. *Proc. Natl. Acad. Sci. USA* **74:**3171–3175.

Chow, L. T., R. E. Gelinas, T. R. Booker, and R. J. Roberts. 1977. An amazing sequence arrangement at the 5′ ends of adenovirus 2 messenger RNA. *Cell* **12:**1–8.

Gelinas, R. E., and R. J. Roberts. 1977. One predominant undecanucleotide in adenovirus late messenger RNAs. *Cell* **11:**533–544.



other during splicing. Assembly of the spliceosome results in base pairing of the snRNA components of the U1 and U2 snRNPs with the 5′ splice site and the intronic branch point, respectively (Fig. 10.8). Dramatic rearrangements of the RNA-RNA interactions within the spliceosome then result is juxtaposition of the 5′ splice site and the branch point for the first transesterification reaction (Fig. 10.8). The RNA of the U5 snRNP may juxtapose the 5′ and 3′ exons to facilitate catalysis of their joining in the second transesterification reaction. However, RNAs of the snRNPs do much more than simply organize the pre-mRNA sequences into a geometry suitable for transesterification. It has long been suspected that the spliceosome might be an RNA enzyme (or **ribozyme**), and several observations

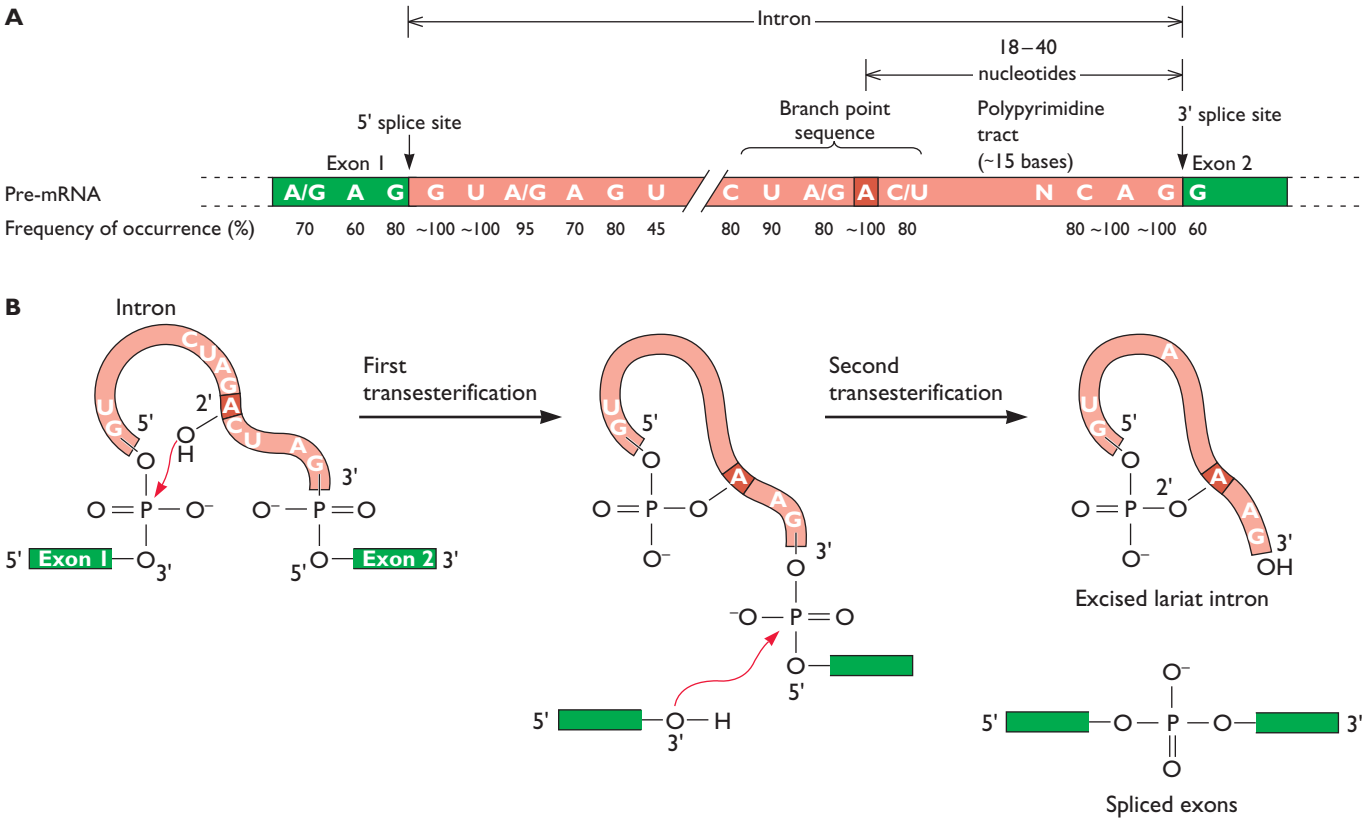


Figure 10.7 Splicing of pre-mRNA. (A) Consensus splicing signals in cellular and viral pre-mRNAs. The most conserved sequences are found at the 5′ and 3′ splice sites at the junctions of exons (green) and introns (pink) and at the 3′ ends of introns. The intronic 5′GU3′ and 5′AG3′ dinucleotides at the 5′ and 3′ ends, respectively, of introns and branch point A (highlighted) are present in all but rare mRNAs made in higher eukaryotes. **(B)** The two transesterification reactions of pre-mRNA splicing. In the first reaction, the 2′ hydroxyl group of the conserved A residue in the intronic branch point sequence makes a nucleophilic attack on the phosphodiester bond at the 5′ side of the GU dinucleotide at the 5′ splice site to produce the intron-3′ exon lariat and the 5′ exon. A second nucleophilic attack by the newly formed 3′ hydroxyl group of the 5′ exon on the phosphodiester bond at the 3′ splice site then yields the spliced exons and the intron lariat.

have provided direct evidence for catalysis by U6 and U2 snRNAs during splicing (Box 10.5).

Although the RNAs of snRNPs play essential roles in splicing as both guides and catalysts, the spliceosome also contains about 150 non-snRNP proteins. One class comprises proteins that package the pre-mRNA substrate, termed heterogeneous nuclear ribonucleoprotein (hnRNP) proteins. Many other splicing proteins contain both RNA-binding and protein-protein interaction domains. Such proteins bind to pre-mRNA sequences within or adjacent to exons, and regulate splicing or exon recognition. Members of one family of such proteins, called the SR proteins because they contain domains rich in serine (S) and arginine (R), act at early stages of splicing to recruit snRNPs to the spliceosome. For example, the SR protein named splicing factor 2 (Sf2, or alternative splicing factor) facilitates binding of U1 snRNP to 5′ splice sites and stabilizes the prespliceosomal complex (Fig. 10.8). Other proteins important for splicing are RNA-dependent helicases, which are thought to catalyze the multiple rearrangements of hydrogen bonding among different snRNAs and the pre-mRNA substrate (Fig. 10.8). Such helicases are generally ATP dependent, and spliceosome assembly and rearrangement depend on energy supplied by ATP hydrolysis.

Splicing of pre-mRNAs is commonly cotranscriptional, and components of the splicing machinery associate with RNA polymerase II. The hyperphosphorylated form of the C-terminal domain of the largest subunit of this enzyme can bind to both SR proteins and spliceosomal snRNPs. Peptide mimics of the C-terminal domain or antibodies raised against it inhibit pre-mRNA splicing *in vivo* or *in vitro*. Furthermore, nontranscribed sequences within RNA polymerase II promoters can dictate whether a particular exon is retained or removed during splicing. As we have

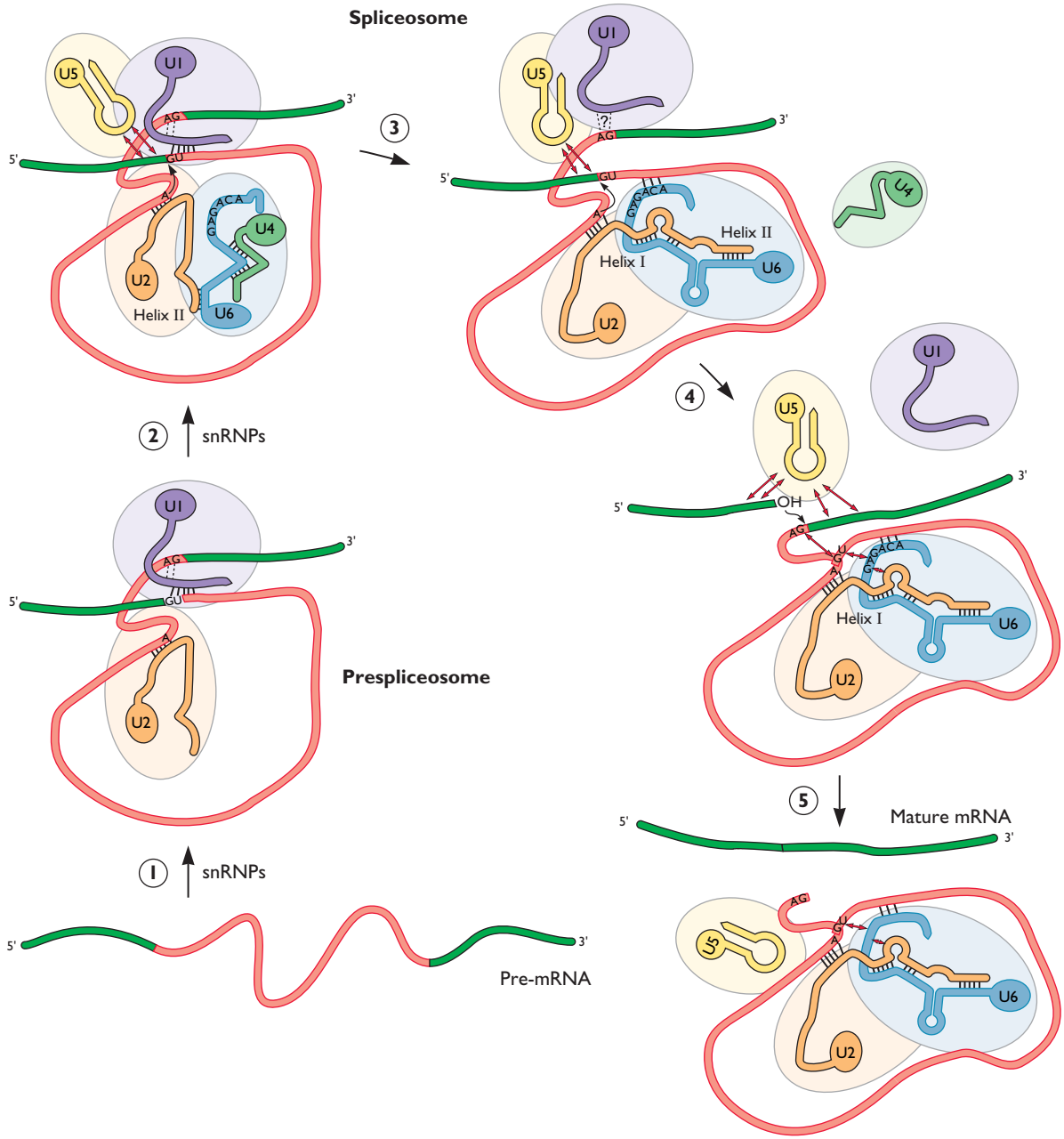


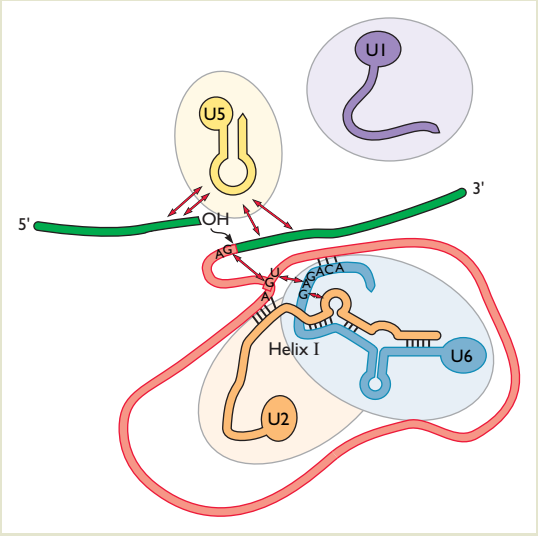
Figure 10.8 RNA-RNA interactions organize substrates and catalysts during splicing. Base pairs are indicated by dashes and experimentally observed or presumed contacts among the RNA molecules by the two-headed arrows. The U1 and U2 snRNAs initially base pair with the 5′ splice site and branch point sequence, respectively, in the pre-mRNA (step 1). The other snRNPs then enter the assembling spliceosome (step 2). The U4 and U6 snRNAs, which are present in a single snRNP, are base paired with one another over an extended complementary region. This snRNP binds to the U5 snRNP, and the snRNP complex associates with that containing the pre-mRNA and U1 and U2 snRNAs to form the spliceosome. RNA rearrangements then activate spliceosomes for catalysis of splicing (step 3). U4 snRNA dissociates from U6 snRNA, which forms hydrogen bonds with both U2 snRNA and the pre-mRNA. The interaction of U6 snRNA with the 5′ splice site displaces U1 snRNA (step 4). One of the U2 sequences hydrogen bonded to U6 snRNA (helix I) is adjacent to the U2 snRNA sequence that is base paired with the pre-mRNA branch point region. The interactions of U2 and U6 snRNAs with each other and with the pre-mRNA therefore juxtapose the branch point and 5′ splice site sequences for the first transesterification reaction (Fig. 10.7B). The U5 snRNA base pairs to sequences in both the 5′ and 3′ exons and may align them for the second transesterification reaction (step 5). The many proteins that participate in spliceosome assembly and activation or that package the pre-mRNA and snRNAs are not shown. Adapted from T. Nilsen, *Cell* **78:**1–4, 1994, with permission.

BOX 10.5 DISCUSSION

RNA catalysis by U2 and U6 snRNAs takes place in the absence of any proteins

In the absence of proteins, U2 and U6 snRNA synthesized *in vitro* base pair to form a stable structure with a three-dimensional conformation similar to that thought to be present at the active site of the spliceosome (shown schematically in the figure). This U2-U6 snRNA complex binds specifically to a short, synthetic RNA molecule containing the sequence of an intron branch point. The branch point adenosine is activated when the substrate RNA is bound to the snRNA complex, and its 2′-OH group attacks a specific phosphodiester bond in U6 RNA to form an unusual phosphotriester bond. This reaction is analogous to the first transesterification during pre-mRNA splicing by the spliceosome. It requires RNA sequences necessary for authentic splicing, including a specific base-pairing interaction between U2 and U6 snRNAs and a particular U6 RNA sequence. These observations therefore provide direct evidence for RNA catalysis of the first splicing reaction.

Valadkhan, S., and J. L. Manley. 2001. Splicing-related catalysis by protein-free snRNAs. *Nature* **413:**701–707.



seen, association of components of the 5′ capping and 3′ polyadenylation systems with the C-terminal domain of RNA polymerase is necessary for these processing reactions. The synthesis of pre-mRNA and its complete processing are therefore coordinated as a result of association of specific proteins needed for each processing reaction with the C-terminal domain of RNA polymerase II. Such a transcription and RNA-processing machine is analogous to, but much more complex than, that of vaccinia virus described above.

Production of Stable Viral Introns

Cells infected by certain herpesviruses provide a remarkable exception to the rule that viral intron-containing pre-mRNAs are processed exactly as their cellular counterparts. As discussed in Chapter 8, the most abundant viral RNAs detected in neurons latently infected by herpes simplex viruses are the 2.0-kb major latency-associated transcripts (LATs). These nuclear RNAs not only lack 3′ poly(A) sequences, but also exhibit the properties of excised introns: they are not linear molecules and contain the branch points characteristic of the intron lariats excised from pre-mRNAs during splicing. Although no viral products are necessary for synthesis of the LAT intron, the processing of LAT pre-mRNA is much more efficient in sensory ganglia, in which herpes simplex virus type 1 establishes latent infection (Box 10.6). In contrast to typical introns removed from other viral and cellular pre-mRNAs, these herpesviral RNAs are remarkably stable, with a half-life of 24 h, and accumulate to high concentrations in the nuclei of latently infected cells. The presence of a branch point sequence that is not a close match to the consensus (Fig. 10.7A) is an important determinant of such stability. As discussed in Chapter 8, various functions have been ascribed to this unusual product of viral RNA processing.

Alternative Splicing of Viral Pre-mRNA

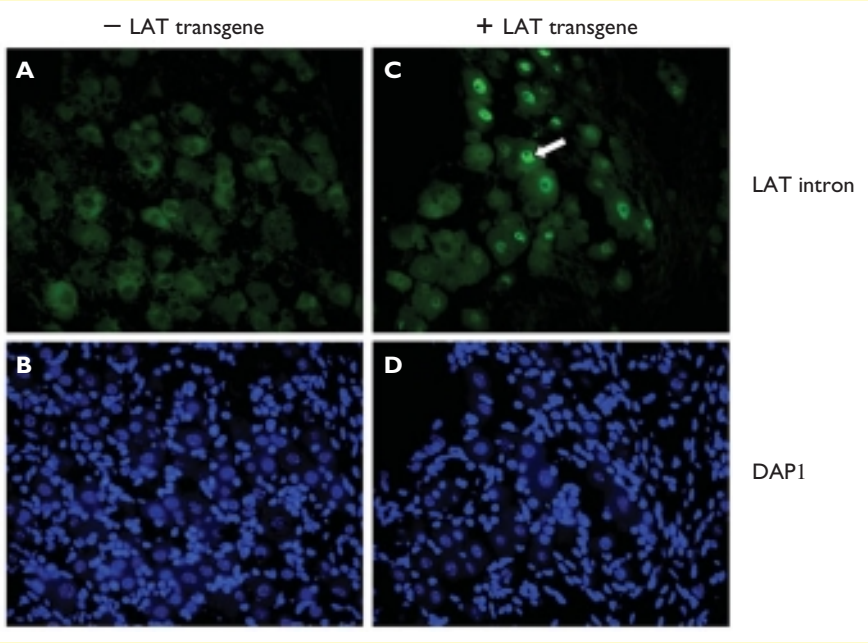
Many viral and cellular pre-mRNAs contain multiple exons. Splicing of the majority removes all introns and joins all exons in the order in which they are present in the substrate pre-mRNA. Such **constitutive splicing** (Fig. 10.9A) produces a single mature mRNA. However, numerous cellular and many viral pre-mRNAs yield more than one mRNA as a result of the splicing of different combinations of exons, a process termed **alternative splicing**. Several different types of alternative splicing can be defined (Fig. 10.9B). Alternative splicing generally leads to the synthesis of mRNAs that differ in their protein-coding sequences.

BOX 10.6 EXPERIMENTS

Synthesis of the herpes simplex virus latency-associated transcript intron is tissue specific

The production and processing of the latency-associated transcript (LAT) in different organs and tissues were examined by creation of a line of transgenic mice carrying in their genomes the region of the herpes simplex virus type 1 genome spanning the LAT coding sequence and promoter. This approach allowed LAT RNA synthesis and processing to be examined in any organ or tissues (including those not infected by herpes simplex virus) and in the absence of other viral sequences or promoters. Expression of the LAT gene was detected in several organs and tissues including brain, liver, and kidney. However, as shown in the figure, the processed LAT intron was observed by fluorescent *in situ* hybridization only in neurons of the sensory ganglia, in which the virus establishes latency, such as dorsal root and trigeminal ganglia. Quantitative assays to measure the relative concentrations of unspliced and spliced LAT RNAs confirmed that splicing to produce the LAT intron is much more efficient in sensory ganglia than in other tissues.

Gussow, A. M., N. V. Giorgani, R. K. Tran, Y. Imai, D. L. Kwiatkowski, G. R. Full, T. P. Margolis, and D. C. Bloom. 2006. Tissue-specific splicing of the herpes simplex virus type latency-associated transcript (LAT) intron in LAT transgenic mice. *J. Virol.* **80:**9414–9423.



Trigeminal neurons from LAT nontransgenic mice **(A)** or LAT transgene siblings **(C)** were hybridized to a digoxigenin-containing complementary RNA specific for spliced LAT RNA, incubated with fluorescein-labeled anti-digoxigenin antibodies, and examined by fluorescent microscopy. **(B and D)** DNA staining of the fields shown in panels A and C, respectively. The many nuclei that do not contain the LAT intron **(C and D)** are those of glial cells. Reprinted from A. M. Gussow et al., *J. Virol.* **80:**9414–9423, 2006, with permission.

The most obvious advantage of this mechanism is that it can expand the limited coding capacity of viral genomes. For example, the early genes of polyomaviruses, as well as the adenoviral E1A and E4 genes, each specify two or more proteins as a result of splicing of primary transcripts at alternative 5′ or 3′ splice sites. Alternative splicing can also be important for temporal regulation of viral gene expression, or the control of a crucial balance in the production of spliced and unspliced-mRNAs. In many cases, alternative splicing of viral pre mRNAs is coupled with other RNA-processing reactions, or regulated by viral proteins. These more complex phenomena are considered in later sections.

Examples of Alternative Splicing of Viral Pre-mRNAs

Cellular differentiation regulates splicing of papillomaviral late pre-mRNAs. The late proteins of bovine papillomavirus type 1 are synthesized efficiently only in highly differentiated keratinocytes. Productive replication of viral DNA and assembly and release of virions are also restricted to these outer cells in an epithelium. Alterations in splicing are crucial for production of the mRNA encoding the major capsid protein, the L1 mRNA (Fig. 10.10A). *In situ* hybridization studies have shown that this mRNA is made only in fully differentiated cells (Fig. 10.10B). Production of the L1 mRNA requires activation of an alternative 3′ splice site that is not recognized in undifferentiated cells. Several *cis*-acting sequences in the viral late pre-mRNA govern the choice of 3′ splice sites (Fig. 10.10C). These sequences are bound by cellular splicing proteins, including SR proteins. Analogous sequences regulate splicing of human papillomavirus late pre-mRNAs. It has therefore been proposed that terminal differentiation of keratinocytes is accompanied by changes in the activity or abundance of these cellular proteins, such that L1 mRNA can be synthesized.

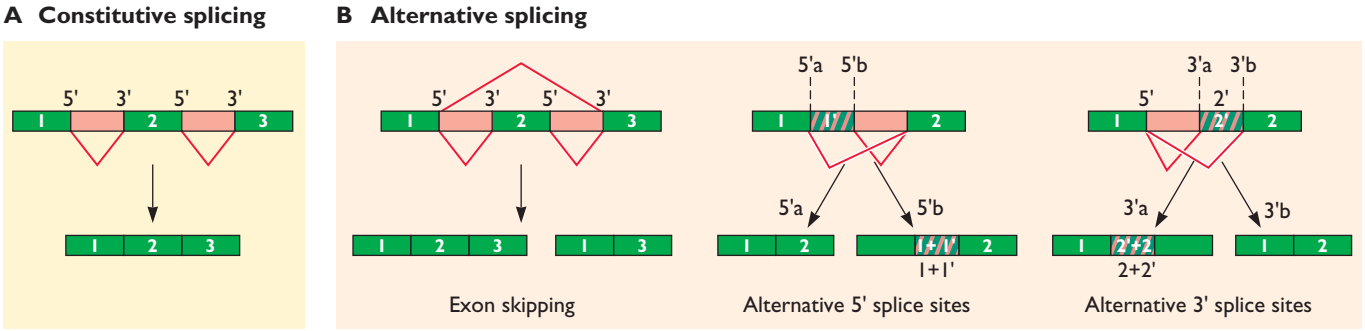


Figure 10.9 Constitutive and alternative splicing. (A) In constitutive splicing all exons (green) are joined sequentially and all introns (pink) are excised. **(B)** Alternative splicing occurs by several mechanisms. In exon skipping, the 3′ splice site of exon 2 is sometimes ignored, so that this exon is not included in some fraction of the spliced mRNA molecules. Alternatively, one of two 5′ splice sites (5′a and 5′b) in exon 1 or one of two 3′ splice sites (3′a and 3′b) in exon 2 are recognized. Recognition of different 5′ and 3′ splice sites produces alternatively spliced simian virus 40 early and adenoviral major late (Fig. 10.11) mRNAs, respectively.

Production of spliced and unspliced RNAs essential for virus replication. The expression of certain coding sequences in retroviral genomes (Gag and Pol) (Appendix, Fig. 19.8) and orthomyxoviruses (M1 and NS1) (Appendix, Fig. 7B) depends on an unusual form of alternative splicing that produces both spliced and unspliced mRNAs: normally all pre-mRNAs that contain introns are fully spliced in mammalian cell nuclei. This phenomenon has been well studied in retrovirus-infected cells, in which the viral genome is produced as unspliced viral transcripts.

In cells infected by retroviruses with simple genomes, such as avian leukosis virus, a full-length, unspliced transcript of proviral DNA serves as both the genome and the mRNA for the capsid proteins and viral enzymes, while a singly spliced mRNA specifies the viral envelope protein (Fig. 10.11A). Retrovirus production depends rather critically on the maintenance of a proper balance between the proportions of unspliced and spliced RNAs: modest changes in splicing efficiencies cause replication defects (Fig. 10.11A). This phenomenon has been used as a genetic tool to select for mutations that affect splicing control. Such mutations arise in different splicing signals at the 3′ splice site, and alter the efficiency of either the first or second step in the splicing reaction. Features that maintain the proper splicing balance include a suboptimal 3′ splice site, and a splicing enhancer in the adjacent exon. A negative regulatory sequence located more than 4000 nucleotides upstream of the 3′ splice site is also important. This sequence, which is bound by both U1 snRNP and specific cellular proteins (Fig. 10.11A), has been proposed to act as a "decoy" 5′ splice site: it forms a complex with the 3′ splice site for production of Env mRNA, but one that does not participate in splicing reactions. This sequence also stimulates polyadenylation.

The splicing of human immunodeficiency virus type 1 pre-mRNA is necessarily much more complex, as more than 40 alternatively spliced mRNAs are made in infected cells. Nevertheless, alternative splicing is also regulated by specific sequences that promote or repress recognition or utilization of splice sites, and by the degree of conformity of 3′ splice sites to the optimal sequence.

Coordination between Polyadenylation and Splicing

Although described separately in previous sections, capping, polyadenylation, and splicing of a pre-mRNA by cellular components are not independent reactions. Rather, one processing reaction governs the efficiency or specificity of another. For example, interaction of the nuclear cap-binding protein with the 5′ end of a pre-mRNA facilitates both removal of the 5′-terminal intron and efficient cleavage at the 3′ poly(A) addition site. Similarly, the presence of a 3′ poly(A) addition signal generally stimulates removal of the intron closest to it. However, splice sites at other positions can suppress recognition of polyadenylation signals. Inhibition of polyadenylation at specific sites is essential for expression of the genes of some retroviruses, and is an important device for the regulation of adenoviral gene expression.

Suppression of recognition of retroviral poly(A) addition sites. The proviral DNAs of retroviruses contain long terminal repeat sequences at either end, both of which contain a poly(A) addition signal (Fig. 10.11B).

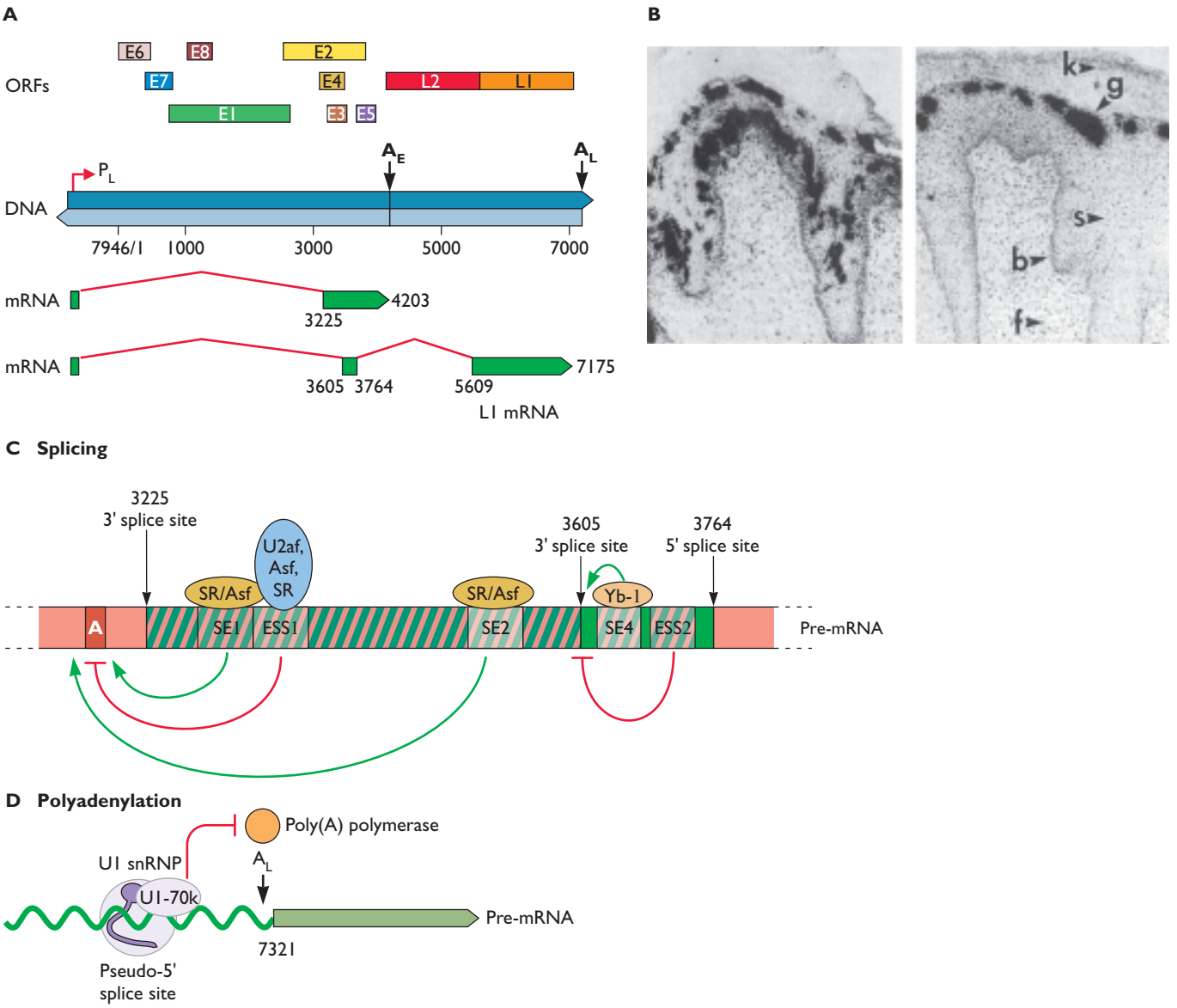
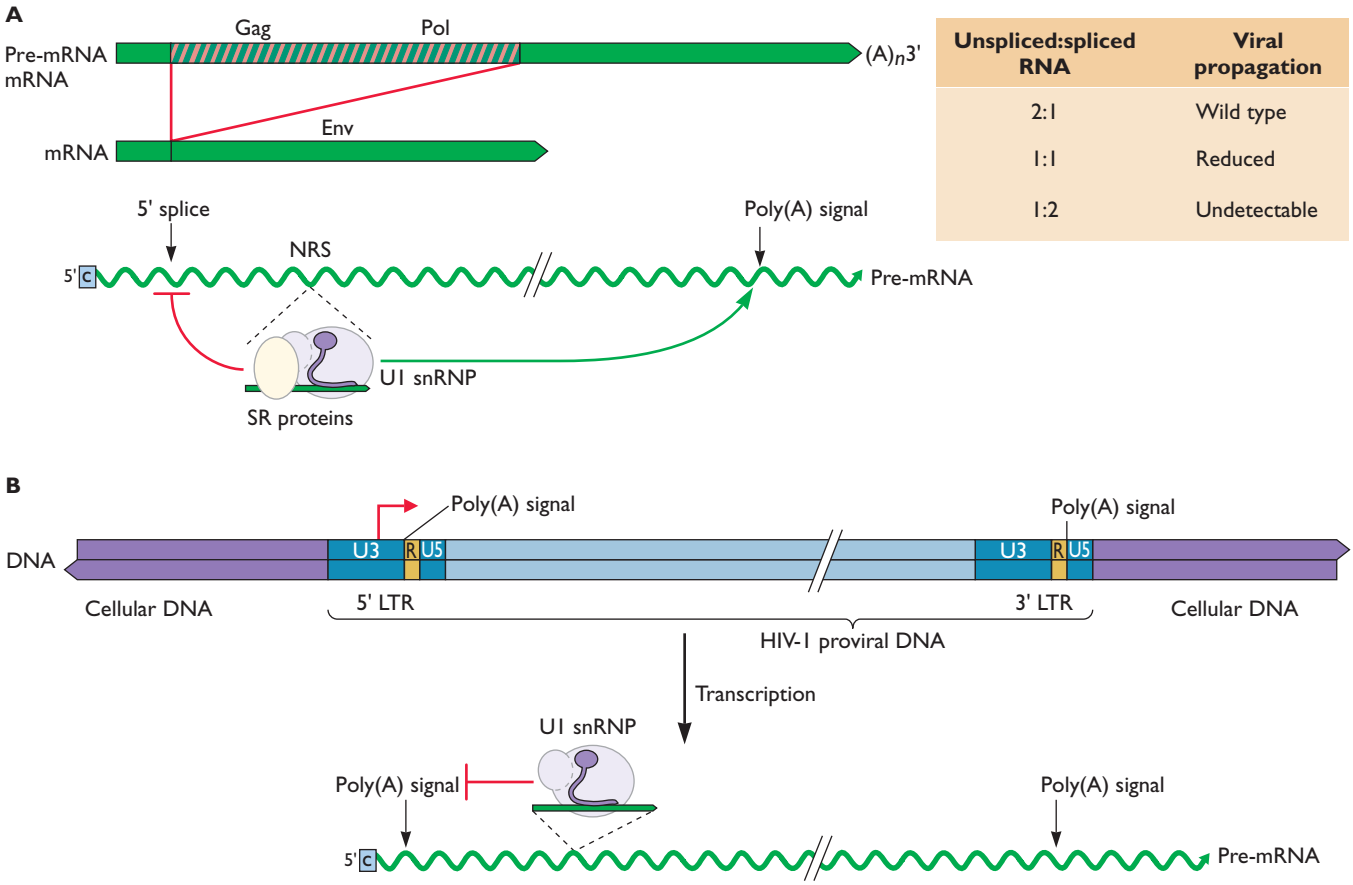


Figure 10.10 Alternative polyadenylation and splicing make active control of the production of bovine papillomavirus type 1 late mRNAs. (A) The circular bovine papillomavirus type 1 genome is represented in linear form, with open reading frames (ORFs) shown above. Two of the many mRNAs made from transcripts from the late promoter (P_L) are shown to illustrate the changes in recognition of splice sites and of poly(A) addition sites necessary to produce the L1 mRNA. Synthesis of this mRNA depends on recognition of a 3′ splice site at position 3605, rather than that at 3225, which is used during the early phase of infection. Polyadenylation of pre-mRNAs must also switch from the early (A_E) to the late (A_L) polyadenylation site. **(B)** *In situ* hybridization of bovine fibropapillomas to probes that specifically detect mRNAs spliced at the 3225 3′ splice site (left) or at the 3605 site (right). The cell layers of the fibropapilloma are indicated in the right panel. Abbreviations: k, keratin horn; g, granular cell layer; s, spinous cell layer; b, basal cell layer; f, fibroma. Note the production of late mRNA spliced at the 3605 3′ splice site only in the outermost layer (g) of fully differentiated cells. From S. K. Barksdale and C. C. Baker, *J. Virol.* **69:**6553–6556, 1995, with permission. Courtesy of C. C. Baker, National Institutes of Health. **(C)** Mechanisms that regulate splicing to produce L1 mRNA, which are specific to highly differentiated keratinocytes of the granular cell layer. The sequences that control alternative splicing at the 3225 and 3605 3′ splice sites are located between these splice sites. The splicing enhancers, SE1, SE2, and SE4, are recognized by cellular SR and other splicing proteins. The SE1 enhancer and the adjacent sequence that inhibits splicing at the 3605 3′ splice site, termed exonic splicing suppressor (ESS1), are thought to facilitate recruitment of U2-associated protein (U2af) and recognition of the branch point sequence upstream of the 3225 3′ splice site. SE2 is located very close to the 3605 3′ splice site and may block access to the branch point for splicing at this site until keratinocytes differentiate. **(D)** Inhibition of polyadenylation at the A_E site by the binding of U1 snRNP to a pseudo-5′ splice site located nearby in the primary transcript (see the text). Such inhibition is the result of binding of the U1 snRNP 70k subunit to poly(A) polymerase.



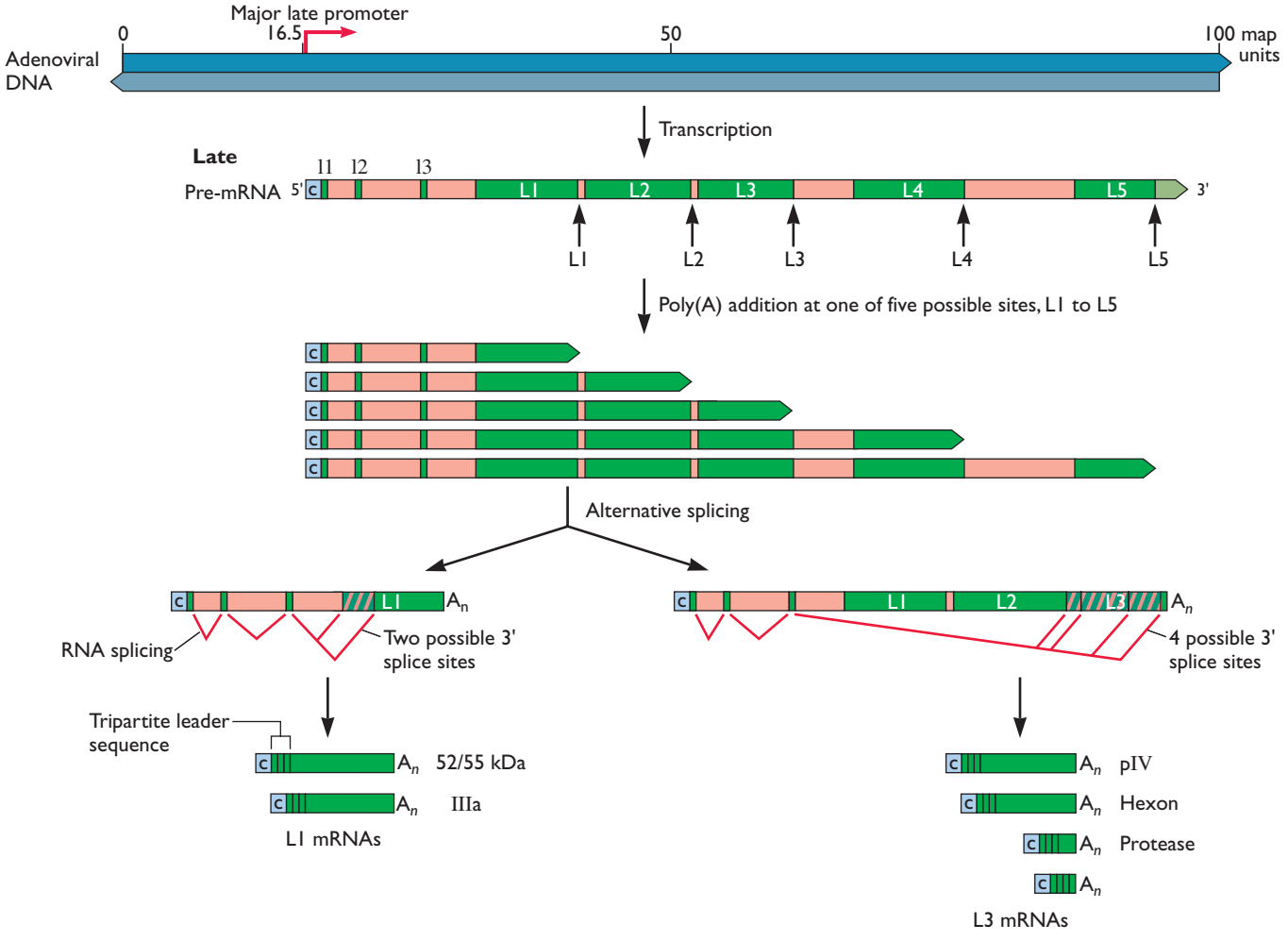
Unspliced:spliced RNA	Viral propagation
2:1	Wild type
1:1	Reduced
1:2	Undetectable

Figure 10.11 Control of RNA-processing reactions during retroviral gene expression. (A) Balanced production of spliced and unspliced mRNAs is illustrated for avian leukosis virus. A single 3′ splice site is recognized in about one-third of the primary transcripts to produce spliced mRNA encoding the Env protein. The Gag and Pol proteins are synthesized from unspliced transcripts. Even a twofold reduction in the ratio of unspliced to spliced mRNA impairs virus reproduction (right). Shown below is the negative regulatory sequence (NRS) located within Gag coding sequences, which is bound by U1 snRNP and SR proteins. This sequence is believed to act as a "decoy" 5′ splice site (to inhibit splicing [red bar]). It also stimulates polyadenylation (green arrow), by an unknown mechanism. **(B)** Suppression of poly(A) site recognition. Utilization of the 5′ polyadenylation site in primary transcripts of human immunodeficiency virus type 1 proviral DNA is inhibited by binding of U1 snRNP to the major 5′ splice site located 195 nucleotides downstream. The ability of the U1 snRNP protein U1a to bind to both poly(A) polymerase and cleavage-polyadenylation specificity protein suggests that the U1 snRNP might inhibit their activity.

Transcription of some proviral DNAs, such as that of Rous sarcoma virus, initiates downstream of the long terminal repeat sequence encoding the essential 5′AAUAAA3′ polyadenylation sequence. Consequently, a functional poly(A) addition site is present only at the 3′ ends. However, many other retroviral transcripts carry complete signals for this modification at both their 5′ and 3′ ends. Nevertheless, poly(A) is added to only the 3′ ends of these pre-mRNAs. At least two mechanisms ensure that the correct poly(A) addition signal of human immunodeficiency virus type 1 pre-mRNA is recognized. Sequences present only at the 3′ end of the pre-mRNA stimulate polyadenylation *in vitro* and *in vivo*, by facilitating binding of Cpsf to the nearby 5′AAUAAA3′ sequence. In addition, recognition of the 5′ poly(A) signal is suppressed by the 5′ splice site lying immediately downstream (Fig. 10.11B). Such inhibition is thought to be mediated by U1 snRNP, which can bind to the 5′ splice site. A related mechanism, binding of U1 snRNP to a pseudo 5′ splice site, appears to suppress recognition of the poly(A) addition site for production of bovine papillomavirus type 1 late mRNAs until the keratinocyte host cell is fully differentiated (Fig. 10.10C).

Alternative polyadenylation and splicing of adenoviral late pre-mRNAs. The production of adenoviral major late mRNAs exemplifies complex alternative splicing and polyadenylation at multiple sites in a pre-mRNA. The major late pre-mRNA contains the sequences of at least 15 different mRNAs. These mRNAs fall into five families (L1 to L5) defined by which of five polyadenylation sites is recognized (Fig. 10.12). The frequency with which each site is used must therefore be regulated to allow production of all major late mRNAs. For example, high efficiency polyadenylation at the L1 site would prevent efficient synthesis of L2 to L5 mRNAs, just as efficient splicing of retroviral pre-mRNAs would preclude production of the essential, unspliced transcripts. During the late phase of adenovirus infection, each of the five polyadenylation sites directs 3′-end formation with approximately the same efficiency. The mechanism responsible for such balanced recognition of multiple poly(A) addition sites is not fully understood. However, the activities of cellular polyadenylation proteins are altered as infection proceeds (see "Posttranscriptional

Figure 10.12 Alternative polyadenylation and splicing of adenoviral major late transcripts. During the late phase of adenoviral infection, major late primary transcripts extend from the major late promoter almost to the right end of the genome. They contain the sequences for at least 15 mRNAs and are polyadenylated at one of five sites, L1 to L5, as a result of decreased activity of cleavage stimulatory protein (see the text). The tripartite leader sequence, present at the 5′ ends of all late mRNAs, is assembled by the splicing of three short exons, l1, l2, and l3. This sequence is then ligated to alternative 3′ splice sites. Such joining of the spliced tripartite leader sequence to an mRNA sequence has been reported to take place after polyadenylation of pre-mRNA. Polyadenylation therefore appears to determine which 3′ splice sites can be utilized during the final splicing reaction.



Regulation of Viral or Cellular Gene Expression by Viral Proteins" below).

As discussed previously, all major late mRNAs contain the 5′-terminal tripartite leader sequence. The splicing reactions that produce this sequence from three small exons (Box 10.4) can take place before polyadenylation of the primary transcript. The final splicing reaction joins the tripartite leader sequence to one of many mRNA sequences (Fig. 10.13). Each primary transcript therefore yields only a **single** mRNA, even though it contains the sequences for many, and most of its sequence is discarded. It remains something of a mystery why the majority of adenoviral late mRNAs are made by this bizarre mechanism. However, one contributing factor may be that it ensures that each major late mRNA molecule carries the 5′-terminal tripartite leader sequence, which is important for efficient translation late in the infectious cycle (Chapter 11).

Editing of Viral mRNAs

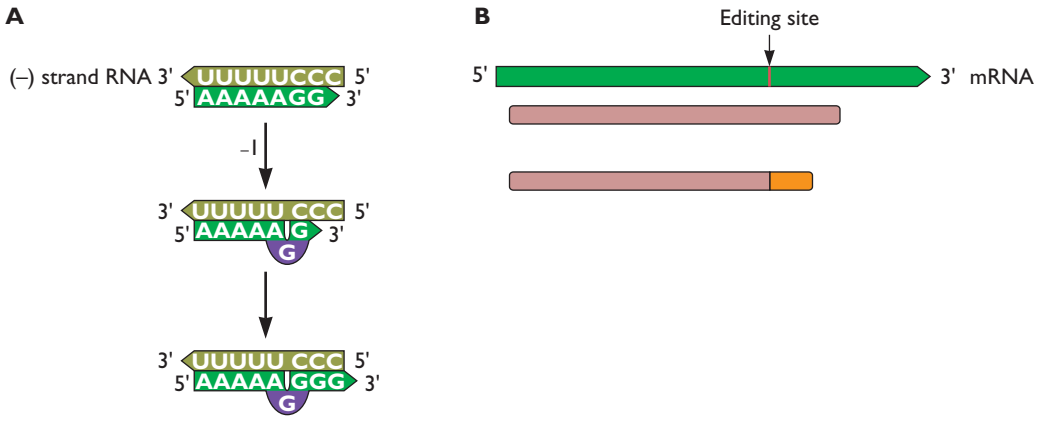
The term "RNA editing" was first coined in 1980 to describe the origin of uridine residues that are not encoded in the DNA template in a mitochondrial mRNA of trypanosomes. Since this modification was discovered, RNA editing has been identified in many different eukaryotes, as well as in some important viral systems.

Viral mRNAs are edited by either insertion of nucleotides not directly specified in the template during synthesis or alteration of a base *in situ*. Both mechanisms result in an mRNA sequence that differs from that encoded in the viral genome, altering the sequence and function of the protein specified by edited mRNA. Consequently, RNA editing has the potential to make an important contribution to regulation of viral gene expression.

Editing during Pre-mRNA Synthesis

mRNAs of *Paramyxoviridae* (e.g., measles and mumps viruses) and *Filoviridae* (e.g., Ebola virus) are edited during their synthesis. Among paramyxoviruses, this modification occurs only in the mRNAs that encode the RNA-dependent RNA polymerase, the P protein. During mRNA synthesis, the viral RNA polymerase inserts one or two G residues at specific positions in a fraction of the RNA molecules. The genomic RNA template contains a polypyrimidine sequence at the RNA-editing site. Insertion of G residues is therefore thought to occur by a reiterative copying mechanism (Fig. 10.13A), analogous to that by which the viral RNA polymerases synthesize 3′ poly(A) tails. The observation that increased stability of the nascent RNA-template RNA duplex just upstream of the editing site reduces the frequency of editing, and vice versa, is consistent with this mechanism.

Figure 10.13 Cotranscriptional editing of measles virus mRNAs. (A) Proposed mechanism. The viral polymerase pauses near a junction of U_n and C_n sequences in the template virion RNA after two C residues of the template have been copied into G residues in the nascent mRNA. As a result of such pausing, some fraction of viral RNA polymerase molecules and their attached nascent mRNA chains slip backward, such that additional nucleotides are incorporated when RNA synthesis resumes. The most stable structure is formed when the measles virus RNA polymerase slips backward by one (−1) position. Consequently, one G residue is incorporated into the viral mRNA when RNA synthesis resumes. **(B)** The measles virus P gene. The unedited mRNA contains a continuous open reading frame (pink) for the P protein. The addition of one G residue at the editing site changes the translational reading frame to one (yellow) that contains a termination codon. The edited mRNA specifies the V protein, which differs from the P protein in its C-terminal sequence.



Because insertional editing of both measles and mumps virus mRNAs alters the translational reading frame, paramyxoviral P genes encode two distinct proteins (Fig. 10.13B). Similarly, RNA editing determines whether an Ebola virus gene is expressed as a full-length glycoprotein that is required for virus entry, or as a truncated secreted protein, and modulates the cytotoxicity of the virus (Box 10.7).

Editing following mRNA Synthesis

Production of two proteins from a single coding sequence. One form of posttranscriptional editing of mRNAs is accomplished by cellular enzymes that deaminate adenine bases in double-stranded RNA regions to form inosine (I). One such enzyme has been implicated in editing of the mRNA of hepatitis delta satellite virus. Two forms of the protein encoded in the genome of this agent, called hepatitis delta antigen, are synthesized in infected cells. Both are necessary for satellite virus reproduction. The large delta antigen, which is required for assembly and inhibits RNA replication, is made when editing converts an UAG termination codon to the UGG tryptophan codon (Fig. 10.14). As many as 50% of satellite virus mRNA molecules are modified at this site, but few other sequences in the viral RNA are edited. The mechanisms that restrict the action of the adenosine deaminase, which exhibits little specificity *in vitro*, have not been elucidated. The larger form of hepatitis delta antigen inhibits editing *in vivo*. This property is important for synthesis of the essential, smaller form of the protein, which is translated from unedited mRNA molecules (Fig. 10.14). It also prevents production of too high a concentration of large delta antigen and consequently inhibition of replication of the satellite virus.

Double-stranded RNA adenosine deaminases may play a broader role in the biology of RNA viruses. Before the discovery of these enzymes, it was assumed that nucleotide changes in viral RNA genomes arise solely by the introduction of incorrect nucleotides by RNA polymerases, or by RNA recombination (Chapter 6). However, several changes can now be attributed to editing. For example, many of the genomic RNAs of defective measles viruses

BOX 10.7 DISCUSSION

RNA editing regulates the cytotoxicity of Ebola viruses

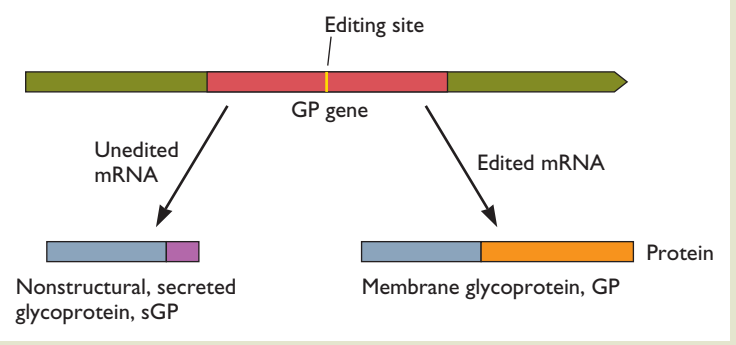
The ~19,000-nucleotide (−) strand RNA genome of Ebola virus (a member of the *Filoviridae*) contains an editing site within the coding sequence for glycoprotein (GP) mRNA. This site comprises seven consecutive U residues and resembles the viral polyadenylation signal. Editing is therefore thought to take place cotranscriptionally, by reiterative copying, as during synthesis of paramyxoviral mRNA (Fig. 10.13). As shown in the figure, the products of the edited and unedited GP mRNAs share an N-terminal sequence (blue), but carry different C-terminal sequences, because introduction of an additional A residue by editing changes the reading frame. The protein specified by edited mRNA, GP, is the viral envelope glycoprotein and localizes to the plasma membrane of infected cells. In contrast, the protein synthesized from the unedited mRNA (sGP) is secreted.

When the editing site was eliminated by mutation of cloned DNA from which infectious virus can be recovered, sGP was not made, as expected. The concentration of GP increased, also as anticipated, but most of the protein accumulated as an immature precursor in the endoplasmic reticulum. Furthermore, and unexpectedly, these alterations in production of the GP proteins were accompanied by severe cytotoxicity: the mutant virus formed small plaques because of the earlier than normal death of infected cells.

These observations indicate that maintenance of a proper ratio of the secreted and membrane-associated forms of the viral glycoprotein is necessary to limit the cytotoxicity of Ebola virus. The secreted glycoprotein is therefore likely to make an important contribution to pathogenicity, by indirectly increasing virus reproduction and spread.

Volchkov, V. E., V. A. Volchkova, E. Mühlberger, L. V. Kolesnikova, M. Weik, O. Dolnik and H.-D. Klenk. 2001. Recovery of infectious Ebola virus from complementary DNA: RNA editing of the GP gene and viral cytotoxicity. *Science* **291:**1965–1969.

(Top) The editing site in the GP gene of the viral RNA genome. **(Bottom)** The differences between the sGP and GP proteins specified by unedited and edited mRNAs, respectively.



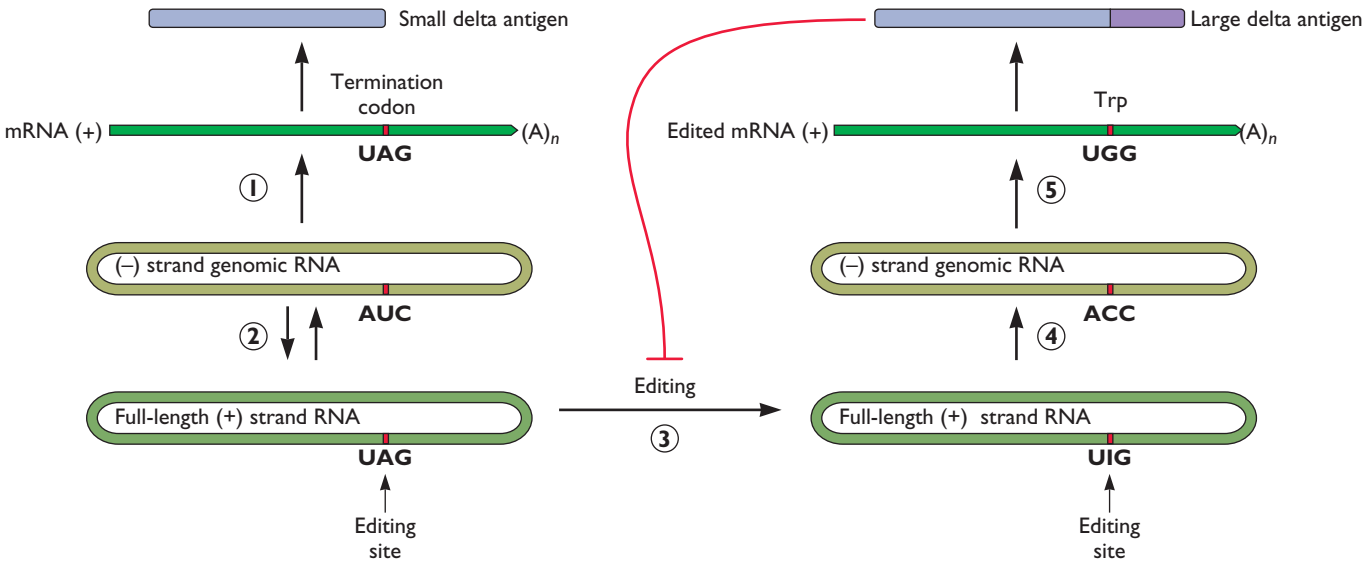


Figure 10.14 Editing of hepatitis delta satellite virus RNA by double-stranded RNA adenosine deaminase. The mRNA synthesized from genomic (–) strand RNA specifies the small delta antigen (step 1), which is required for replication of the genome (2). Double-stranded RNA adenosine deaminase acts on the full-length (+) RNA to convert a specific A residue to I (3). Because I base pairs with C, the genome (–) strands copied from edited full-length (+) strands contain a C residue (4). Such edited (–) RNA is therefore copied into (+) mRNA that contains a UGG codon (tryptophan) at the editing site (5), rather than the UAG stop codon. As a result, the mRNA made from edited RNA specifies the large delta antigen, which contains a 19-amino-acid C-terminal extension (purple). This protein inhibits RNA editing (red bar) and genome replication, and is needed for association of the hepatitis delta satellite viral genome with envelope proteins of its helper virus, hepatitis B virus.

isolated from the brains of patients who died of subacute sclerosing panencephalitis appear to have been edited by cellular adenosine deaminases.

Recent studies suggest that, like their cellular counterparts, transcripts of viral DNA genomes can also be edited by adenine deaminases. Transcripts of the k12 region of the human herpesvirus 8 genome are edited efficiently at just one site, both in infected cells and by adenine deaminase 1 *in vitro*. This modification appears to regulate the function of the kaposin protein that is encoded within the k12 region: this protein exhibits transforming activity only when it is made from the unedited coding sequence.

Editing as a powerful antiviral defense mechanism. Other cellular enzymes, known as Apobec3s, edit RNA by deamination of cytidine to uridine. Inhibition of the activity of such enzymes is important for the successful replication of several viruses. This phenomenon was discovered through efforts to elucidate the function of the human immunodeficiency virus type 1 accessory protein Vif. This protein is required for efficient replication *in vivo*, and in certain types of human cells in culture, which proved to contain Apobec3G. In the absence of Vif, this editing enzyme becomes incorporated into virions. Although it normally edits specific cellular mRNAs, Apobec3G catalyzes cytidine deamination during the first stage of reverse transcription, synthesis of (–) strand complementary DNA (cDNA). Such editing both triggers degradation of the (–) strand, presumably because of the abnormal presence of U residues, and results in hypermutation of proviral coding sequences. It therefore serves as an effective antiviral defense. Vif targets Apobec3G for degradation by the proteasome, and hence prevents its incorporation into assembling virus particles.

Export of RNAs from the Nucleus

Any mRNA made in the nucleus must be transported to the cytoplasm for translation. Other classes of RNA, including small cellular and viral RNAs made by RNA polymerase III, must also enter the cytoplasm permanently (e.g., transfer RNAs [tRNAs]) or transiently (snRNAs). The export of viral mRNAs is mediated by the host cell machinery and, in most cases, is indistinguishable from export of analogous cellular RNAs. However, transport to the cytoplasm of unusual mRNA molecules is a critical step in some viral life cycles. In this section, we describe the cellular export machinery and the mechanisms that ensure export of such viral mRNA substrates.

The Cellular Export Machinery

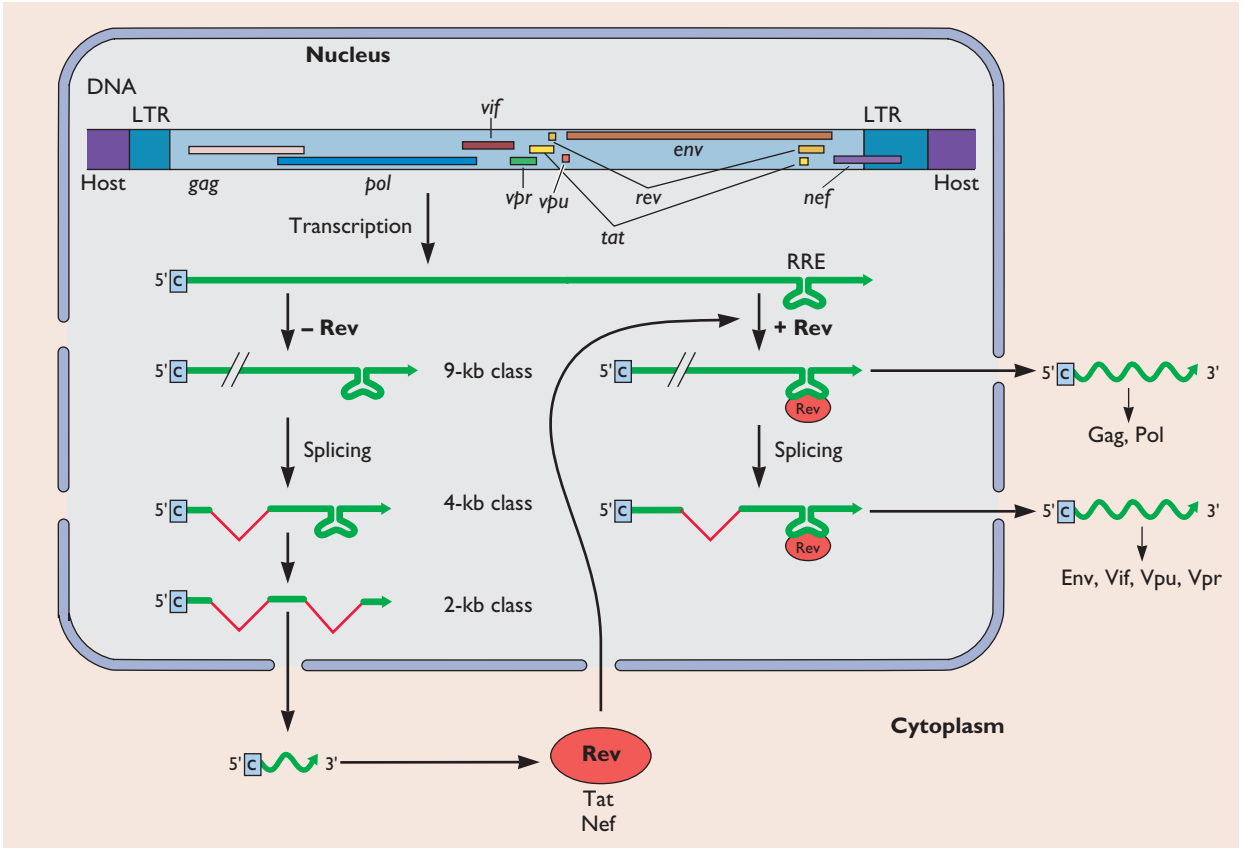
The substrates for mRNA export are not naked RNA molecules, but rather ribonucleoproteins. Some of the proteins travel to the cytoplasm with the mRNA, but are then displaced and return to the nucleus. Consequently, such hnRNP proteins shuttle between the nucleus and cytoplasm. One such protein, the abundant hnRNP-A1 protein, contains a short sequence that directs both export and import. This sequence was the first nuclear export signal to be identified. A different kind of nuclear export signal is found in proteins that direct export of certain viral mRNAs and small cellular RNAs (see "Export of Viral mRNA" below). In fact, export of RNA molecules (with the exception of tRNAs) is directed by sequences present in the proteins associated with them.

Like proteins entering the nucleus, RNA molecules travel between nuclear and cytoplasmic compartments via the nuclear pore complexes described in Chapter 5. Numerous genetic, biochemical, and immunocytochemical studies have demonstrated that specific nucleoporins (the proteins from which nuclear pore complexes are built) participate in nuclear export. Export of RNA molecules also shares several mechanistic features with import of proteins into the nucleus. Substrates for nuclear export or import are identified by specific protein signals and some soluble proteins, including the small guanosine nucleotide-binding protein Ran, function in both import and export. And RNA export, like protein import, is mediated by receptors that recognize nuclear export signals and direct the proteins, and ribonucleoproteins that contain them, to and through nuclear pore complexes.

Export of Viral mRNA

All viral mRNAs made in infected cell nuclei carry the same 5′- and 3′-terminal modifications as cellular mRNAs that are exported. Furthermore, many viral mRNAs, like their cellular

Figure 10.15 Regulation of export of human immunodeficiency virus type 1 mRNAs by the viral Rev protein. Before the synthesis of Rev protein in the infected cell, only fully spliced (2-kb class) viral mRNAs are exported to the cytoplasm (left). These mRNAs specify viral regulatory proteins, including Rev. The Rev protein enters the nucleus, where it binds to an RNA structure, the Rev-responsive element (RRE) present in unspliced (9-kb class) and singly spliced (4-kb class) viral mRNAs. This interaction induces export to the cytoplasm of the RRE-containing mRNAs, from which virion structural proteins and enzymes are made (right). The Rev protein therefore alters the pattern of viral gene expression as the infectious cycle progresses.



counterparts, are produced by splicing of intron-containing precursors. Cellular pre-mRNAs that contain introns and splice sites ordinarily are retained in the nucleus, at least in part because they remain associated with spliceosomes. Furthermore, a protein complex that marks mature mRNAs for export is assembled on the RNA only during splicing. Therefore the pre-mRNAs are either spliced to completion and exported, or degraded within the nucleus. However, replication of retroviruses, herpesviruses, and orthomyxoviruses requires production of mRNAs that are incompletely spliced, or not spliced at all, and their export from the nucleus for translation and/or virion assembly. Efforts to address the question of how the normal restrictions on cellular mRNA export are circumvented in cells infected by these viruses have provided important insights into the complex process of export of macromolecules from the nucleus.

The Human Immunodeficiency Virus Type 1 Rev Protein Directs Export of Intron-Containing mRNAs

The human immunodeficiency virus type 1 Rev protein is by far the best understood of the viral proteins that modulate mRNA export from the nucleus. This protein and related proteins of other lentiviruses promote export of the unspliced and partially spliced viral mRNAs. Rev binds specifically to an RNA sequence termed the **Rev-responsive element** that lies within an alternatively spliced intron of viral pre-mRNA (Fig. 10.15). The Rev-responsive element is some 250 nucleotides in length and forms several stem-loops (Fig. 10.16A), one of which contains a high-affinity binding site for the arginine-rich RNA-binding domain of Rev (Box 10.8). Export of RNAs that contain the Rev-responsive element depends on the formation of Rev-protein oligomers on this sequence, and a leucine-rich nuclear export signal present in Rev.

The C-terminal domain of Rev contains a short nuclear export signal (Fig. 10.16B) that is sufficient to induce export of heterologous proteins. In the oligomeric Rev-RNA complex, the nuclear export signals of the protein become organized on the surface. One cellular protein that binds to the nuclear export signal of Rev is exportin-1 (also known as Crm-1) (Fig. 10.17). Exportin-1, which binds simultaneously to Rev and the GTP-bound form of Ran, is the **receptor** for Rev-dependent export of the human immunodeficient type 1 RNAs bound to it. The viral protein therefore functions as an **adapter**, directing viral, intron-containing mRNAs to a preexisting cellular export receptor. Translocation of the complex containing viral RNAs, Rev and cellular proteins through the nuclear pore complex to the cytoplasm requires specific nucleoporins. In the cytoplasm, hydrolysis of GTP bound to Ran by a Ran-specific GTPase-activating protein present only in the cytoplasm induces dissociation of the

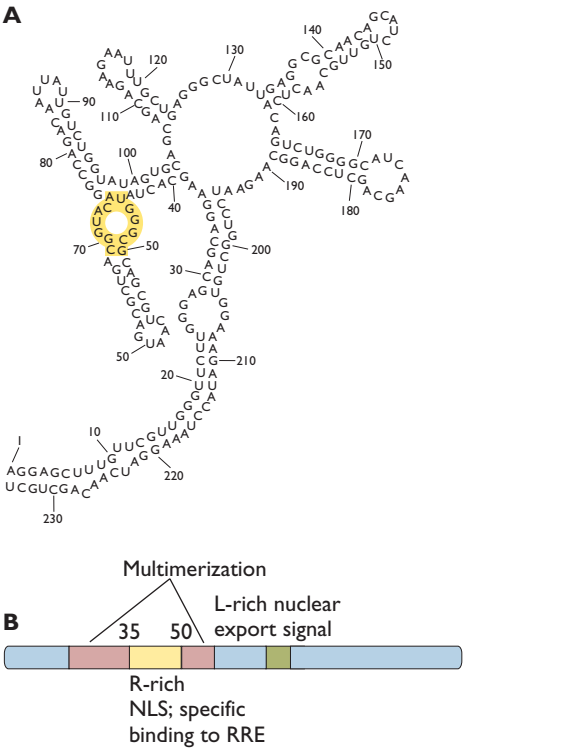


Figure 10.16 Features of the Rev-responsive element and Rev protein. (A) Predicted secondary structure of the 234-nucleotide Rev-responsive element, with the high-affinity binding site for Rev shaded in yellow. **(B)** The functional organization of the Rev protein.

export complex. Rev then shuttles back into the nucleus via a typical nuclear localization signal (Fig. 10.17B), where it can pick up another cargo RNA molecule.

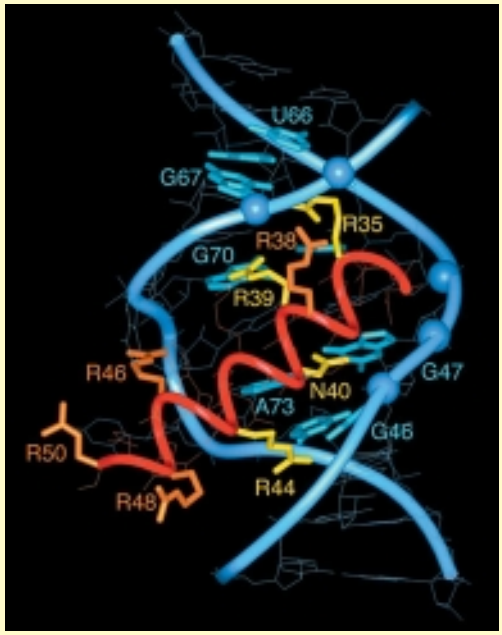
Other cellular proteins required for Rev-dependent RNA export include an ATP-dependent RNA helicase (Ddx3), which may catalyze reorganization of RNA molecules for transit though the nuclear pore, and human Rev-interacting protein (hRip). As the latter protein is needed for release of Rev-associated RNA from the nuclear periphery into the cytoplasm, its discovery identified a previously unknown reaction in Rev-dependent export. This reaction may be a useful target for antiviral drugs, as loss of hRip neither results in mislocalization of cellular mRNAs or proteins, nor impairs cell viability.

Perhaps the most interesting aspect of Rev-dependent RNA export is the exit of mRNAs by a pathway that normally does not handle such cargo, but rather exports small RNA species and proteins of the host cell. The Rev nuclear export signal is similar to, and can be functionally replaced by, that of the cellular protein TfIIIa. This protein binds specifically to 5S rRNA and is required for export of this cellular RNA from the nucleus. Peptides containing the

BOX 10.8 EXPERIMENTS

Structure of a Rev peptide bound to the high-affinity binding site of the Rev-responsive element, determined by nuclear magnetic resonance methods

Comparison of the high-resolution structures of a Rev peptide bound to the high-affinity site and free Rev has provided important mechanistic insights into how specific RNA sequences and structural features are recognized. The Rev peptide corresponding to amino acids 34 to 50 forms an α-helix (red) that binds to the major groove of the RNA (blue), with phosphates contacted by Rev shown as spheres. The bases shown are invariant in RNA molecules selected from a large population for high-affinity binding to Rev *in vitro*. The major groove of an A-form helix is too narrow to accommodate an α-helix. However, in the Rev-RNA complex, the groove is widened by formation of two purine-purine base pairs and distortion of the RNA backbone. The purine-purine pairs are not observed in the solution structure of the RNA alone, indicating that binding of Rev induces a substantial conformational change. The Rev protein penetrates deeply into the major groove, and interacts extensively with the RNA over three to four turns of the α-helix. These interactions include base-specific contacts, for example, via the three arginine and one asparagine residues shown in yellow, and numerous contacts with the phosphate backbone of the RNA, via residues colored orange. From J. L. Battiste et al., *Science* **273:**1547–1551, 1996, with permission. Courtesy of J. R. Williamson, The Scripps Research Institute.



Rev nuclear export signal inhibit export of 5S rRNA, but not of mRNAs. The human immunodeficiency virus type 1 Rev protein therefore circumvents the normal restriction on the export of intron-containing pre-mRNAs from the host cell nucleus by diverting such viral mRNAs to a cellular pathway that handles unspliced RNAs.

RNA Signals Can Mediate Export of Intron-Containing Viral mRNAs by Cellular Proteins

The genomes of many retroviruses do not encode analogous proteins to Rev, even though unspliced viral mRNAs must reach the cytoplasm. These unspliced viral mRNAs contain specific sequences that promote export. Because they must function by means of cellular proteins, such sequences were termed **constitutive transport elements** (CTEs). The first such sequence was found in the 3′ untranslated region of the genome of Mason-Pfizer monkey virus.

Even low concentrations of RNA containing the Mason-Pfizer monkey virus CTE inhibit export of mature mRNAs when microinjected into *Xenopus* oocyte nuclei, but CTE RNA does **not** compete with Rev-dependent export. This observation indicated that this retroviral RNA sequence is recognized by components of a cellular mRNA export pathway. A search for such proteins led to the first identification of a mammalian protein mediating mRNA export: the human Tap protein binds specifically to the CTE and is essential for export from the nucleus of the unspliced viral RNAs and spliced cellular mRNAs. Tap proved to be the human homolog of a yeast protein that is essential for mRNA export.

The pathway of Tap-dependent mRNA export has not yet been fully elucidated, but the Ran-GTP protein does not participate. The direct and specific binding of Tap to the CTE of unspliced retroviral RNAs appears to bypass a cellular process that ensures that export is normally strictly coupled with splicing (Fig. 10.18). The Tap protein can bind only nonspecifically and with low affinity to cellular pre-mRNAs, but it does interact with components of a protein complex that is deposited on cellular mRNAs as they are

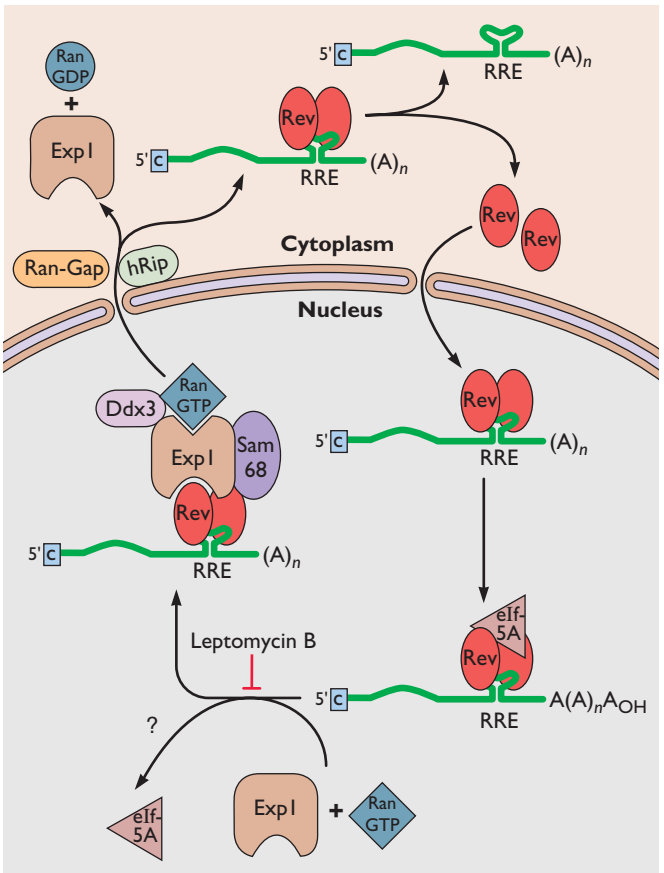


Figure 10.17 Mechanism of Rev protein-dependent export. The cellular nuclear proteins exportin-1 (Exp1), the GTP-bound form of Ran (Ran-GTP), and the 68-kDa Src-associated protein in mitosis (Sam68) have been implicated in Rev-dependent mRNA export, for example by analysis of the effects of dominant negative forms of the proteins. In the presence of Ran-GTP, Rev binds to exportin-1. This protein is related to the import receptors described in Chapter 5, and interacts with nucleoporins. The complex, containing Rev, exportin-1, and Ran-GTP bound to the Rev-responsive element in RNA, is translocated through the nuclear pore complex to the cytoplasm via interactions of exportin-1 with nucleoporins, such as Can/Nup14 and Nup98. Translocation may be facilitated by the action of Ddx3, an ATP-dependent RNA helicase. The Sam68 protein can bind to the Rev nuclear export signal, but does not appear to shuttle between nucleus and cytoplasm. It may therefore act prior to docking of the viral RRE-containing RNA complex at the nuclear pore. The human Rev-interacting protein (hRip) appears to act following translocation, as it is essential for efficient release of Rev-associated RNA into the cytoplasm. Hydrolysis of GTP bound to Ran to GDP induced by the cytoplasmic Ran GTPase-activating protein (Ran-Gap) is presumed to dissociate the export complex, releasing viral RNA for translation or virion assembly, and Ran, exportin-1, and other proteins for reentry into the nucleus.

spliced, such as the Ref/Aly protein. Indirect recruitment of Tap to an mRNA, which couples export to splicing, is circumvented in the case of retroviral pre-mRNAs containing CTEs: such RNAs are recognized directly by Tap, allowing export of unspliced RNAs from the nucleus.

Control of the Balance between Export and Splicing

The successful replication of retroviruses and orthomyxoviruses depends on production of spliced mRNAs in addition to export to the cytoplasm of unspliced RNAs. The relative efficiencies of splicing and export maintain a finely tuned balance in the production of spliced and unspliced viral RNAs. On one hand, splicing of viral pre-mRNA must be inefficient to allow export of the essential, intron-containing mRNAs (Fig. 10.11A). Indeed, increasing the efficiency of splicing of human immunodeficiency virus type 1 pre-mRNA, by replacing the natural, suboptimal splice sites with efficient ones, leads to complete splicing of all pre-mRNA molecules before Rev can recognize and export the unspliced mRNA to the cytoplasm. On the other hand, when unspliced RNAs remain in the nucleus (e.g., before Rev is made in infected cells), they are eventually spliced to completion. Efficient export is therefore required to place unspliced mRNA into the cytoplasm for translation or incorporation into virus particles.

Export of Single-Exon Viral mRNAs

Most of the viral mRNAs made in cells infected by hepadnaviruses, herpesviruses, or orthomyxoviruses are not spliced. In contrast to the retroviral mRNAs described in previous sections, these viral mRNAs do not retain introns. Rather, the viral genes that encode them contain no introns and the RNAs **cannot** be spliced. We therefore designate such mRNAs as **single-exon mRNAs** to distinguish them from those that retain introns.

Because such viral mRNAs do not contain splice sites, they should not become trapped in the nucleus by a spliceosome retention mechanism. On the other hand, they have few counterparts in uninfected mammalian cells growing under normal conditions, a property that raises the question of how viral, single-exon mRNAs are transported efficiently to the cytoplasm. Recent experiments have identified both viral proteins and RNA sequences that promote export of such viral mRNA, analogous to the retroviral Rev protein and constitutive transport elements, respectively.

The herpes simplex virus type 1 early mRNA encoding thymidine kinase contains a sequence that directs export by cellular components. This sequence is sufficient to induce efficient, cytoplasmic accumulation of unspliced β-globin mRNA when fused to it. Furthermore, its recognition by the cellular hnRNP L protein correlates with RNA accumulation in the cytoplasm. It may therefore direct the viral mRNA to a cellular pathway for export of the rare, cellular mRNAs that are not spliced. This same pathway may be responsible for export of hepadnaviral mRNAs. The efficient cytoplasmic accumulation of these viral mRNAs depends on a conserved sequence, termed the **posttranscriptional regulatory element**, which

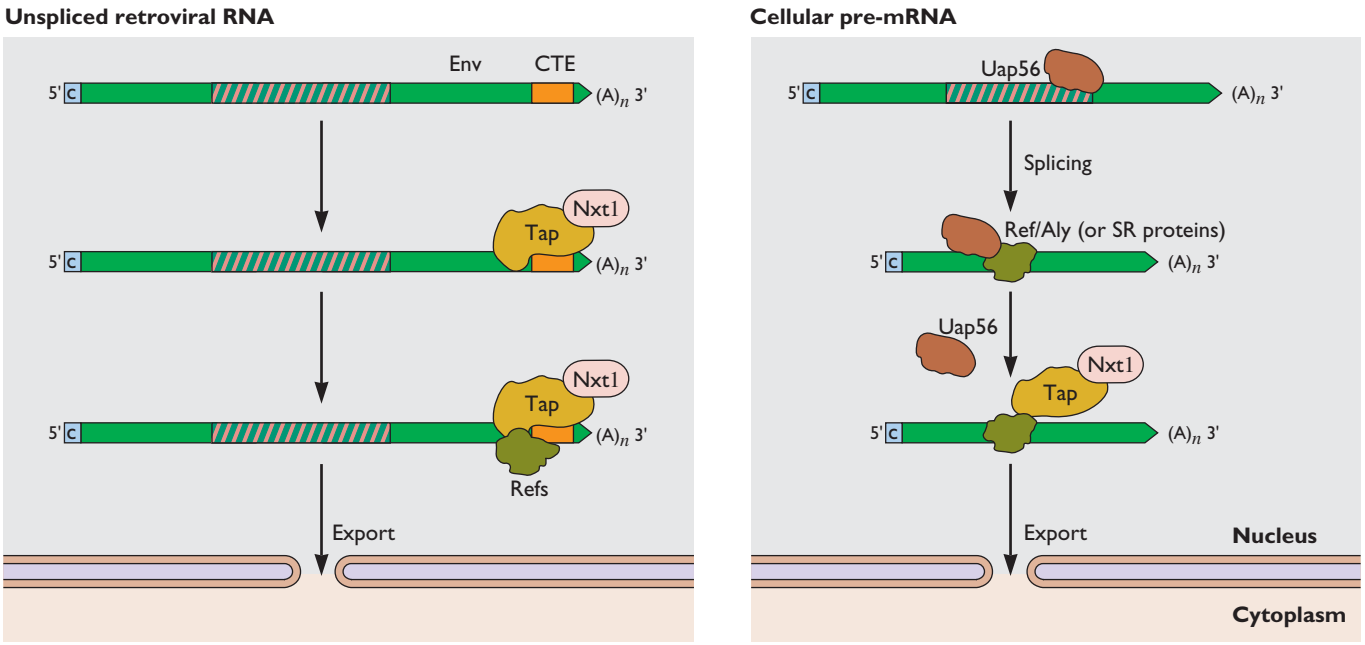


Figure 10.18 Export of unspliced RNA of retroviruses with simple genomes and cellular mRNAs from the nucleus. Export of unspliced, primary transcripts of many retroviruses (left) depends on the constitutive transport element (CTE) in the RNA. This sequence is recognized by the cellular Tap subunit of the export receptor dimer Tap-Nxt1, which is then bound by proteins that mark mRNAs as appropriate substrates for export, such as Ref. A variety of experimental approaches, inducing genetic studies of yeast, have indicated that Tap is an essential component of a cellular mRNA export pathway. However, Tap does not bind to cellular mRNAs (right) with high affinity, but rather becomes associated with them via interactions with specific proteins, such as Ref/Aly and certain SR proteins that are deposited on the cellular mRNA during splicing. In this way, spliced mRNAs are marked for export. The direct interaction of Tap with retroviral CTEs therefore appears to bypass the mechanism(s) that couple splicing of cellular mRNAs with their export.

can function in the absence of viral proteins. Functionally analogous sequences have been identified in one of the uncommon mammalian mRNAs that lacks introns (a histone 2A mRNA), suggesting that hepadnaviral mRNAs leave the nucleus via a cellular pathway that handles a minor fraction of the mRNA traffic in uninfected cells.

The efficient export of the majority of herpesviral single-exon mRNAs requires the viral ICP27 protein. This protein, like the products of all immediate-early genes, is made at the beginning of the infectious cycle. It is a nuclear phosphoprotein that contains a leucine-rich nuclear export signal resembling that of the human immunodeficiency virus Rev protein, and shuttles between the nucleus and the cytoplasm. ICP27 specifically facilitates the export of viral single-exon mRNAs: such mRNAs do not enter the cytoplasm efficiently in cells infected by mutant viruses that do not direct the synthesis of ICP27, but export of viral spliced mRNAs continues normally. This viral protein has been reported to bind to viral RNAs in infected cells. It contains different types of RNA-binding motifs in its N-terminal and C-terminal portions, both of which are necessary for optimal replication of the virus. A considerable body of genetic evidence indicates that the RNA-binding motifs of ICP27 contribute to efficient cytoplasmic accumulation of viral single-exon mRNAs. An unusual property of the ICP27 protein is that it contains both a binding site for Tap and a leucine-rich nuclear export signal that can mediate export by the exportin-1 pathway. The function of the latter is not yet clear, but the results of various experimental approaches indicate that ICP27 functions as an adapter of export of viral single-exon mRNAs via the Tap pathway (Box 10.9). Whether this pathway mediates export of all such viral mRNAs is not clear: it has been reported that export of some mRNAs is inhibited by the exportin-1 inhibitor, leptomycin B.

Posttranscriptional Regulation of Viral or Cellular Gene Expression by Viral Proteins

The genomes of several viruses encode proteins that regulate one or more RNA-processing reactions. These proteins play crucial roles in temporal regulation of viral gene expression, or in inhibition of the production of cellular mRNAs (Table 10.3).

BOX 10.9 DISCUSSION

Multiple lines of evidence indicate that the herpes simplex virus type 1 ICP27 protein engages the cellular Tap-Nxt1 export pathway

The nucleocytoplasmic shuttling of the ICP27 protein under the direction of its leucine-rich nuclear export signal originally suggested that this herpesviral protein, like Rev, engages the exportin-1-mediated export pathway of the host cell. However, shuttling of ICP27 between the nucleus and cytoplasm and export of viral mRNA in microinjected *Xenopus* oocytes are not sensitive to the expotin-1 inhibitor leptomycin B. This drug also had no effect on 1CP27 shuttling in herpes simplex virus type 1-infected cells. Rather, several observations indicate that ICP27 directs viral single exon mRNAs to the Tap-Nxt1 export pathway:

- ICP27 interacts with Tap/Nxt1 in infected cells.
- Overproduction of the Ref/Aly proteins, which are components of the Tap-Nxt1 pathway (Fig. 10.18) in infected cells, increased the efficiency of export of several viral late mRNAs.
- ICP27-dependent export of viral single exon mRNAs from *Xenopus* oocyte nuclei was blocked by a high concentration of a retroviral constitutive transport element, to which Tap binds (Fig. 10.18).
- Overproduction of a dominant negative derivative of Tap, which lacks C-terminal sequences that interact with nucleoporins, inhibited export of viral single exon mRNA in infected cells.

Temporal Control of Viral Gene Expression

Regulation of Alternative Polyadenylation by Viral Proteins

The frequencies with which alternative poly(A) addition sites within specific viral pre-mRNAs are utilized change during infection by adenoviruses, herpesviruses, and papillomaviruses. As discussed previously, the polyadenylation of bovine papillomavirus type 1 late mRNA is activated by a specific complement of cellular proteins found only in fully differentiated cells of the epidermis. In contrast, viral proteins have been implicated in regulation of polyadenylation in cells infected by the larger DNA viruses.

Despite its name, the adenoviral major late promoter is active during the early phase of infection, prior to the onset of viral DNA synthesis. The major late pre-mRNAs made during this period are polyadenylated predominantly at the L1 mRNA site (Fig. 10.12), even though they also contain the L2 and L3 3′ processing sites. Such selective recognition of the L1 site depends on the cellular cleavage stimulatory protein (Cstf), which binds to the U/GU-rich sequence 3′ to the cleavage site (Fig. 10.4). As infection continues, the activity of this cellular protein decreases. The recognition of the other four polyadenylation sites present in major late pre-mRNA synthesized during the late phase (Fig. 10.12) is much less dependent on Cstf. It is therefore likely that these poly(A)-addition signals compete more effectively with the L1 site for components of the polyadenylation machinery later in the infectious cycle. In the case of the L3 polyadenylation site, a downstream sequence is also required.

Experiments in which the synthesis of adenoviral mRNAs from truncated major late transcription units were

Table 10.3 Viral proteins that regulate RNA-processing reactions

Virus	Protein(s)	Functions
Adenovirus		
Human adenovirus type 2	E4 ORF4	Induces dephosphorylation of cellular SR proteins by protein phosphatase 2A; relieves inhibition of L1 pre-mRNA splicing at the IIIa 3′ splice site by phosphorylated SR proteins present early in infection
	E1B 55-kDa–E4 ORF6	The complex inhibits export of fully processed cellular mRNAs and induces selective export of viral late mRNAs
	L4 33 kDa	Promotes alternative splicing to produce L1 IIIa mRNA
Herpesvirus		
Herpes simplex virus type 1	ICP27	Stimulates polyadenylation of viral late mRNAs with suboptimal sequences at their polyadenylation sites; inhibits splicing of intron-containing cellular and probably viral mRNAs; promotes export of viral single-exon mRNAs
Retrovirus		
Human immunodeficiency virus type 1	Rev	Mediates export of unspliced and incompletely spliced viral mRNAs

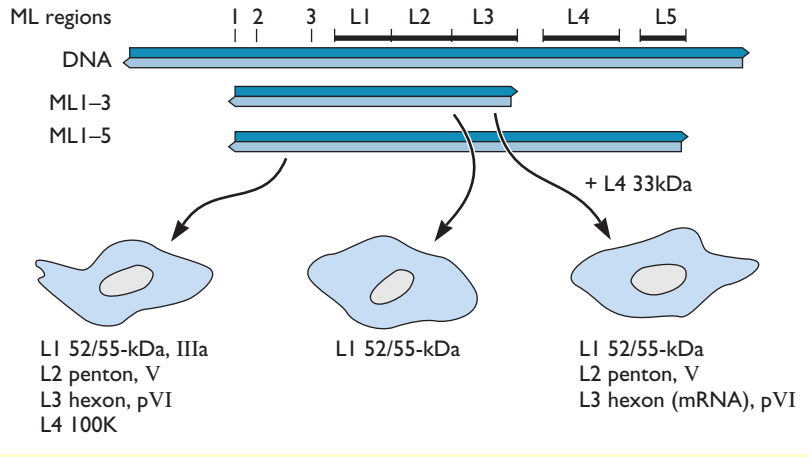
BOX 10.10 EXPERIMENTS

A single adenoviral protein controls the early-to-late switch in major late RNA processing

In efforts to develop cell lines stably producing adenoviral late proteins, plasmids containing various segments of the major late (ML) transcription unit under the control of an inducible promoter were introduced into human cells. The plasmid ML1-5 supported very efficient expression of all the ML coding sequences and synthesis of the full set of the ML proteins. In contrast, only the L1 52/55-kDa protein was synthesized efficiently in cells containing the ML1-3 plasmid, even through the L2 and L3 coding sequences were present (see figure). Examination of the cytoplasmic concentrations of processed ML mRNAs showed that only the L1 52/55-kDa mRNA was made efficiently in cells containing the ML1-3 plasmid, as is also the case during the early phase of infection. These observations implied that one or more viral proteins encoded in the region of the genome present in the ML1-5 but not in the ML1-3 plasmid induce the early-to-late switch in processing of ML pre-mRNA. In fact, synthesis of the L4 33-kDa protein in cells containing the ML1-3 plasmid allowed production of the L1 IIIa and the L2 and L3 proteins. This viral protein significantly stimulated the synthesis of fully processed hexon mRNA, but did not alter the nuclear concentration of ML pre-mRNA. It was therefore concluded that the L4 33-kDa protein is necessary and sufficient to switch processing of the ML pre-RNA from the early to the late pattern.

Farley, D. C., J. L. Brown, and K. N. Leppard. 2004. Activation of the early-late switch in adenovirus type 5 major late transcription unit expression by L4 gene products. *J. Virol.* **78**:1782–1791.

The major late (ML) coding regions (L1-L5) of the Ad5 genome are shown to scale at the top, with the regions in the ML1-3 and ML1-5 plasmids introduced into human cells shown below. 1, 2, and 3 indicate the positions of the three segments of the tripartite leader sequence. The proteins made in cells containing these plasmids, and the ML1-3 plasmid plus a vector directing synthesis of the viral L4 33-kDa protein, are indicated below.



examined (Box 10.10) have established that synthesis of the viral L4 33-kDa protein is essential for the switch to the late pattern of gene expression. It is not yet known whether this protein modulates the activity of Cstf or other components of the polyadenylation machinery. As the L4 33-kDa protein is itself encoded within the major late transcription unit, it is thought that the small quantities produced initially during the late phase activate efficient production of all major late mRNAs.

Some herpesviral late pre-mRNAs contain poly(A) addition signals that function poorly in the absence of the ICP27 protein, at least in part because the downstream U/GU-rich sequences are not optimal. This viral protein appears to stimulate binding of Cstf to such suboptimal sites, and consequently increase the rate of polyadenylation.

Viral Proteins Can Regulate Alternative Splicing

Some viral proteins that regulate pre-mRNA splicing alter the balance among alternative splicing reactions at specific points in the infectious cycle. For example, the ratios of alternatively spliced mRNA products of several adenoviral pre-mRNAs change with the transition into the late phase of infection. This phenomenon has been studied most extensively using the L1 mRNAs. The L1 pre-mRNA, the product of major late transcription and polyadenylation during the early phase of infection, can be spliced at one of two alternative 3′ splice sites (Fig. 10.19). However, only the L1 mRNA that specifies the 52/55-kDa protein is made prior to the onset of viral DNA synthesis. At such early times in the infectious cycle, binding of cellular SR proteins to a negative regulatory sequence located immediately upstream of the branch point for the L1 IIIa mRNA blocks its recognition (Fig. 10.19). A viral early protein encoded within the E4 transcription unit induces dephosphorylation of the SR proteins, which are highly phosphorylated in uninfected cells. Such dephosphorylated SR proteins lose the ability to repress recognition of the 3′ splice site of the L1 IIIa mRNA. Overproduction

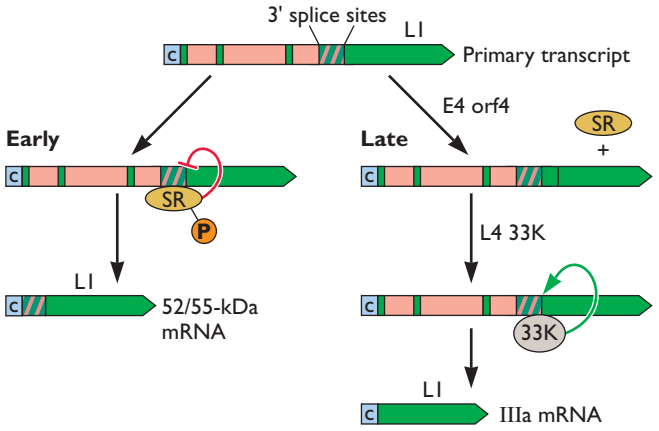


Figure 10.19 Regulation of alternative splicing of adenoviral major late L1 pre-mRNA. The polyadenylated L1 pre-mRNA contains alternative 3′ splice sites, for the 52/55-kDa protein and protein IIIa **(left)**. During the early phase of infection, only the 3′ splice site for the 52/55-kDa protein is utilized, because binding of SR proteins to the pre-mRNA blocks recognition of the 3′ splice site for production of the mRNA for protein IIIa **(right)**. An E4 protein induces dephosphorylation of these cellular proteins by protein phosphatase 2. This modification inhibits binding of the SR proteins to the pre-mRNA. However, efficient utilization of the IIIa mRNA 3′ splice site (during the late phase) requires the viral L4 33-kDa protein, which activates splicing via an infected cell-specific splicing enhancer.

of the SR protein Sf2 in adenovirus-infected cells impairs not only synthesis of the L1 IIIa mRNA, but also the temporal shift in splicing of early E1A and E1B pre-mRNAs, as well as viral replication. This observation indicates that dephosphorylation of cellular SR proteins makes a major contribution to posttranscriptional regulation of adenoviral gene expression. However, other mechanisms are also important. For example, efficient production of the L1 IIIa mRNA depends on a splicing enhancer (Fig. 10.19) that is active only in adenovirus-infected cells (or extracts prepared from them). The viral L4 33-kDa protein stimulates IIIa mRNA production in infected cells, and is sufficient to activate splicing via this enhancer in *in vitro* reactions. This protein also stimulates splicing at other suboptimal 3′ splice sites in major late pre-mRNAs, such as those that produce the L2 mRNAs for proteins V and pre-VII.

Regulation of mRNA Export

Even though all are encoded within a single proviral transcription unit, the regulatory and virion proteins of human immunodeficiency virus type 1 are made sequentially in infected cells, as a result of regulation of mRNA export by the Rev protein. As described previously, this protein regulates a switch in viral gene expression from early production of viral regulatory proteins to a later phase, in which virion components are made (Fig. 10.15).

Viral proteins that modulate mRNA export may play subsidiary, but nonetheless crucial, roles in temporal regulation of viral gene expression. For example, the transcriptional program described in Chapter 8 results in efficient transcription of herpes simplex virus late genes only following initiation of viral DNA synthesis in infected cells. Because all but one of the late mRNAs comprises a single exon, their efficient entry into the cytoplasm and the synthesis of viral late proteins requires ICP27. Consequently, this posttranscriptional regulator is essential for putting the viral transcriptional program into effect. Similarly, as discussed in the previous section, the complete panoply of adenoviral major late gene products can be produced only when the viral L4 33-kDa protein induces the switch to the late pattern of processing of these pre-mRNAs.

Viral Proteins Can Inhibit Cellular mRNA Production

All viral mRNAs are translated by the protein synthesis machinery of the host cell. Inhibition of production of cellular mRNAs can therefore favor this essential step in viral replication. Several mechanisms of selective inhibition of cellular RNA processing operate in virus-infected cells.

Inhibition of Polyadenylation and Splicing

The influenza virus NS1 protein can inhibit both polyadenylation and splicing of cellular pre-mRNAs. This viral protein comprises an N-terminal RNA-binding domain and a C-terminal segment that is required for inhibition of polyadenylation. The C-terminal domain contains binding sites for both Cpsf and PabII (Fig. 10.5). Its interaction with Cpsf inhibits polyadenylation of cellular mRNAs in experimental systems, and NS1 is required for inhibition of cellular mRNA polyadenylation and cytoplasmic accumulation in virus-infected cells. These properties suggest that NS1 could increase the intranuclear concentrations of cellular pre mRNAs, thereby facilitating cap snatching (Chapter 6) and indirectly inhibiting translation of cellular mRNAs. However, cellular protein synthesis is inhibited effectively when NS1 is not made. Under these circumstances, infected cells produce larger quantities of IFN mRNAs and of other cellular mRNAs encoding proteins with antiviral activities. Inhibition of processing of such cellular mRNAs may therefore contribute to the circumvention of host cellular defenses, a critical function of the NS1 protein (see Volume II, Chapter 3).

In addition to its other activities, the herpes simplex virus protein ICP27 inhibits splicing of cellular pre-mRNAs. This protein induces redistribution of spliceosomal snRNPs from a pattern of diffuse speckles in the nucleus to a limited number of sites with a punctate pattern. The viral protein colocalizes with snRNPs at the latter sites, and may bind directly to them. Such abnormal, intranuclear distribution of essential splicing components might contribute

to the inhibition of splicing, but ICP27 also inhibits splicing in *in vitro* reactions. Direct interaction of the viral protein with components of the spliceosome inhibits splicing at an early step in assembly. Genetic analyses have shown that disruption of cellular RNA processing by ICP27 leads to inhibition of cellular protein synthesis. Moreover, this function is genetically separable from the role of the protein in efficient production of viral late mRNAs. Because herpesviral genes generally lack introns, inhibition of splicing is an effective strategy for the selective inhibition of cellular gene expression.

Inhibition of Cellular mRNA Export

To facilitate production of viral mRNAs. In contrast to the other viruses considered in this section, adenovirus infection disrupts cellular gene expression by inhibition of export of cellular mRNAs from the nucleus. During the late phase of infection, the great majority of newly synthesized mRNAs entering the cytoplasm are viral in origin. Synthesis and processing of cellular pre-mRNAs are unaffected, but these mRNAs are not exported and are degraded within the nucleus. Viral mRNAs are exported selectively, a phenomenon that is important for efficient synthesis of late proteins. When selective viral mRNA export is prevented by mutations in the viral genome, both the quantities of late proteins made in infected cells and the virus yield are reduced substantially. These same phenotypes are seen in herpes simplex virus-infected cells synthesizing an altered ICP27 protein that is defective only for the inhibition of pre-mRNA splicing. These properties emphasize the importance of posttranscriptional inhibition of cellular mRNA production for efficient virus reproduction.

The preferential export of late mRNAs in adenovirus-infected cells requires two viral early proteins, the E1B 55-kDa and E4 Orf6 proteins. These proteins are found both free and in association with one another in infected cell nuclei. The complex is responsible for the regulation of mRNA export. Both the E1B and the E4 proteins contain leucine-rich nuclear export signals and can shuttle between the nucleus and cytoplasm. Perhaps surprisingly, such shuttling is dispensable for regulation of mRNA export. The E1B 55-kDa and E4 Orf6 proteins associate with several cellular proteins, including cullin 5 and elongins B and C, to form an infected-cell-specific E3 ubiquitin ligase that targets specific proteins for degradation by the proteasome. The results of recent studies indicate that this activity of the viral proteins is required for regulation of mRNA export. However, the cellular export proteins that might be targeted for proteasomal degradation have yet to be identified.

The selectivity of mRNA export in adenovirus-infected cells is especially puzzling, because the viral mRNAs possess all the characteristic features of cellular mRNAs and are made in the same way. One hypothesis is that the viral early-protein complex recruits nuclear proteins needed for export of mRNA to the specialized sites within the nucleus at which the adenoviral genome is replicated and transcribed. As a result of such sequestration, export of cellular mRNAs would be inhibited.

To block cellular antiviral responses. As we have seen, the genomes of many RNA viruses encode all the enzymes necessary for synthesis and processing of viral mRNAs in the cytoplasm. Infection by some of these viruses results in inhibition of nuclear export. One well-characterized example is inhibition of a major mRNA export pathway in cells infected by the rhabdovirus vesicular stomatitis virus. This effect is mediated by binding of the viral matrix (M) protein to the cellular export protein Rae1. This protein normally shuttles between the nucleus and cytoplasm and binds to both Tap/Nxf1 and a cellular nuclear pore protein. As the M protein is made in the cytoplasm, it is likely to sequester Rae1 in that compartment. While the consequent disruption of cellular mRNA export probably facilitates inhibition of host cell protein synthesis, it also appears to block an important anti-viral defense: expression of the cellular Rae1 and Nup98 genes is induced by interferon, a potent antiviral cytokine (see Volume II, Chapter 3).

Picornaviruses also disrupt trafficking from the nucleus to the cytoplasm. The poliovirus 2A protease induces relocation of particular nuclear proteins to the cytoplasm. Such redistribution correlates with loss of structure from the central channel of the nuclear pore, and cleavage of specific nucleoporins (e.g., Nup153). The small leader (L) protein of encephalomyocarditis virus, a member of the cardiovirus group within the *Picornaviridae*, binds to Ran-GTPase, an essential component of pathways of Ran-dependent nuclear export and import. The phenotypes of mutants with deletions in the L gene suggest that inhibition of Ran dependent trafficking by L protein both tempers the interferon antiviral response, and contributes to inhibition of cellular protein synthesis.

Regulation of Turnover of Viral and Cellular mRNAs in the Cytoplasm

Individual mRNAs may differ in the rate at which they are translated, and also in such properties as cytoplasmic location and stability. Indeed, the intrinsic lifetime of an mRNA can be a critical parameter in the regulation of gene expression.

In the cytoplasm of mammalian cells, the lifetimes of specific mRNAs can differ by as much as 100-fold. This property is described in terms of the time required for 50% of the population of the mRNAs to be degraded under conditions in which replenishment of the cytoplasmic pool is blocked, the **half-life** of the mRNA. Many mRNAs are

very stable, with half-lives exceeding 12 h. As might be anticipated, such mRNAs encode proteins needed in large quantities throughout the lifetimes of all cells, such as structural proteins (e.g., actin) and ribosomal proteins. At the other extreme are very unstable mRNAs with half-lives of less than 30 min. This class includes mRNAs specifying regulatory proteins that are synthesized in a strictly controlled manner in response to cues from external or internal environments of the cell, such as cytokines, and proteins that regulate cell proliferation. The short lifetimes of these mRNAs ensure that synthesis of their products can be shut down effectively once they are no longer needed. Specific sequences that signal the rapid turnover of the mRNAs in which they reside, such as a 50- to 100-nucleotide AU-rich sequence within the 3′ untranslated region, have been identified, as have several mechanisms of mRNA degradation. The majority of mammalian mRNAs are degraded by the pathway summarized in Figure 10.20, in which deadenylation of the 3′ end of the mRNA triggers decapping of the 5′ end by decapping protein 2. The removal of this protective structure renders the RNA susceptible to rapid 5′→3′ exonucleolytic degradation.

The stabilities of viral mRNAs have not been examined in much detail, in part because many viral infectious cycles are completed within the normal range of mRNA half-lives. Nevertheless, it is clear that viral proteins that induce RNA degradation play an important role in selective expression of viral genes in cells infected by large DNA viruses. Regulation of the stability of specific viral or cellular mRNAs has also been implicated in the permanent changes in cell growth properties (transformation) induced by some viruses. Furthermore, RNA-mediated induction of degradation of specific mRNAs, a widespread phenomenon known as RNA interference, is thought to contribute to host antiviral defense mechanisms (see next section).

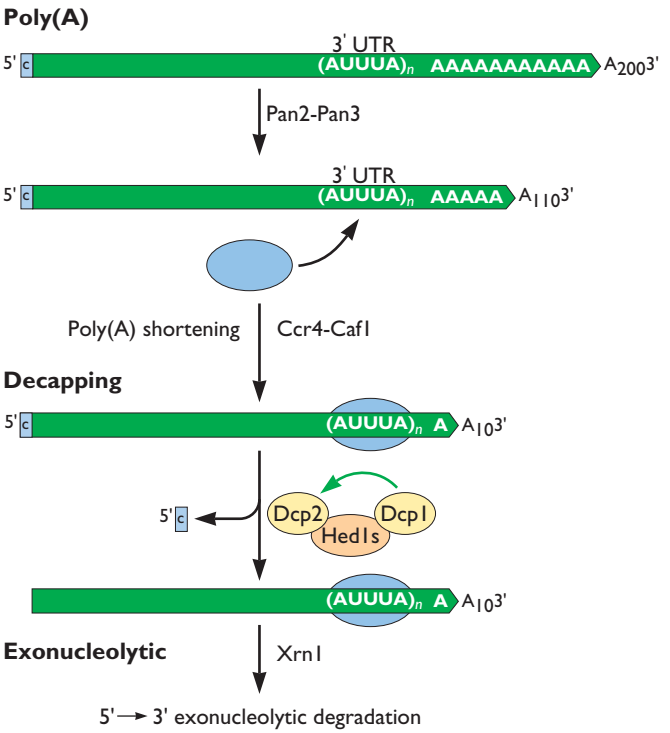


Figure 10.20 Destabilization of cellular mRNAs by 5′AUUUA3′ sequences, repeated in the 3′ untranslated regions (UTR). Such sequences, which are present in short-lived mRNAs encoding cytokines and proteins that regulate cell growth and division, are specifically recognized by one or more proteins. Some proteins stabilize mRNAs upon binding to 5′AUUUA3′ sequences. Binding of others, depicted by the blue oval, appears to induce shortening of the poly(A) tail, in a two-step process catalyzed by different deadenylases, the Pan and Ccr4-Caf1 complexes. Shortening of the poly(A) tail to <110 nucleotides triggers decapping by the enzyme-decapping protein 2 (Dcp2). This reaction is stimulated by Dcp1, which interacts with Dcp2 via the Hedls protein. The exact way in which the decapping enzyme is recruited to target mRNAs, and the contribution of shortening of the poly(A) tail, are not fully understood. The decapped mRNA is then degraded by 5′→3′ exonucleases, such as Xrn1.

Regulation of mRNA Stability by Viral Proteins

The virion host shutoff protein (Vhs) of herpes simplex virus type 1 reduces the stability of mRNAs in infected cells. As its name implies, Vhs is a structural protein of the virion. It is present at low concentrations in the tegument and therefore is delivered to infected cells at the start of the infectious cycle, before viral gene expression begins. It remains in the cytoplasm, where it mediates degradation of some cellular mRNAs. The Vhs protein is an RNase that cleaves mRNA endonucleolytically. The removal of cellular mRNAs at the start of infection facilitates viral gene expression, presumably by reducing or eliminating competition from cellular mRNAs during translation.

The Vhs protein specifically targets mRNAs, probably by virtue of its binding to the translation initiation protein eIF4H, an interaction that stimulates the activity of the viral enzyme. However, Vhs cannot distinguish viral mRNAs from their cellular counterparts. It therefore induces degradation of both viral and cellular mRNAs. Although more Vhs protein is made in infected cells once its coding sequence is expressed during the late phase of infection, the protein is sequestered in the tegument of assembling virus particles by interaction with the viral VP16 protein. As a result, the activity of Vhs decreases as the infection cycle progresses. This mechanism presumably contributes to the efficient synthesis of viral proteins characteristic of the late phase of infection.

The genomes of poxviruses, such as vaccinia virus, also contain the coding sequence for enzymes that induces

degradation of viral and cellular mRNAs. The D10 and D9 proteins are not, however, RNases. Rather, they are decapping enzymes that share a motif with the cellular Dcp2 decapping enzyme. Like their cellular counterpart, the viral enzymes hydrolyze the cap to release ^{m7}GDP. It is clear from the results of genetic experiments that the D10 protein induces rapid turnover of viral and cellular mRNAs, and hence facilitates inhibition of cellular protein synthesis in infected cells. It has been suggested that turnover of viral mRNAs may facilitate the production of specific sets of viral proteins during the successive phases of the infectious cycle, with the D9 and D10 enzymes acting early and late in infection.

Regulation of mRNA Stability in Transformation

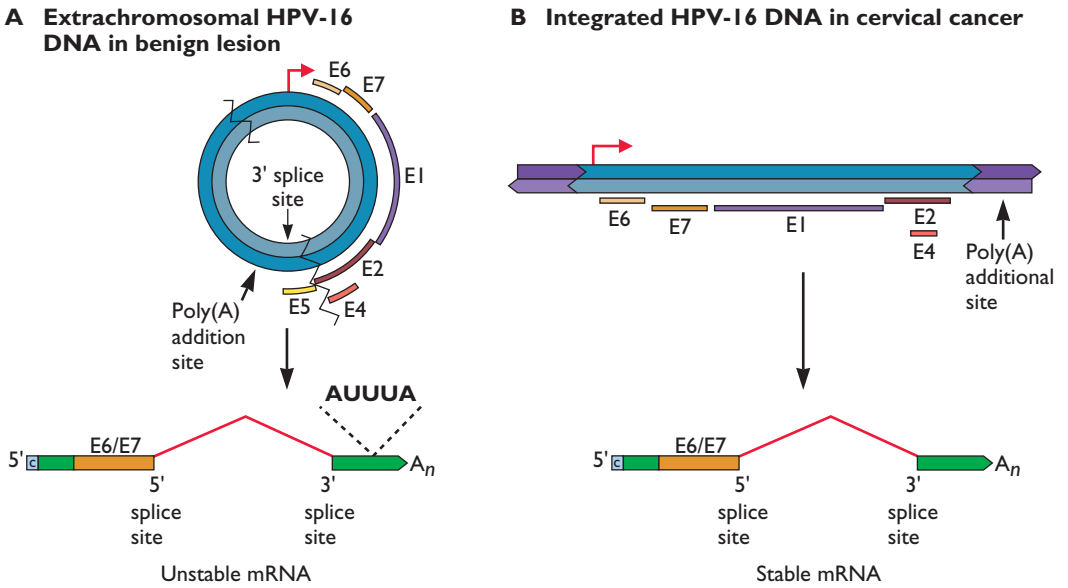
Stabilization of specific viral mRNAs appears to be important in the development of cervical carcinoma associated with infection by high-risk human papillomaviruses, such as types 16 and 18. As discussed in Volume II, Chapter 7, the E6 and E7 proteins of these viruses induce abnormal proliferation of the cells in which they are made. In benign lesions, the circular human papillomavirus genome is not integrated (Fig. 10.21). The E6 and E7 mRNAs that are synthesized from such templates contain destabilizing, AU-rich sequences in their 3′ untranslated regions and possess short lifetimes. This property, as well as repression of transcription from the viral promoter from which the viral pre-mRNAs are transcribed, results in low, steady-state concentrations of the E6 and E7 mRNAs. In cervical carcinoma cells, the viral DNA is integrated into the cellular genome. Such reorganization of viral DNA frequently disrupts the sequences encoding the E6 and E7 mRNAs, such that their 3′ untranslated regions are copied from cellular DNA sequences (Fig. 10.21). These hybrid mRNAs therefore lack the destabilizing AU-rich sequences and are more stable. The increase in the stability of the viral mRNAs encoding the papillomaviral transforming proteins accounts, at least in part, for their higher concentrations in tumor cells.

Production and Function of Small RNAs That Inhibit Gene Expression

Small Interfering RNAs, Micro-RNAs, and Their Synthesis

In the early 1990s, attempts to produce more vividly purple petunias by creation of transgenic plants carrying an additional copy of the gene for the enzyme that makes

Figure 10.21 Stabilization of human papillomavirus type 16 mRNAs upon integration of the viral genome into cellular DNA. (A) In benign lesions, the viral genome is maintained as an extrachromosomal, circular episome. Transcription of such viral DNA and pre-mRNA processing produce various alternatively spliced mRNAs containing the E6 and/or E7 protein-coding sequences, but, as illustrated, all contain destabilizing 5′AUUUA3′ sequences in their 3′ untranslated regions. **(B)** In cervical carcinoma cells, the viral genome is integrated into cellular DNA (purple) such that the viral genome is disrupted upstream of the E6/E7 mRNA 3′ splice site. The mRNAs encoding these viral proteins are therefore made by using 3′ splice and polyadenylation sites transcribed from adjacent cellular DNA, and they lack the destabilizing sequence.



the purple pigment often resulted in white flowers. It is now clear that this seemingly esoteric observation represented the first example of a previously unknown mechanism of posttranscriptional regulation of gene expression, called RNA interference or RNA silencing, that is ancient, and widespread in eukaryotes. Our understanding of the mechanisms and functions of RNA interference, as well as its exploitation as an experimental tool, have advanced at a remarkably rapid pace. Indeed, Andrew Fire and Craig Mello were awarded the Nobel Prize in physiology or medicine in 2006, just 8 years after their groundbreaking study of the mechanism of RNA interference was published.

RNA interference is mediated by small RNA molecules (typically 21 to 28 nucleotides in length) that induce mRNA degradation or inhibit translation. The two main types of these regulatory RNA molecules are distinguished by how they are synthesized. Small interfering RNAs (siRNAs), such as those first discovered in plants, are initially processed by endonucleolytic cleavage of double-stranded RNAs by cytoplasmic dicer enzymes (Fig. 10.22). The double-stranded RNA precursors are formed by base pairing of transcripts that contain complementary sequences, such as the (+) and (−) strand RNAs synthesized in cells infected by many viruses with RNA genomes. Micro-RNAs (miRNAs) are also processed from longer precursors. However, these precursors are capped and polyadenylated transcripts synthesized by RNA polymerase II, in which self-complementary sequences form imperfect hairpin structures (Fig. 10.22). Although the catalog of mammalian miRNAs is far from complete, it is clear that genes encoding them are often clustered. This arrangement allows synthesis of transcripts containing multiple miRNA sequences. Such transcripts are initially processed by endonucleolytic cleavage in the nucleus to liberate pre-miRNAs, imperfect hairpins of 60 to 80 nucleotides. Further processing of pre-miRNAs occurs following export to the cytoplasm, where they are cleaved by dicer enzymes.

In the case of both siRNAs and miRNAs, the products of dicer cleavage are largely double-stranded, with two unpaired bases at the 3′ ends. These RNAs are then unwound from one 5′ end (Box 10.11) and one strand becomes tightly associated with a member of the Argonaute (Ago) family of proteins in the effector ribonucleoprotein, termed the RNA-induced silencing complex, Risc. In these complexes, the small RNA acts as a "guide," identifying the target mRNA by base pairing to specific sequences within it. This complex then induces mRNA cleavage or inhibition of translation, depending in part on the degree of complementarity between the siRNA or miRNA and the mRNA: perfect base pairing usually results in mRNA cleavage. The complement of proteins present in the Risc is also likely to be important. Most organisms that have been examined contain multiple Ago proteins (eight have been identified in humans), and they appear to fulfill different functions. Silencing complexes that contain human Ago2 cleave mRNAs with which the small guide RNA base pairs, but Ago1, Ago3, and Ago4 do not possess such RNase activity.

The introduction of small double-stranded RNAs analogous to the products formed by dicer during production of siRNAs has proved to be a very valuable experimental tool. Such exogenous RNAs are incorporated into Riscs with high efficiency, allowing the experimenter to inhibit expression of particular genes by targeting siRNAs to degrade the corresponding mRNA. However, mammalian cells generally synthesize miRNAs, rather than siRNAs, and the genomes of several DNA viruses contain sequences coding for miRNAs.

Viral Micro-RNAs

The first viral miRNAs were identified in 2004, by cloning and sequencing of small RNAs made in cells latently infected by Epstein-Barr virus. Subsequently, miRNAs of a number of other viruses have been described. Such RNAs are typically identified by combining computational methods that screen viral genomes for sequences with the properties of pre-miRNAs (Fig. 10.22) with assays for detection of viral RNAs, such as hybridization of low-molecular-weight RNAs isolated from infected cells to viral microarrays. Such approaches have identified significant numbers of miRNAs encoded by the genomes of alpha-, beta-, and gammaherpesviruses, as well as by polyomaviral genomes. With the exceptions described below, the functions of these recently recognized viral miRNAs are not known.

Simian Virus 40 miRNAs That Facilitate Immune Modulation

The genome of the polyomavirus simian virus 40 contains the sequence for a single pre-miRNA, which is transcribed as part of the late pre-mRNA (Fig. 10.23). Unusually, both RNA strands produced by processing of this miRNA precursor by the pathway shown in Figure 10.22 are stable, indicating they are both incorporated into effector silencing complexes. The miRNAs are synthesized late in infection, and induce cleavage and degradation of the mRNA for the early-gene product, large T antigen (Fig. 10.23). Mutations designed to disrupt the pre-miRNA secondary structure prevented both viral miRNA synthesis and large-T mRNA degradation, but did not alter the yield of infectious virus. However, the susceptibility of killing of infected cells by cytotoxic T cells specific for large T antigen was reduced significantly. As such cells are important for clearing infected cells from host animals (see Volume II, Chapter 4), this observation suggests that the viral miRNAs facilitate survival of simian virus 40-infected cells in the face of immune surveillance mechanisms.

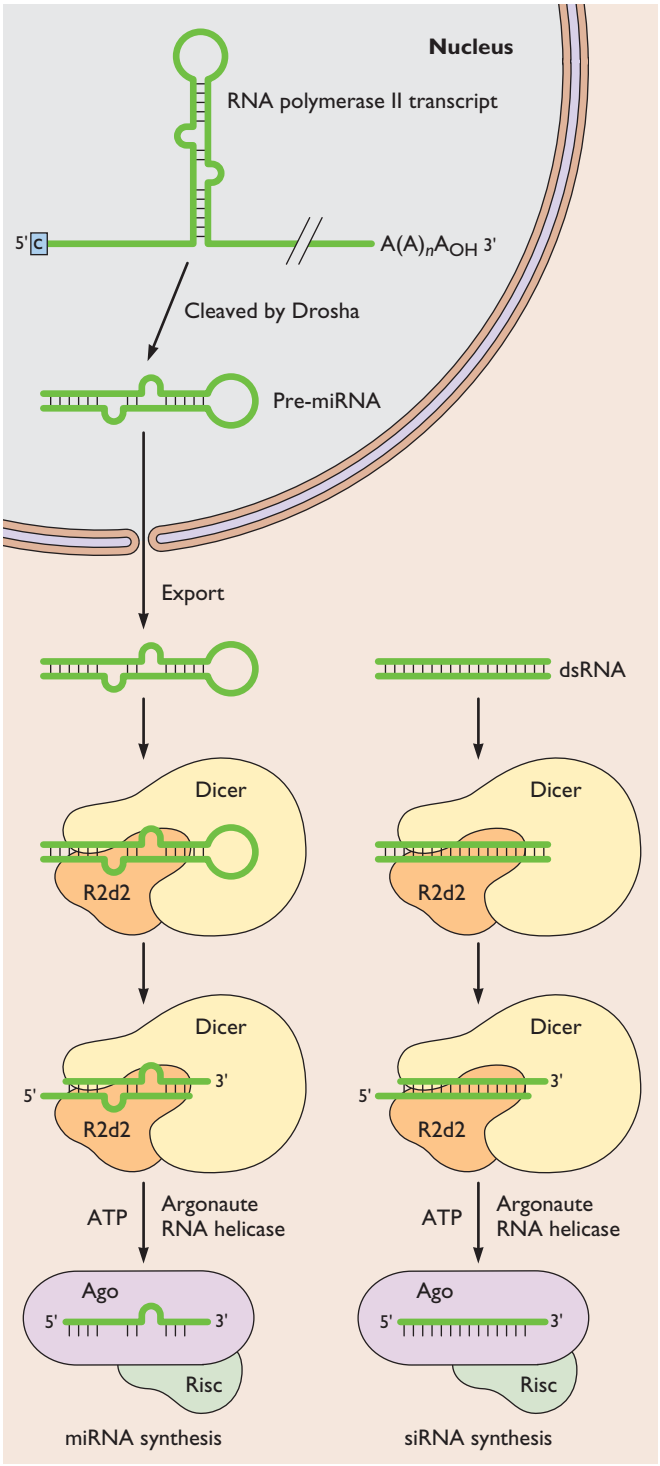


Figure 10.22 Synthesis of siRNAs and miRNAs. As shown, siRNAs are processed from cytoplasmic double-stranded RNAs, whereas the precursors of miRNAs, which are transcripts made by RNA polymerase II, must undergo initial processing in the nucleus. They are then exported to the cytoplasm via the export receptor exportin-5. In both cases, cleavage by Dicer enzymes produces short, largely double-stranded RNAs with unpaired nucleotides at their 3′ ends. Upon unwinding of these duplexes, one RNA strand becomes tightly associated with an Argonaute (Ago) protein (and others) in the RNA-induced silencing complex (Risc). The other strand is degraded.

Latency-Associated miRNAs of Herpesviruses

It is striking that pre-miRNA coding sequences have been identified in regions of several alpha-, beta-, and gammaherpesviral genomes that are expressed in latently infected cells. For example, 12 miRNAs are made in cells latently infected by human herpesvirus type 8. As discussed in Volume II, Chapter 7, this virus is a causative agent of Kaposi's sarcoma and B-cell lymphoma, and the viral gene products made in latently infected transformed cells are of considerable interest. The 12 latency-associated miRNAs are processed from three overlapping transcripts synthesized from viral promoters active in latently infected cells. Indeed, each transcript contains all the pre-miRNA sequences. Ectopic expression of the viral miRNA coding region reduced substantially the concentrations of eight cellular mRNAs for proteins that participate in regulation of proliferation, immune responses and apoptosis. The mRNA encoding thrombospondin 1 is of particular interest, in part because it is a target of several of the latency-associated miRNAs. In addition, an important function of this protein is to block angiogenesis (proliferation of new blood vessels), which is a hallmark of Kaposi's sarcomas.

Viral Gene Products That Block RNA Interference

Recent studies have identified proteins (or RNAs) of a wide range of viruses that counter host cell RNA interference by various mechanisms. Most are RNA-binding proteins that are believed to sequester miRNAs or their precursors. Such viral gene products are discussed in more detail in Volume II, Chapter 3, as they function to subvert an important antiviral defense mechanism.

Perspectives

Many of the molecular processes required for replication of animal viruses, including such virus-specific reactions as synthesis of genomic mRNAs and RNAs from an RNA template, were foretold by the properties of the bacteriophages that parasitize bacterial cells. In contrast, the covalent modifications necessary to produce functional mRNAs in eukaryotic cells were without precedent when discovered in viral systems. Study of the processing of viral RNAs has yielded much fundamental information about the mechanisms of capping, polyadenylation, and splicing. More recently, viral systems have provided equally important insights into export of mRNA from the nucleus to the cytoplasm. Perhaps the most significant lesson learned

BOX 10.11 DISCUSSION

How the guide strand of siRNAs is identified

During formation of RNA-induced silencing complexes, one strand of the double-stranded siRNA, the guide strand, is retained while the second (often called the passenger strand) is destroyed. siRNAs contain many different sequences, raising the question of how guide and passenger strands are distinguished.

The answer came from efforts to identify siRNAs that are most effective in inducing mRNA cleavage when introduced into cells. It was observed that the 5′ end of the guide RNA forms less thermodynamically stable base pairs than the 3′ end. Naturally occurring siRNAs and miRNAs exhibit this same asymmetry. As base pairs at the ends of double-stranded nucleic acids transiently break and re-form (they are said to "breathe"), this sequence might favor recognition of the transiently single-stranded 5′ end of the guide strand. Regardless, the less stable base pairs at the 5′ end of the guide strand favor unwinding from that end, which requires Dicer and the double-stranded RNA-binding protein, R2d2. Subsequent studies demonstrated that R2d2 binds to the siRNA end that contains the most stable base pairs and therefore determines the orientation of the dicer-R2d2 heterodimer on the duplex siRNA.

Khvorava, A., A. Reynolds, and S. D. Juyasena. 2003. Funtional siRNAs and MiRNAs exhibit strand bias. *Cell* **115:**209–216.

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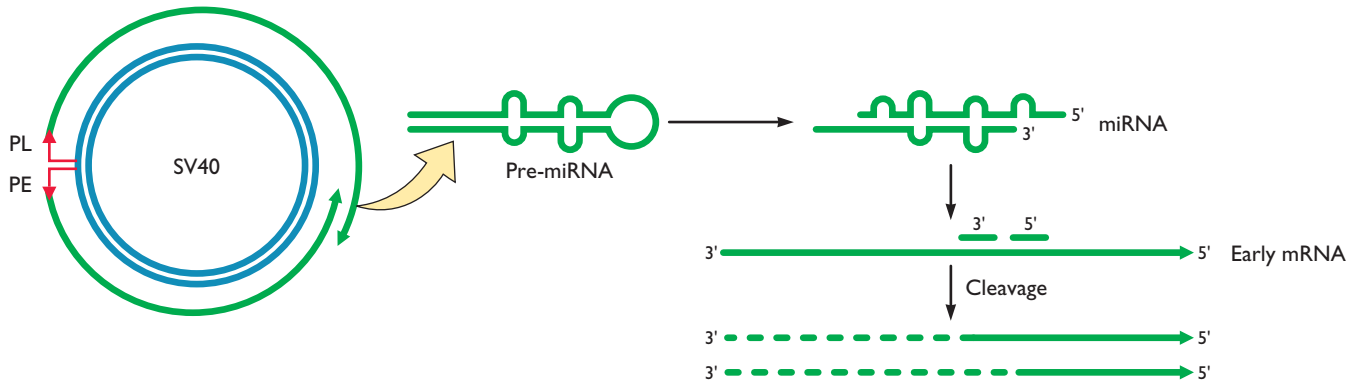
from the study of viral mRNA processing is the importance of these reactions in the regulation of gene expression.

Regulation of viral RNA processing can be the result of differences in the concentrations or activities of specific cellular components in different cell types, or actively induced by viral gene products. Several mechanisms by which viral proteins or RNAs can regulate or inhibit polyadenylation or splicing reactions, export of mRNA from the nucleus, or mRNA stability have been quite well characterized. However, our understanding of regulation of viral gene expression via RNA-processing reactions is far from complete; the mechanisms of action of several crucial viral regulatory proteins have not been fully elucidated, and many of the specific mechanisms deduced by using experimental systems have yet to be confirmed in virus-infected cells. Furthermore, exploration of the physiological functions of viral miRNAs that target viral or cellular mRNAs for degradation (or inhibit translation) began only very recently.

Among the greatest challenges for the future is a full understanding of the recently identified physical and functional couples among the reactions that produce mRNAs in eukaryotic cell nuclei. The intimate relationships among synthesis, capping, polyadenylation, and splicing of pre-mRNAs suggest that the transcription, RNA processing, and export machineries are organized within the nucleus to optimize all reactions in the production of a functional mRNA. Further study of viral mRNA production via these processes seems likely to be as valuable in addressing such complex issues as many viral systems were in the initial elucidation of RNA-processing reactions.

Figure 10.23 The miRNAs of simian virus 40. The circular simian virus 40 genome is shown at the left, with the positions of the early (P_E) and late (P_L) promoters and the primary transcripts indicated. As shown, the 3′ ends of early and late pre-mRNAs are encoded by opposite strands of the same sequence. Downstream of its polyadenylation site (arrowhead), the late pre-RNA contains an pre-miRNA sequence, that is processed to a 57-nucleotide pre-miRNA and then to two miRNAs, designated 3′ and 5′. Both are perfectly complementary to specific sequences in the early mRNAs which encode T antigen and induce its cleavage.



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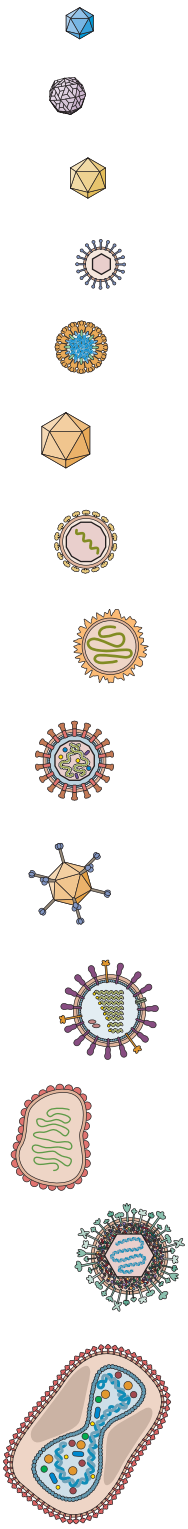

11

Control of Translation

The difficulty lies, not in the new ideas, but in escaping old ones.…
JOHN MAYNARD KEYNES

Introduction

Viruses are obligate intracellular parasites; their replication is dependent on components of the host cell. Some aspects of viral multiplication depend more on cellular contributions than do others. For example, all viral genomes encode at least one protein needed for viral nucleic acid synthesis. However, viruses are dependent on the host cell for the translation of their messenger RNAs (mRNAs) because most viral genomes do not encode any part of the translational machinery (Box 11.1). Viral infection often results in modification of the host's translational apparatus so that viral mRNAs can be translated selectively.

Studies of virus-infected cells have contributed much to our understanding of translation and its regulation. Before the advent of recombinant DNA technology, infected cells were a ready source of large quantities of relatively pure mRNA for *in vitro* studies of protein synthesis. The 5′ cap structure was identified on a viral RNA, and new translation initiation mechanisms, such as internal ribosomal entry, were discovered during studies of infected cells. Our understanding of how the activity of the multisubunit cap-binding complex can be regulated originated from the finding that one of its subunits is cleaved in infected cells.

Translation is a universal process in which proteins are produced from mRNA templates read in the 5′ → 3′ direction, and the growing polypeptide chain is synthesized from the amino to the carboxy terminus. Each amino acid is specified by a genetic code consisting of three bases, a **codon**, in the mRNA. Translation takes place on **ribosomes**, and **transfer RNAs (tRNAs)** are the adapter molecules that link specific amino acids with individual codons in the mRNA. This chapter explores the basic mechanisms by which translation occurs in eukaryotic cells, the many ways that viral mRNAs are translated to confer expanded coding capacity in genomes of limited size, and how translation is regulated in infected cells.

BOX 11.1 DISCUSSION

Viral contributions to the translational machinery

Analysis of the nucleic acid of the largest DNA viruses challenges the belief that no viral genomes encode any part of the translational machinery. The 330- to 380-kbp DNA genome of viruses that infect the unicellular green alga *Chlorella* encode 10 to 15 tRNAs. These viral tRNAs are produced in infected cells, and some of them are aminoacylated, suggesting that they function during protein synthesis. These viral genomes also encode a homolog of elongation protein 3 that is produced in infected cells. The 1,180-kbp DNA genome of *Mimivirus*, the largest known virus, encodes six tRNAs, four aminoacyl tRNA synthetases, release protein eRF1, elongation protein eF-Tu, initiation protein 1, and eIF4E.

These remarkable observations show that parts of the cellular translational machinery can be replaced by viral gene products. Why viral genomes encode such gene products is not known. One possibility is that these genes are relics of an ancestral translation machinery that has been lost during viral evolution. An alternative is that these genes were recently acquired and assist in modifying the translation apparatus to favor the production of viral proteins. For example, the use of viral tRNAs may compensate for the low abundance of some tRNAs in host cells, allowing more efficient replication.

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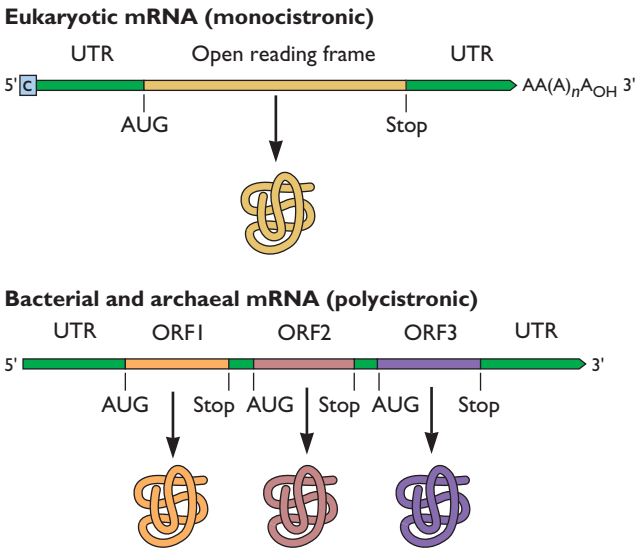
Mechanisms of Eukaryotic Protein Synthesis

General Structure of Eukaryotic mRNA

Most eukaryotic mRNAs, with the exception of organelle and certain viral mRNAs, begin with a 5′ 7-methylguanosine (m^7G) **cap structure** (Fig. 11.1; see also Fig. 10.2). It is joined to the second nucleotide by a 5′-5′ phosphodiester linkage, in contrast to the 5′-3′ bonds found in the remainder of the mRNA. The unique cap structure directs pre-mRNAs to processing and transport pathways, regulates mRNA turnover, and is required for efficient translation by the 5′-end-dependent mechanism. Most eukaryotic mRNAs contain **5′ untranslated regions**, which may vary in length from 3 to more than 1,000 nucleotides, although they are typically 50 to 70 nucleotides in length. Such 5′ untranslated regions often contain secondary structures (e.g., hairpin loops [see Fig. 6.2]) formed by base pairing of the RNA. These double-helical regions must be unwound to allow passage of ribosomal 40S subunits during translation.

Figure 11.1 Structure of eukaryotic and bacterial/archaeal mRNAs. UTR, untranslated region; AUG, initiation codon; ORF, open reading frame; Stop, termination codon. Adapted from G. M. Cooper, *The Cell: a Molecular Approach* (ASM Press, Washington, DC, and Sinauer Associates, Sunderland, MA, 1997), with permission.



Translation begins at an **initiation codon** and ends at a **termination codon**. The termination codon is followed by a **3′ untranslated region**, which can regulate initiation, translation efficiency, and mRNA stability. At the very 3′ end of the mRNA is a stretch of adenylate residues known as the **poly(A) tail**, which is added to nascent pre-mRNA. The poly(A) tail is necessary for efficient translation, and may promote interactions among proteins that bind both ends of the mRNA.

Most bacterial and archaeal mRNAs are **polycistronic**: they encode several proteins, and each open reading frame is separated from the next by an untranslated spacer region. The vast majority of eukaryotic mRNAs are **monocistronic**; i.e., they encode only a single protein (Fig. 11.1). A small number of eukaryotic mRNAs are functionally polycistronic, and different strategies have evolved for synthesizing multiple proteins from a single mRNA. Members of the virus family *Dicistroviridae* are unique because their virions contain true bicistronic mRNAs.

The Translation Machinery

Ribosomes

Mammalian ribosomes, the sites of translation, are composed of two subunits designated according to their sedimentation coefficients, 40S and 60S (Fig. 11.2). The 40S subunit comprises an 18S rRNA molecule and 30 proteins, while the 60S subunit contains three rRNAs (5S, 5.8S, and 28S rRNAs) and 50 proteins. Actively growing mammalian cells may contain approximately 10 million ribosomes.

Remarkably, the catalytic activity of ribosomes is carried out largely by RNA, not protein. After removal of 95% of the ribosomal proteins, the 60S ribosomal subunit can still catalyze the formation of peptide bonds; the peptidyl transferase center, where peptide bonds are formed, contains only RNA. The ribosome is the largest known RNA catalyst. The protein components of ribosomes are thought to help fold the rRNAs properly, so that they can fulfill their catalytic function, and to position the tRNAs.

tRNAs

tRNAs are adapter molecules that align each amino acid with its corresponding codon on the mRNA. Each tRNA is 70 to 80 nucleotides in length and folds into a highly base-paired L-shaped structure (Fig. 11.2B). This shape is thought to be required for the appropriate interaction between tRNA and the ribosome during translation. The adapter function of tRNAs is carried out by two distinct regions of the molecule. At their 3′ ends, all tRNAs have the sequence 5′-CCA-3′, to which amino acids are covalently linked by **aminoacyl-tRNA synthetase**. Each of these enzymes recognizes a single amino acid and the correct tRNA. At the opposite end of the tRNA is the **anticodon loop**, which base pairs with the mRNA template. The accuracy of protein synthesis is maintained by two different proofreading mechanisms: faithful incorporation of amino acids depends on the specificity of codon-anticodon base pairing, as well as on the correct attachment of amino acids to tRNAs by aminoacyl-tRNA synthetases.

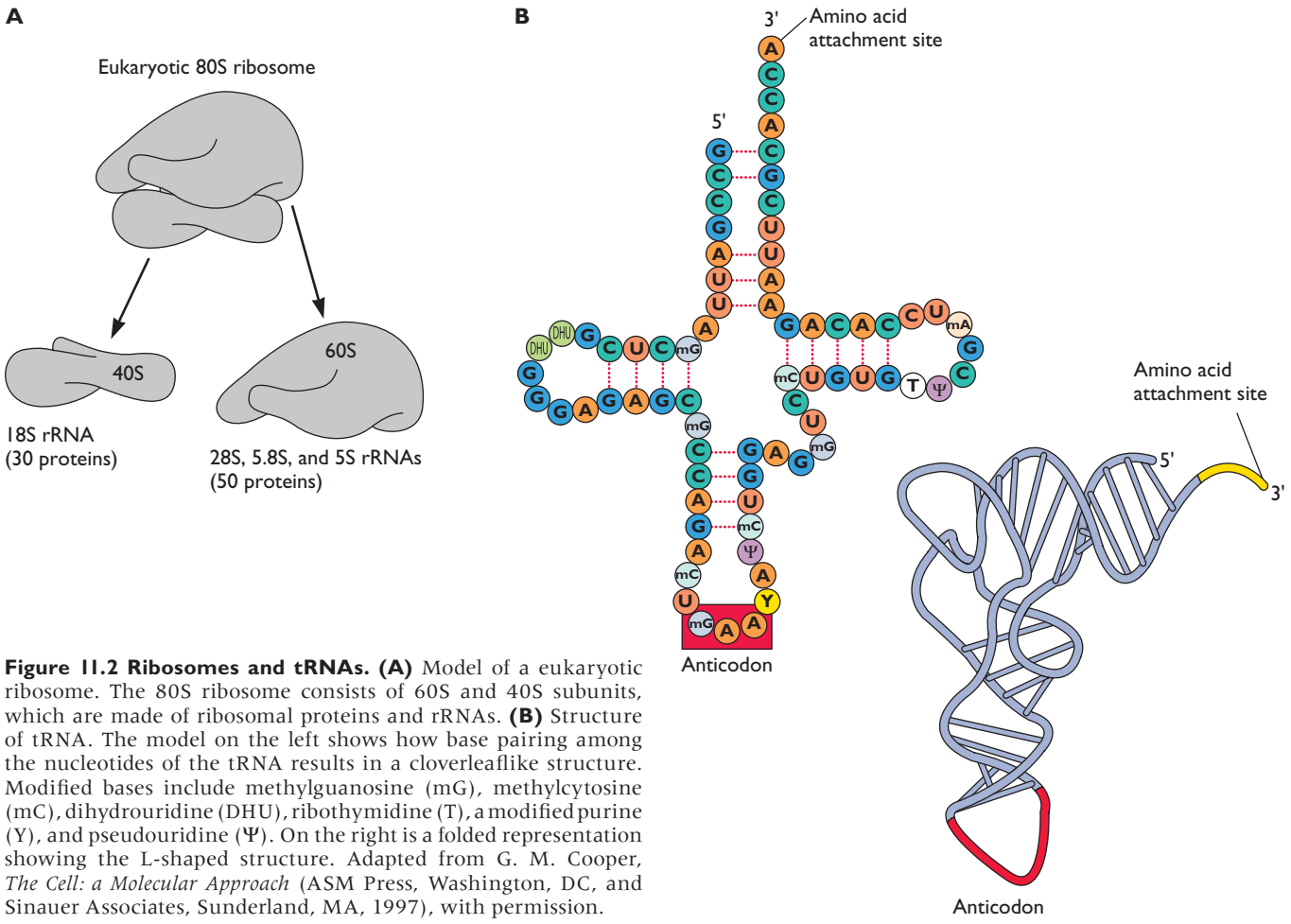


Figure 11.2 Ribosomes and tRNAs. (A) Model of a eukaryotic ribosome. The 80S ribosome consists of 60S and 40S subunits, which are made of ribosomal proteins and rRNAs. **(B)** Structure of tRNA. The model on the left shows how base pairing among the nucleotides of the tRNA results in a cloverleaflike structure. Modified bases include methylguanosine (mG), methylcytosine (mC), dihydrouridine (DHU), ribothymidine (T), a modified purine (Y), and pseudouridine (Ψ). On the right is a folded representation showing the L-shaped structure. Adapted from G. M. Cooper, *The Cell: a Molecular Approach* (ASM Press, Washington, DC, and Sinauer Associates, Sunderland, MA, 1997), with permission.

Table 11.1 Mammalian translation proteins[a]

Name	Subunit	Function
Initiation proteins		
eIF1		Enhances initiation complex formation at the appropriate AUG initiation codon; destabilizes aberrant 48S complexes
eIF1A		Enhances initiation complex formation at the appropriate AUG initiation codon; affects ribosome dissociation and release of 60S subunit; promotes Met-tRNAi binding to 40S, promotes scanning
eIF2		Binds Met-tRNAi, and GTP; associates with eIF3, eIF5
	α	Affects eIF2B binding by phosphorylation
	β	Binds Met-tRNAi; associates with eIF2B, eIF5
	γ	Binds GTP, Met-tRNAi; GTPase
eIF2B		eIF2 recycling
	α	Recognition of P-eIF2
	β	Binds GTP; recognition of P-eIF2
	γ	Binds ATP; recognition of P-eIF2
	δ	Guanine exchange
	ε	Guanine exchange
eIF3		Dissociates 80S ribosomes; promotes Met-tRNAi and mRNA binding to 40S ribosomal subunit
	k	
	j	40S ribosomal subunit, dissociation of mRNA after termination, multifactor complex[b] assembly
	i	
	h	
	g	Binds mRNA, eIF4B
	f	May bind mTor and S6k1[c]
	e	
	d	Binds mRNA
	c	Binds 40S ribosomal subunit; ternary complex; mRNA; AUG recognition; multifactor complex assembly
	b	Binds 40S ribosomal subunit; ternary complex; mRNA; multifactor complex assembly; scanning
	a	Binds 40S ribosomal subunit; eIF4B; ternary complex; mRNA; multifactor complex assembly
eIF4AI		ATPase, RNA helicase
eIF4AII		ATPase, RNA helicase
eIF4B		Binds RNA; promotes mRNA-40S ribosomal subunit interaction; promotes helicase activity of eIF4A
eIF4E		Binds m^7G cap of mRNA
eIF4GI		Binds eIF4E, eIF4A, eIF3, Mnk1, Pab1p, Paip-1, RNA
eIF4GII		Binds eIF4E, eIF4A, eIF3, Mnk1, Pab1p, Paip-1, RNA
eIF4H		Binds RNA; stimulates ATPase and helicase of eIF4A
eIF5		Promotes GTPase activity of eIF2
eIF5B		Ribosome-dependent GTPase; required for 60S ribosomal subunit joining
eIF6		Binds to 60S ribosomal subunit; promotes 80S ribosome dissociation
Elongation proteins		
eEF1A		GTP-dependent binding of aminoacyl-tRNAs; GTPase
eEF1B		Guanine nucleotide exchange on eEF1A
	α	GTP nucleotide exchange activity
	β	GTP nucleotide exchange activity
	γ	Anchors eEF1 to cytoskeleton or membranes
eEF2		GTPase; promotes translocation of peptidyl-tRNA from A site to P site
Termination proteins		
eRF1		Recognizes termination codons; promotes ribosome-catalyzed peptidyl-tRNA hydrolysis; interacts with peptidyl transferase of 60S ribosomal subunit and A site of ribosome
eRF3		GPTase; associates with eRF1 and Pab1p

[a]Modified from N. Sonenberg et al., *Translational Control of Gene Expression* (Cold Spring Harbor Press, Cold Spring Harbor, NY, 2000).
[b]multifactor complex comprises eIF3, eIF1, eIF5, and ternary complex.
[c]S6k1, ribosomal protein S6 kinase.

Translation Proteins

Many nonribosomal proteins are required for eukaryotic translation (Table 11.1). Some form multisubunit complexes containing as many as 11 different proteins, while others function as monomers. Translation can be separated experimentally into three distinct stages: initiation, elongation, and termination. The proteins that participate at each stage are named eukaryotic initiation, elongation, and termination proteins. These proteins are designated in the same way as their bacterial and archaeal counterparts, with the prefix "e" to distinguish them. The amino acid sequences of these proteins are conserved from yeasts to mammals, indicating that the mechanisms of translation are similar throughout eukaryotes.

Initiation

The majority of regulatory mechanisms function during initiation because this is the rate-limiting step in the translation of most mRNAs (see "Regulation of Translation during Viral Infection" below). At least 11 initiation proteins participate in this energy-dependent process. The end result is formation of a complex containing the mRNA, the ribosome, and the initiator Met-tRNA$_i$ in which the reading frame of the mRNA has been set. The 80S ribosome, which is the predominant species in cells, must be dissociated because it is the 40S subunit that participates in initiation. Three initiation proteins, eIF1A, eIF3, and eIF6 (Table 11.1), promote such dissociation.

There are two mechanisms by which ribosomes bind to mRNA in eukaryotes. In 5′-end-dependent initiation, by which the majority of mRNAs are translated, the initiation complex binds to the 5′ cap structure and moves, or scans, in a 3′ direction until the initiating AUG codon is encountered. In 5′-end-dependent initiation, the initiation complex binds at, or just upstream of, the initiation codon. Internal ribosome entry sites were first discovered in picornavirus mRNAs, and are now known to be present in some cellular mRNAs.

5′-End-Dependent Initiation

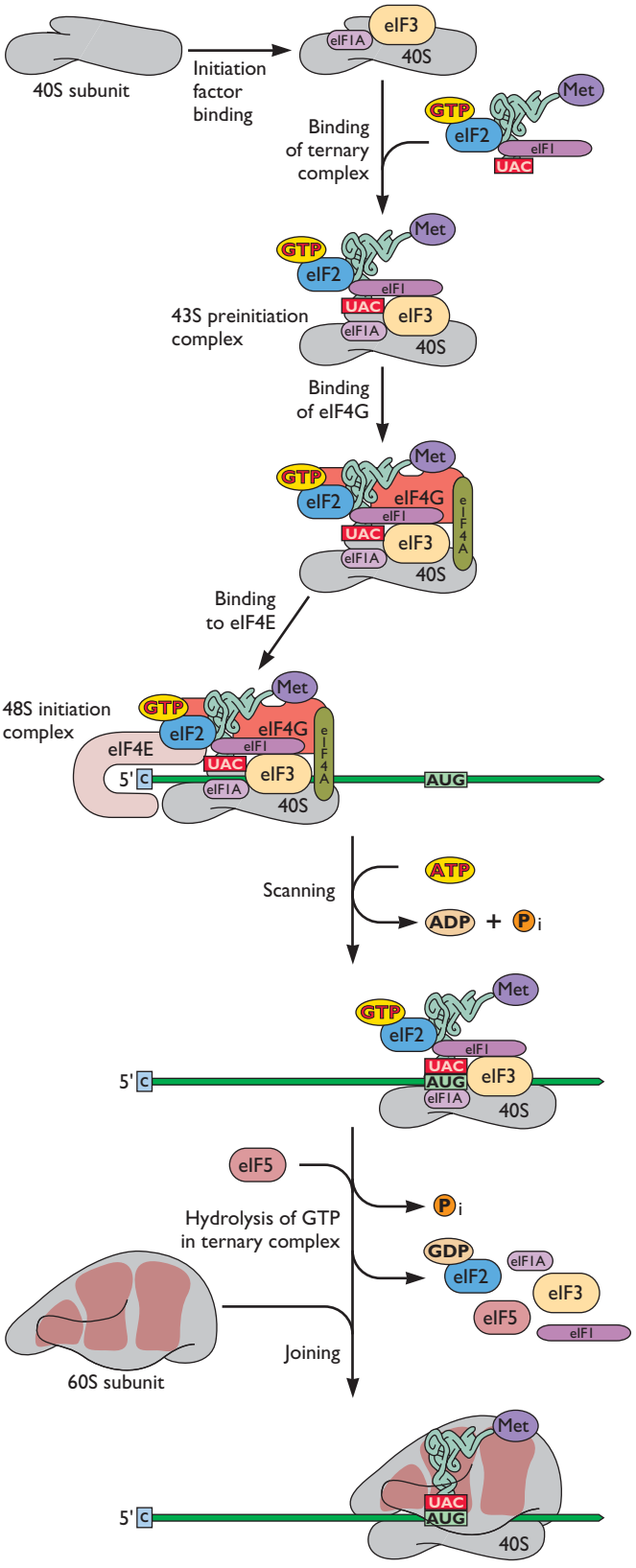
How ribosomes assemble at the proper end of mRNA. The first step in the 5′-end-dependent initiation pathway is recognition of the m^{7}G cap by the cap-binding protein, eIF4E (Fig. 11.3). eIF4G acts as a scaffold between the cap structure and the 40S subunit, which associates with the mRNA via an interaction of eIF3 with the C-terminal domain of eIF4G. This important adapter molecule was first discovered as the target of proteolytic cleavage in poliovirus-infected cells that results in the abrupt termination of host protein synthesis. The ability of eIF4G to bind RNA is also important for recruitment of the 40S ribosomal subunit. After binding near the cap, the 40S ribosomal subunit moves in a 3′ direction on the mRNA in a process called **scanning**. When the 40S subunit reaches the AUG initiation codon, GTP is hydrolyzed and initiation proteins are released, allowing the 60S ribosomal subunit to associate with the 40S subunit, forming the 80S initiation complex.

Role of the poly(A) tail in initiation. The presence of a poly(A) tail can stimulate mRNA translation. This surprising effect is a consequence of interactions between proteins associated with the 5′ and 3′ ends of the mRNA, promoting 40S subunit recruitment. Such interactions were first demonstrated in the yeast *Saccharomyces cerevisiae*, in which poly(A)-tail-binding protein, Pab1p, is required for efficient mRNA translation. Stimulation of translation by poly(A), which requires Pab1p, occurs by enhancing the binding of 40S ribosomal subunits to mRNA. Pab1p interacts with the N terminus of eIF4G (Fig. 11.4). Alteration of the Pab1p-binding site on eIF4G destroys stimulation of translation by poly(A). These results have led to a model in which Pab1p, bound to the poly(A) tail, interacts with eIF4G bound to the 5′ cap, perhaps stabilizing the interaction and assisting in recruitment of 40S subunits (Fig. 11.4). A consequence of these interactions is that the 5′ and 3′ ends of the mRNA are brought into close proximity.

Many viral mRNAs, such as the mRNA of barley yellow dwarf luteovirus, lack a 5′-terminal cap and 3′ poly(A) sequence. Nevertheless, the ends of these mRNAs are brought together by base pairing between discrete sequences in the 5′ and 3′ untranslated regions. Translation of mRNA of the flavivirus dengue virus, which has a 5′ cap structure but lacks a 3′ poly(A) sequence, may also depend on complementarity between the untranslated regions.

The juxtaposition of mRNA ends might be a mechanism to ensure that only intact mRNAs that contain 5′ cap and poly(A) are translated. Such structures could also stabilize mRNA by preserving the interaction among the translation initiation proteins associated with both ends. Translation reinitiation might also be stimulated by such an arrangement: once the ribosome terminates translation, it might be rapidly repositioned at the AUG initiation codon rather than dissociating into the two subunits.

VPg-dependent ribosomal recruitment. The 40S ribosomal subunit appears to be brought to the mRNAs of members of the *Potyviridae* and the *Caliciviridae* via interactions with VPg, the small protein linked to the first base of the RNA (Fig. 6.11). VPg of the plant virus turnip mosaic virus binds eIF4E, thereby recruiting eIF4G, eIF3, and the 40S ribosomal subunit to the mRNA. In cells infected with members of the *Caliciviridae*, VPg binds both eIF4E and eIF3. Such interactions may also facilitate selective translation of viral mRNAs over cellular mRNAs, although the mechanisms involved have not been elucidated.



The role of mRNA secondary structure in translation. Translation efficiency is reduced by the presence of a stable secondary structure in the mRNA 5′ untranslated region, particularly when the structure is near the 5′ terminus. There are at least two reasons for this effect. If RNA secondary structure is adjacent to the 5′ cap, it can inhibit binding of the 40S ribosomal subunit. In addition, the presence of secondary structure blocks ribosome movement toward the initiation codon.

The ATP-dependent RNA helicase activity of eIF4A is enhanced by eIF4B and eIF4H (Fig. 11.3). Such activity is thought to unwind regions of double-stranded RNA (dsRNA) near the 5′ end of the mRNA, allowing the 43S preinitiation complex to bind. The helicase may also migrate in a 3′ direction, unwinding dsRNA and enabling ribosomes to scan. mRNAs with less secondary structure in the 5′ untranslated region have a reduced requirement for RNA helicase activity during mRNA translation and hence are less dependent on the cap structure through which the helicase is brought to the mRNA. Dependence of translation on the cap can be measured experimentally by determining the effect on protein synthesis of cap analogs such as m^7GDP and m^7GTP. These compounds competitively inhibit 5′-end-dependent initiation by binding to eIF4E and preventing it from associating with capped mRNAs. For example, the 5′ untranslated region of alfalfa mosaic virus RNA segment 4 is largely free of secondary structure, and translation of this mRNA is quite resistant to inhibition by cap analogs.

Choosing the initiation codon. For over 90% of mRNAs, translation initiation occurs at the 5′-proximal AUG codon. If the 5′-proximal AUG codon is mutated

Figure 11.3 5′-cap-dependent assembly of the initiation complex. Initiation proteins eIF3 and eIF1A bind to free 40S subunits to prevent their association with the 60S subunit, while interaction of eIF6 (not shown) with the larger subunit prevents it from associating with the 40S subunit. eIF4F, which consists of three proteins—eIF4A, eIF4E, and eIF4G—binds the cap via the eIF4E subunit, and the ribosome binds a ternary complex containing eIF2, GTP, and Met-tRNA$_i$, forming a 43S preinitiation complex. The ribosome then binds eIF4G via eIF3. Alternatively, eIF4G may first join the 43S preinitiation complex and then bind the mRNA via eIF4E bound to the cap. The 40S subunit then scans down the mRNA until the AUG initiation codon is reached. eIF1 and eIF1A are required for selection of the correct AUG initiation codon. eIF5 triggers GTP hydrolysis, eIF2 bound to GDP is released along with other initiation proteins, and the 60S ribosomal subunit joins the complex. Adapted from G. M. Cooper, *The Cell: a Molecular Approach* (ASM Press, Washington, DC, and Sinauer Associates, Sunderland, MA, 1997), with permission.

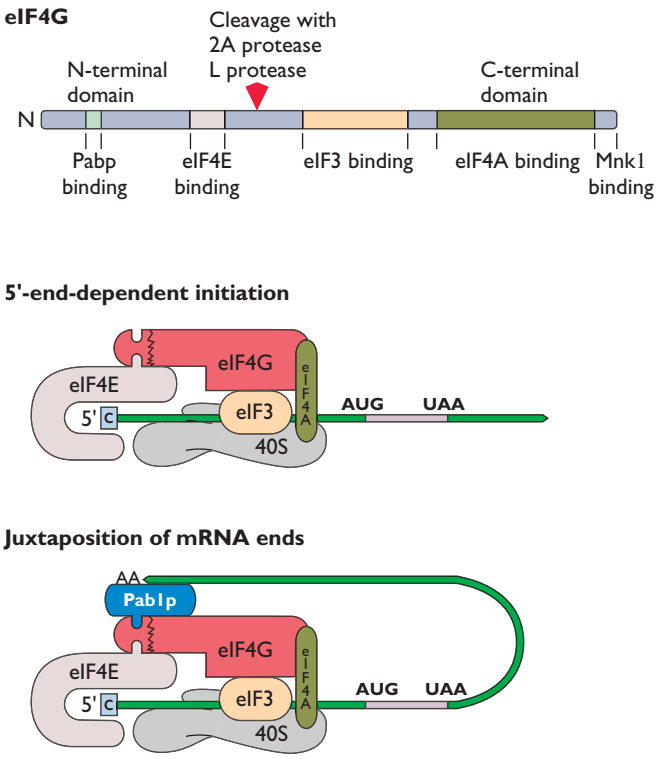


Figure 11.4 5′-end-dependent initiation. (Top) Schematic of eIF4G. Adapted from S. J. Morley et al., *RNA* **3:**1085–1104, 1997, with permission. **(Middle)** Model of initiation complex assembly. eIF4F is brought to the mRNA 5′ end by interaction of eIF4E with the cap structure. The N terminus of eIF4G binds eIF4E, and the C terminus binds eIF4A. The 40S ribosomal subunit binds to eIF4G indirectly via eIF3. **(Bottom)** 5′-end-dependent initiation is stimulated by the poly(A)-binding protein Pab1p, which interacts with eIF4G. This interaction may bring the mRNA ends together and facilitate formation of the initiation complex at the 5′ end. Adapted from M. W. Hentze, *Science* **275:**500–501, 1997, with permission.

so that it cannot serve as an initiation codon, translation starts at the next downstream AUG. Insertion of an AUG codon upstream of the initiating codon causes initiation at the more 5′-proximal site. The efficiency of initiation is influenced by the nucleotide sequence surrounding this codon. Studies of the effects of mutating these sequences have shown that the consensus sequence 5′-GCC**A**C-CAUGG-3′ is recognized most efficiently in mammalian cells: the presence of a purine at the –3 position (boldface) is most important for high levels of translation. However, only 5% of eukaryotic mRNAs contain this ideal consensus sequence; most have suboptimal sequences that result in less efficient translation. This finding indicates that not all mRNAs must be translated maximally, but rather only at levels appropriate for the function of the protein product. If a very poor match to this consensus sequence is present, the AUG codon may be passed over by the ribosome and initiation may occur further downstream (see "The Diversity of Viral Translation Strategies" below).

The results of genetic and biochemical experiments demonstrate that eIF1 plays a role in discrimination during selection of the initiation codon. In the absence of eIF1, the 43S preinitiation complex is unable to discriminate between AUG and AUU or GUG codons. Certain alterations of eIF1 of yeasts enhance initiation at a UUG triplet. eIF1 might influence codon-anticodon pairing, either directly or by modulating the conformation of the 43S complex.

Although AUG is the initiation codon for most proteins, synthesis may also begin at ACG, GUG, and CUG codons in viral and cellular mRNAs, although far less frequently. In all cases, the first amino acid of the protein is a methionine. Precisely how ribosomes recognize non-AUG codons so that methionine is inserted is not known. Because the efficiency of initiation at these sites is always low, non-AUG initiation might be another mechanism for regulating translation efficiency. Not all mRNAs support initiation from a non-AUG site, suggesting that there may be structural elements in the mRNA that regulate translation of such a codon.

Methionine-independent initiation. The structural proteins of some viruses begin not with methionine but with glutamine (CAA), proline (CCU), or alanine (GCU or GCA). Initiation of synthesis of these viral proteins does not require initiator tRNA methionine or the ternary complex because the viral mRNA mimics the structure of tRNA (Fig. 11.5A). The tRNA-like structure occupies the P site of the ribosome, allowing initiation to take place within the A site. These mRNAs require no translation initiation proteins, and can bind ribosomes and induce them to enter the elongation phase of translation. Methionine-independent initiation of the mRNA of turnip yellow mosaic virus is accomplished in a similar way, except that the tRNA-like structure is located in the 3′ untranslated region of the viral RNA (Fig. 11.5B). The tRNA-like structure is aminoacylated with valine, which is incorporated as the first amino acid of the viral polyprotein.

Does the 40S subunit move on the mRNA? Translation of most mRNAs begins at the 5′-proximal AUG codon. How does the ribosome reach this location? In one popular model, the 40S ribosomal subunit migrates along the 5′ untranslated region until it reaches the AUG initiation codon. Experimental proof for this long-standing hypothesis is still lacking. Hydrolysis of ATP is necessary for movement of the 43S complex on RNA containing weak

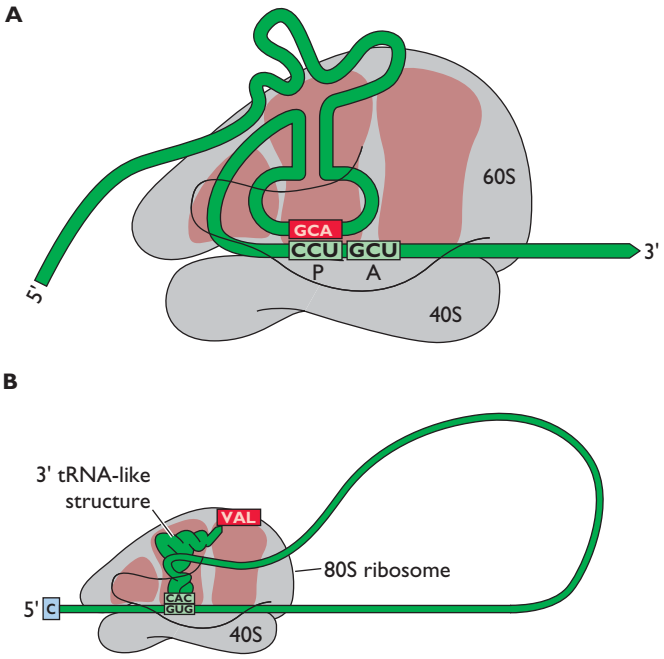


Figure 11.5 Two mechanisms of methionine-independent initiation. (A) The viral mRNA of picornavirus-like viruses of insects mimics the structure of tRNA, which occupies the P site of the ribosome, allowing initiation to take place within the A site. Adapted from M. Bushell and P. Sarnow, *J. Cell Biol.* **158:**395–399, 2002, with permission. **(B)** A tRNA-like structure in the 3′ untranslated region of turnip yellow mosaic virus RNA, aminoacylated with valine, occupies the P site of the ribosome. Adapted from S. Barends et al., *Cell* **112:**123–129, 2003, with permission.

secondary structures. Movement of ribosomes on RNA lacking secondary structure does not require ATP hydrolysis. However, energy is required by the ATP-dependent RNA helicase, not for ribosome movement. In an alternative model, the ribosome does not travel to the initiation codon, but remains at the 5′ cap. The mRNA is threaded through the ribosome, eventually bringing the initiation codon to the P site.

Ribosome shunting. Stable RNA secondary structures in 5′ untranslated regions may inhibit scanning of 40S ribosomes. In some RNAs, such hairpin structures are not inhibitory because ribosomes bypass them. This process, called **ribosome shunting**, may be dependent or independent of viral proteins. Shunting on the 35S cauliflower mosaic virus RNA requires translation of the very short upstream open reading frame on the same viral RNA. In contrast, shunting on adenovirus major late mRNAs occurs in the absence of viral proteins, although its efficiency is increased by the viral L4 100-kDa polypeptide. Translation of cellular inhibitor of apoptosis 2 is also mediated by

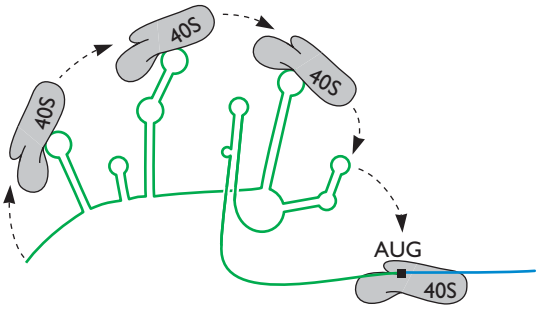


Figure 11.6 Hypothetical model of ribosome shunting. The 40S ribosomal subunit binds to the mRNA by a cap-dependent mechanism and then bypasses large regions of the mRNA with secondary structure to reach the AUG initiation codon. Shunting elements, such as the loops in the figure, and viral or cell proteins may direct ribosome movement.

a ribosome shunt. The mechanism of ribosome shunting remains to be elucidated (Fig. 11.6).

5′-End-Independent Initiation

The internal ribosome entry site. The mRNAs of picornaviruses differ from most host cell mRNAs. They lack the 5′-terminal cap structure, and the 5′ untranslated regions are highly structured and contain multiple AUG codons. Infection of host cells by many picornaviruses results in the inhibition of host cell translation. These observations led to the hypothesis that translation of the mRNA of (+) strand picornaviruses was initiated by an unusual mechanism. It was suggested that the ribosome bound internally, rather than at the mRNA 5′ end. In a key experiment, the 5′ untranslated region of poliovirus mRNA was shown to promote internal binding of the 40S ribosomal subunit, and was termed the **internal ribosome entry site (IRES)** (Box 11.2).

An IRES has been identified in the mRNAs of all picornaviruses, in other viral mRNAs including those of pestiviruses and hepatitis C virus, and in some cellular mRNAs. There is very little nucleotide sequence conservation among these IRESs, with the exception of an oligopyrimidine tract 25 nucleotides upstream of the 3′ end of the IRES. Viral IRESs contain extensive regions of RNA secondary structure (Fig. 11.7). Although such secondary structure is not strictly conserved, it is of extreme importance for ribosome binding. Viral IRESs have been placed in five groups, depending on a variety of criteria, including primary sequence and secondary structure conservation, the location of the initiation codon, and activity in different cell types.

The discovery of the IRES makes even more puzzling the rarity of eukaryotic mRNAs that contain several open reading frames reminiscent of bacterial and archaeal mRNAs (Fig. 11.1). In principle, all the open reading frames of a

BOX 11.2 EXPERIMENTS

Key experiment: discovery of the IRES

The hypothesis that poliovirus mRNA is translated by internal ribosome binding was first tested by examining the translation of mRNAs containing two open reading frames (ORFs) separated by the poliovirus 5′ untranslated region (figure, panel A). These bicistronic mRNAs directed the synthesis of two proteins, but the second ORF was efficiently translated only if it was preceded by the picornavirus 5′ untranslated region. It was concluded that ribosomes bind within the viral 5′ untranslated region, thereby permitting translation of the second ORF. The segment of the 5′ untranslated region that directs internal ribosome entry was called the IRES.

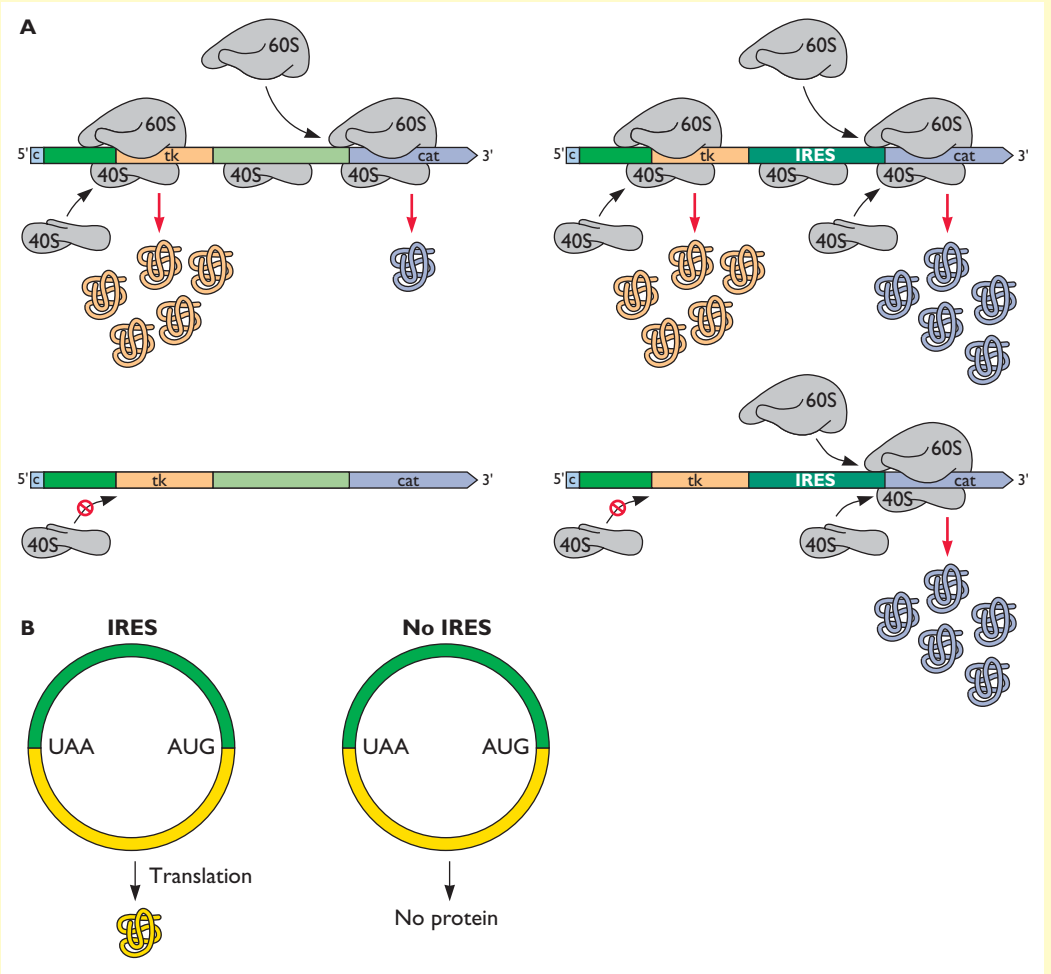
It had long been known that covalently closed circular mRNAs cannot be translated by 5′-end-dependent initiation. Translation by internal ribosome binding, however, should not require a free 5′ end. To test this hypothesis, circular mRNAs with and without an IRES were created. The circular mRNA was translated only if an IRES was present (Figure, panel B). This experiment formally proved that translation initiation directed by an IRES occurs by internal binding of ribosomes and does not require a free 5′ end.

Chen, C. Y., and P. Sarnow. 1995. Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs. *Science* **268:**415–417.

Jang, S. K., H. G. Kräusslich, M. J. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer. 1988. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *J. Virol.* **62:**2636–2643.

Pelletier, J., and N. Sonenberg. 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* **334:**320–325.

Assays for an IRES. (A) Bicistronic mRNA assay. Plasmids were constructed that encode bicistronic mRNAs encoding the thymidine kinase (tk) and chloramphenicol acetyltransferase (cat) proteins separated by a spacer (light green) or a poliovirus IRES (dark green). Plasmids were introduced into mammalian cells by transformation. In uninfected cells containing either plasmid (top lines), both tk and cat proteins were detected, although without an IRES, cat synthesis was inefficient. Translation of cat from this plasmid probably occurs by reinitiation. In poliovirus-infected cells, 5′-end-dependent initiation is blocked (stop sign), and no proteins are observed without an IRES. cat protein is detected in infected cells when the IRES is present, demonstrating internal ribosome binding. Adapted from J. Pelletier and N. Sonenberg, *Nature* **344:**320–325, 1988, with permission. **(B)** Circular mRNA assay for an IRES. Circular mRNAs containing an ORF (yellow) were produced and translated *in vitro*. No protein product was observed unless an IRES was included in the circular mRNA. Adapted from C. Y. Chen and P. Sarnow, *Science* **268:**415–417, 1995, with permission.



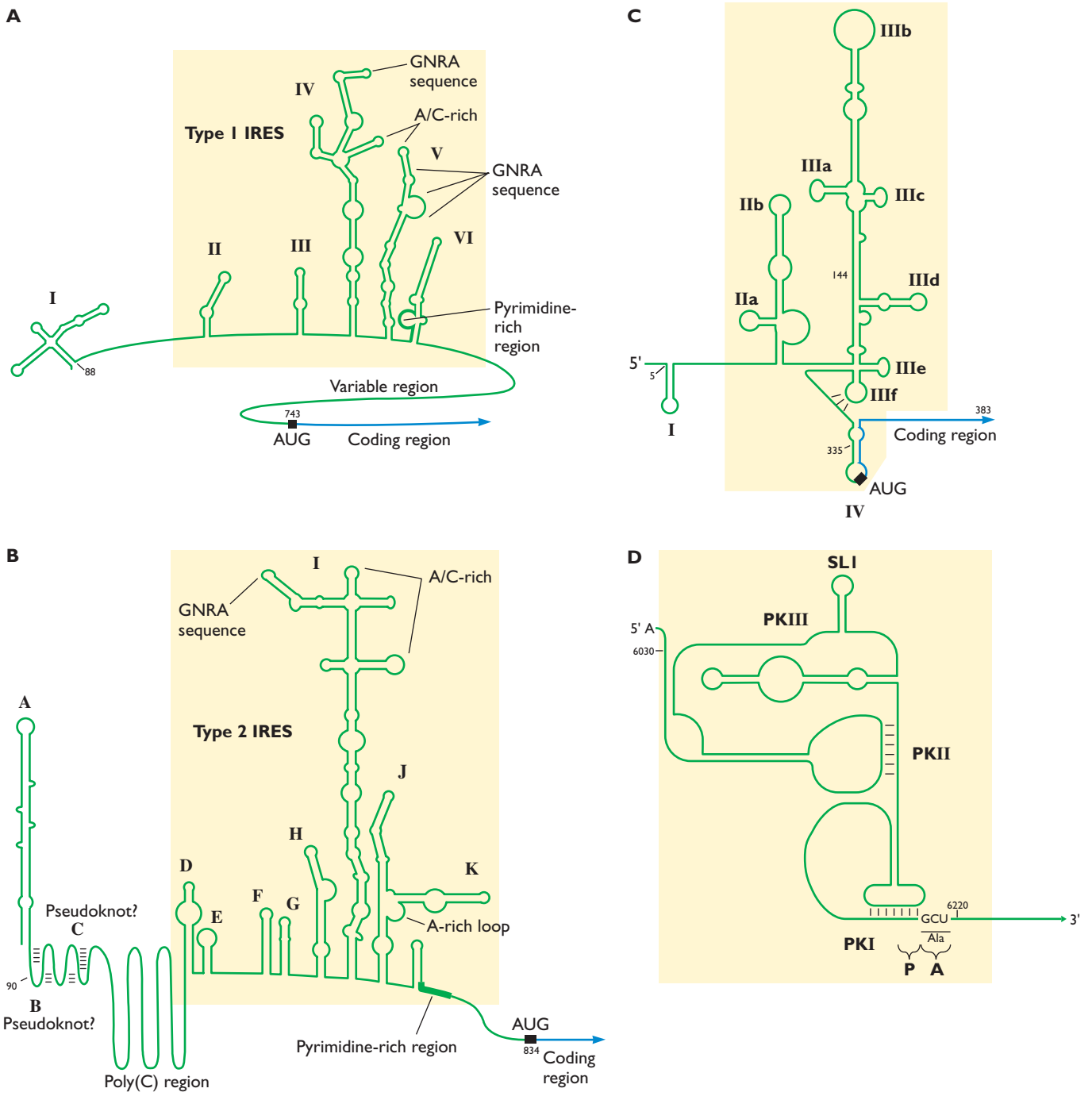


Figure 11.7 Four types of IRES. The 5′ untranslated regions from poliovirus **(A)**, encephalomyocarditis virus **(B)**, hepatitis C virus **(C)**, and cricket paralysis virus **(D)** are shown. The IRES in panels A and B is indicated by yellow shading. Predicted secondary and tertiary RNA structures (RNA pseudoknots) are shown. The poliovirus IRES is a type 1 IRES, which is found in the genomes of enteroviruses and rhinoviruses. The ribosome probably enters the IRES at domains V and VI and scans to the AUG initiation codon, which is located 50 to 100 nucleotides past the 3′ end of the IRES. The type 2 IRES is found in the genomes of aphthoviruses and cardioviruses. The 3′ end of the hepatitis C virus IRES (type 4) extends beyond the AUG initiation codon (black box). The IRES of picornavirus-related viruses of insects (type 5), such as cricket paralysis virus, mimics a tRNA and occupies the P site in the 40S ribosomal subunit. Translation initiates with a non-AUG codon from the A site. THE IRES ends at the initiating codon. A sixth class of IRES element has been identified in the viral RNA of teschoviruses, picornaviruses that infect pigs. These IRESs have similarities to that of hepatitis C virus. The initiation codon of the IRES of hepatitis A virus (type 3, not shown) is located 50 to 100 nucleotides past the 3′ end of the IRES. (A and B) Adapted from S. R. Stewart and B. L. Semler, *Semin. Virol.* **8:**242–255, 1997, with permission. (C) Adapted from S. M. Lemon and M. Honda, *Semin. Virol.* **8:**274–288, 1997, with permission. (D) Adapted from E. Jan and P. Sarnow, *J. Mol. Biol.* **324:**889–902, 2002, with permission.

polycistronic mRNA can be translated in a eukaryotic cell as long as each frame is preceded by an IRES. Nevertheless, only one such naturally occurring polycistronic mRNA has been identified in eukaryotes, and it is not known if an IRES is present. Bicistronic mRNAs produced in the laboratory have been used in the expression and cloning of genes (Box 11.3).

The mechanism of internal initiation. Different sets of translation initiation proteins are required for the function of various IRESs. Internal ribosome binding on the hepatitis A virus IRES requires all of the initiation proteins, including eIF4E. At the other extreme, the intergenic IRES of cricket paralysis virus requires **none** of the translation initiation proteins. However, the

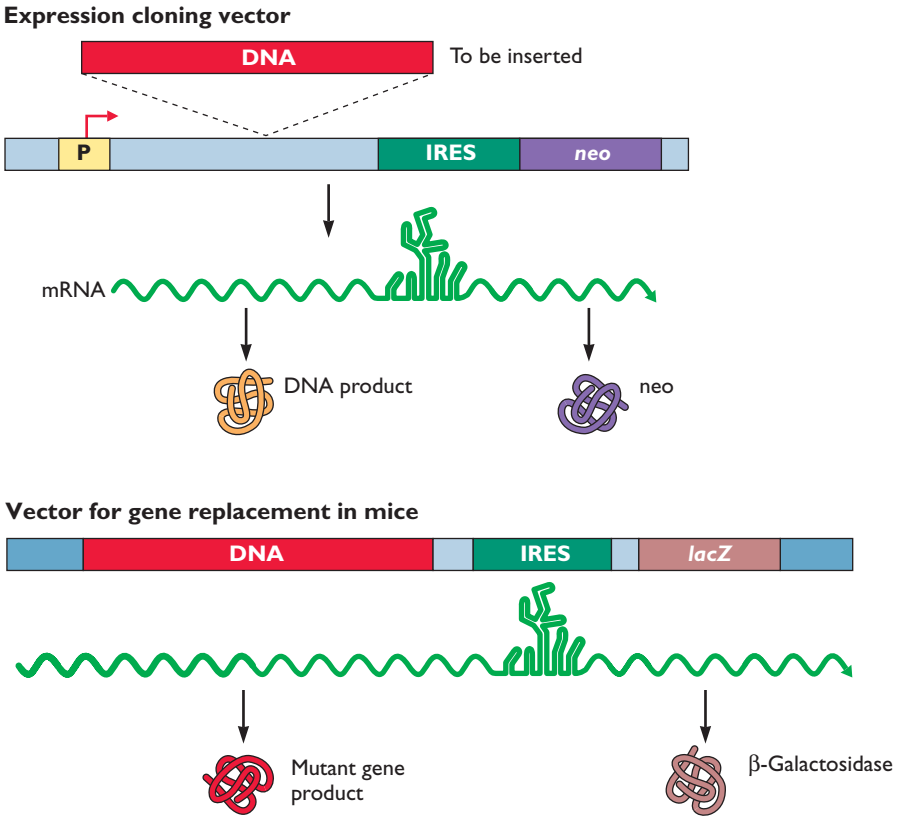
BOX 11.3 BACKGROUND

Use of the IRES in cloning and expression vectors

The IRES has been used widely in the expression and cloning of foreign genes in eukaryotes. One strategy for the expression of genes in mammalian cells is to produce mRNAs in the cytoplasm by using a bacteriophage DNA-dependent RNA polymerase, such as T7 RNA polymerase. Such mRNAs are poorly translated because they are not capped; inclusion of an IRES in the 5′ untranslated region allows them to be translated efficiently.

Another application of the IRES is in the functional cloning of new genes (figure, top panel). A DNA library is made by using a cloning vector that produces a bicistronic mRNA encoding both the desired gene and a selectable marker. The use of a selectable marker on the same mRNA increases the efficiency of screening because most transformants express both genes.

IRESs have also been used in the isolation of mutant mice by homologous recombination in embryonic stem cells. Bicistronic vectors have been designed to produce mRNA encoding the altered protein and β-galactosidase, separated by an IRES (figure, bottom panel). Because β-galactosidase is encoded on the same mRNA as the targeted gene product, it serves as a marker for mRNA expression.



(Top) Design of plasmids for expression cloning. The expression library is created by inserting DNA into a site on the plasmid downstream of a promoter. The foreign DNA is followed by an IRES linked to a selectable marker such as neomycin resistance (neo). The mRNA produced from this plasmid DNA encodes the cloned DNA product and the protein conferring neomycin resistance. The library is introduced into cells that are then screened for expression of the desired gene. **(Bottom)** Vector for gene replacement in mice. In this example, the goal is to replace the gene with a mutant version. The targeting plasmid consists of mutant DNA followed by an IRES and the *lacZ* gene. The flanking light blue bars represent sequences from the mouse gene that mediate homologous recombination. After replacement of the endogenous gene with this synthetic version, mRNA that encodes the mutant gene product as well as the β-galactosidase protein will be produced. The latter can be detected in tissues by staining with the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

activity of most IRESs require a subset of translation initiation proteins.

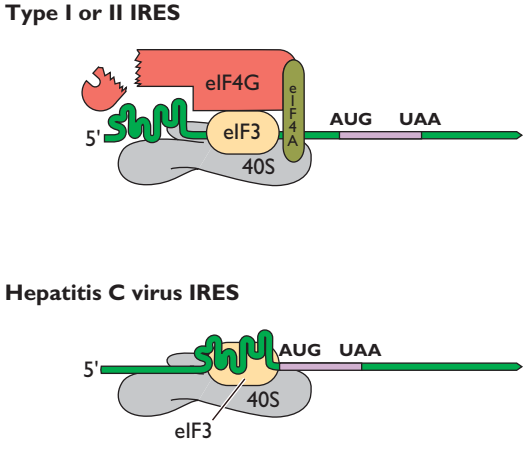
Translation initiation via a type 1 IRES comprises binding of the 40S ribosomal subunit to the IRES, followed by scanning of the subunit to the initiation codon. The 40S subunit may bind directly to the RNA or could be recruited to the IRES by means of interaction with translation initiation proteins (Fig. 11.8). In poliovirus-infected cells, the scaffold eIF4G is cleaved, reducing the translation of most cellular mRNAs. It was therefore assumed that this initiation protein was not required for function of the poliovirus IRES. However, the C-terminal fragment of eIF4G, which contains binding sites for eIF3 and eIF4A (Fig. 11.8), stimulates translation directed by the poliovirus IRES. These results have led to a model in which the 40S ribosomal subunit is recruited to the IRES via interaction with eIF3 bound to the C-terminal domain of eIF4G (Fig. 11.8). It has been reported that IRES function is markedly enhanced in cells in which the poliovirus protease $2A^{pro}$ is synthesized. Because $2A^{pro}$ is responsible for cleavage of eIF4G, such stimulation of translation may be due to production of the C-terminal proteolytic fragment of eIF4G.

The IRESs of hepatitis C virus (Fig. 11.7C), pestiviruses such as bovine viral diarrhea virus and classical swine fever virus, and teschoviruses function very differently from those of other picornaviruses. The formation of the 48S initiation complex on the mRNA is independent of eIF4A, eIF4B, and eIF4F and is highly sensitive to secondary RNA structure around and downstream of the AUG initiation codon. Purified 40S ribosomal subunits bind directly to stem-loop IIId of the hepatitis C virus IRES, and single point mutations in this structure abolish the interaction and block internal initiation. Addition of only Met-$tRNA_i$, eIF2, and GTP is required to form 48S complexes. A dramatic conformational change in the 40S ribosomal subunit occurs when it binds the hepatitis C virus IRES (Fig. 11.9), clamping the mRNA in place and setting the AUG initiation codon within the P site of the ribosome. The IRES also contacts the E site of the ribosome, where the deacylated tRNA is harbored after translocation of the 80S ribosome. Initiation of translation from the IRES of hepatitis C virus and related viruses therefore resembles translation initiation of bacterial mRNAs.

The intergenic IRESs of picornavirus-like viruses of insects form complexes with the 40S ribosome independent of initiation proteins, and translation begins at other than an AUG codon. Initiation from these IRESs is uniquely inhibited by ternary complex (Met-$tRNA_i$–eIF2–GTP) and a high concentration of Met-$tRNA_i$. In cells infected with these viruses, recycling of eIF2-GDP is blocked and the concentration of ternary complex is low, inhibiting cellular mRNA translation, but the activity of the intergenic IRES is not reduced. The secondary structure of the IRES of these viruses mimics an uncharged tRNA, and mutations that destabilize the fold abrogate translation. The tRNA-like structure is recognized and bound by the 40S ribosomal subunit, placing the initiation codon within the A site instead of the P site (Fig. 11.5A). Initiation is therefore dependent on elongation proteins eEF1A and eEF2 and the appropriate aminoacylated tRNAs. Like the IRESs of hepatitis C virus and pestiviruses, IRESs of the picornavirus-like viruses of insects also occupy the E site of the 80S ribosome.

As discussed above, translation of cellular mRNAs is enhanced by the juxtaposition of mRNA ends (Fig. 11.4). Translation of viral mRNAs by internal initiation is also stimulated by this arrangement, which may be established in at least two ways. The 5′ and 3′ ends of the RNA genome of foot-and-mouth disease virus are brought together by RNA-RNA interactions. The mRNA of hepatitis C virus is not polyadenylated and therefore cannot bind Pab1p. The interaction between molecules of polypyrimidine-tract-binding protein that bind both the viral 3′ untranslated region and the IRES may bring together the 5′ and 3′ ends of the mRNA.

Figure 11.8 5′-end-independent initiation. (Top) Initiation in the type I or II IRES does not depend on the presence of a cap structure but requires the C-terminal fragment of eIF4G to recruit the 40S ribosomal subunit via its interaction with eIF3. eIF4G probably binds directly to the IRES. **(Bottom)** The ribosomal 40S subunit binds to the hepatitis C virus IRES without the need for translation initiation proteins. eIF3 also binds the IRES and is thought to be necessary for recruitment of the 60S ribosomal subunit.



Other host cell proteins that contribute to IRES function. The poliovirus IRES functions poorly in reticulocyte lysates, in which most capped mRNAs are translated efficiently (Box 11.4). Reticulocytes are immature red

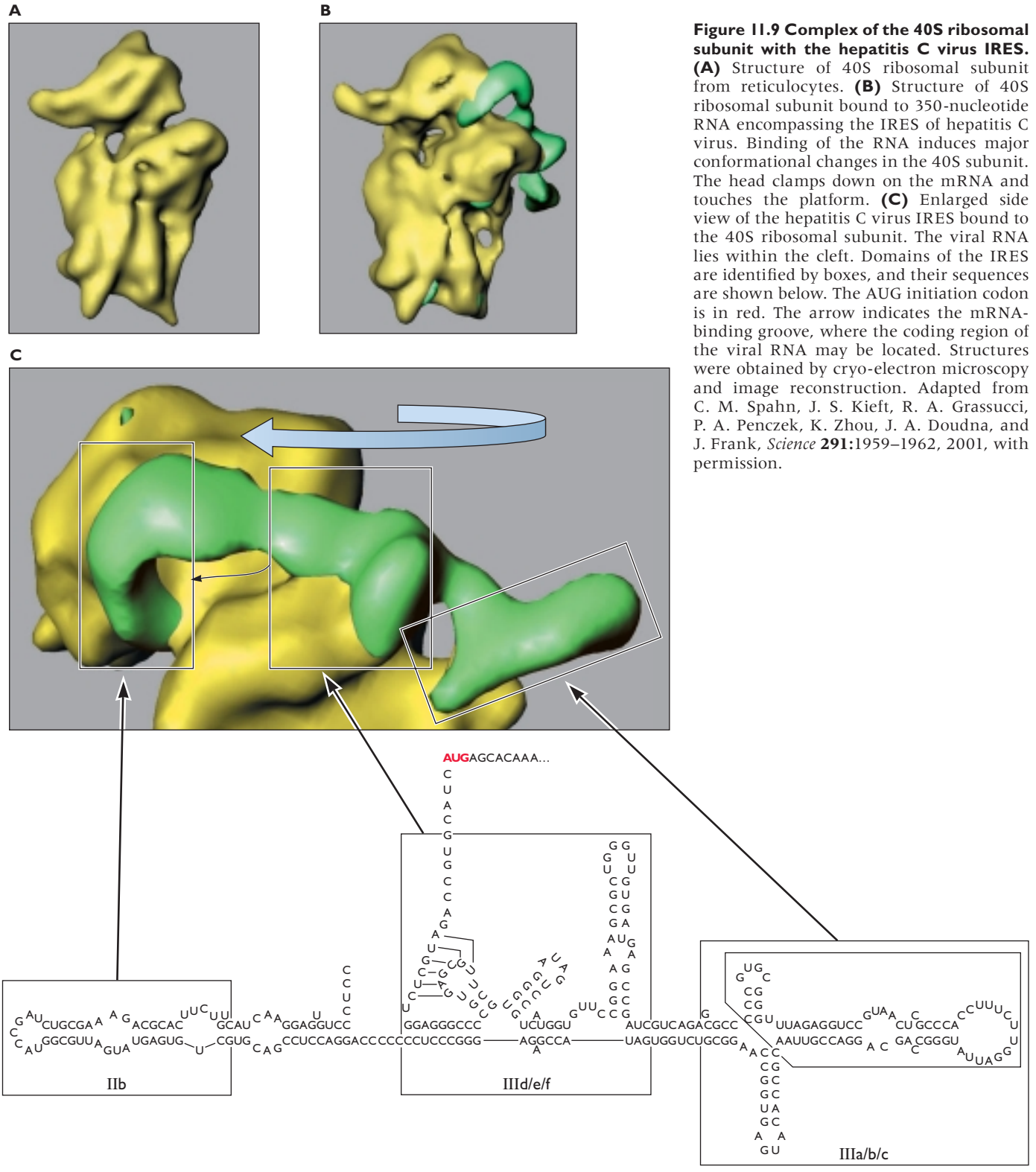


Figure 11.9 Complex of the 40S ribosomal subunit with the hepatitis C virus IRES. **(A)** Structure of 40S ribosomal subunit from reticulocytes. **(B)** Structure of 40S ribosomal subunit bound to 350-nucleotide RNA encompassing the IRES of hepatitis C virus. Binding of the RNA induces major conformational changes in the 40S subunit. The head clamps down on the mRNA and touches the platform. **(C)** Enlarged side view of the hepatitis C virus IRES bound to the 40S ribosomal subunit. The viral RNA lies within the cleft. Domains of the IRES are identified by boxes, and their sequences are shown below. The AUG initiation codon is in red. The arrow indicates the mRNA-binding groove, where the coding region of the viral RNA may be located. Structures were obtained by cryo-electron microscopy and image reconstruction. Adapted from C. M. Spahn, J. S. Kieft, R. A. Grassucci, P. A. Penczek, K. Zhou, J. A. Doudna, and J. Frank, *Science* **291:**1959–1962, 2001, with permission.

BOX 11.4 DISCUSSION

Translation in vitro: *the reticulocyte lysate and wheat germ extract*

Our present understanding of the fundamentals of translation initiation, elongation, and termination, as well as viral translation strategies, would not be possible without the technique of in vitro translation in cell extracts. In this method, cells are lysed and the nuclei are removed by centrifugation. The mRNA is added to the lysate, and the mixture is incubated in the presence of an isotopically labeled amino acid which is incorporated into the translation product.

The ideal extract for *in vitro* translation has two important properties: high translation efficiency and low protein synthesis in the absence of added mRNA. By the early 1970s, cell extracts prepared from Krebs II ascites tumor cells or rabbit reticulocytes were found to translate protein with high efficiency, but the presence of endogenous mRNAs that were also translated complicated the analysis of proteins made from added mRNA. In 1973 a cell extract from commercial wheat germ that had low background levels of protein synthesis, and in which exogenous mRNAs were translated very efficiently, was developed. A few years later, the background in a reticulocyte lysate was eliminated by treatment with micrococcal nuclease, which destroyed the endogenous mRNA. This nuclease requires calcium for its activity, and it was therefore a simple matter of adding a calcium chelator, EGTA [ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], to the reaction to prevent the degradation of exogenously added mRNA.

Wheat germ extract and reticulocyte lysate are still widely used in translation studies, because the cells are abundant and inexpensive and are excellent sources of initiation proteins. Micrococcal nuclease followed by calcium chelation has been successfully used to make mRNA-dependent extracts from many mammalian cell types, although the translation efficiency of such systems does not approach that of wheat germ or reticulocyte lysates. Unfortunately, it has not been possible to prepare consistently translation extracts from normal mammalian tissues, which has hampered the study of tissue-specific translation regulation in virus-infected and uninfected cells.

Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* **67:**247–256.

Roberts, B. E., and B. M. Patterson. 1973. Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. *Proc. Natl. Acad. Sci. USA* **70:**2330–2334.

blood cells that primarily produce hemoglobin; they lack nuclei. Addition of a cytoplasmic extract to reticulocyte lysates restores efficient translation from this IRES. These observations led to the suggestion that ribosome binding to the IRES requires more than translation initiation proteins. Such proteins have been identified by their ability to bind to the IRES and to restore its function in the reticulocyte lysate. One host protein identified by this approach is the La protein, which binds to the 3′ end of the poliovirus IRES. This protein is associated with the 3′ termini of newly synthesized small RNAs, including all nascent transcripts of cellular RNA polymerase III. While predominantly nuclear, La protein is localized to the cytoplasm in poliovirus-infected cells. It is present in low concentrations in reticulocyte lysates, and when added to them it stimulates the function of the poliovirus IRES. Furthermore, depletion of La mRNA by RNA interference reduced poliovirus IRES-dependent translation.

Polypyrimidine-tract-binding protein (Ptb), also called heterogeneous nuclear ribonucleoprotein I (hnRnpI), was also found to bind the poliovirus IRES. This predominantly nuclear protein, a negative regulator of alternative pre-mRNA splicing, is redistributed to the cytoplasm during poliovirus infection. Polypyrimidine-tract-binding protein binds to sequences upstream of the pyrimidine-rich sequence of the poliovirus IRES, and to both the 5′ and 3′ untranslated regions of hepatitis C virus RNA. If this protein is removed from a cell extract, the activity of the poliovirus and hepatitis C virus IRESs is eliminated. However, neither IRES activity is restored when purified Ptb is added to the depleted extracts. These findings suggest that another protein(s) associated with polypyrimidine-tract-binding protein might also be required for IRES function.

Another cellular protein necessary for the activity of the enterovirus and rhinovirus IRES is the cytoplasmic RNA-binding protein, poly(rC)-binding protein 2, originally identified by its ability to bind stem-loop IV of the poliovirus IRES (Fig. 11.7A). Mutations in the poliovirus 5′ untranslated region that abolish binding of poly(rC)-binding protein cause decreased translation *in vitro*. Depletion of poly(rC)-binding proteins from human translation extracts inhibits translation dependent on the IRESs of poliovirus, coxsackievirus B, and rhinovirus, but not on those of encephalomyocarditis virus or foot-and-mouth disease virus. Translation activity of the IRESs was restored by addition of purified poly(rC)-binding protein 2. This protein binds to, and functions cooperatively during internal initiation with, SRp20, a protein that is essential for constituitive splicing and regulation of alternative splice site utilization. Cleavage of poly(rC)-binding protein 2 is thought to enable a switch from translation to replication during poliovirus infection (Chapter 6).

No single host cell protein that is essential for the function of all viral IRESs has been identified. Many of these

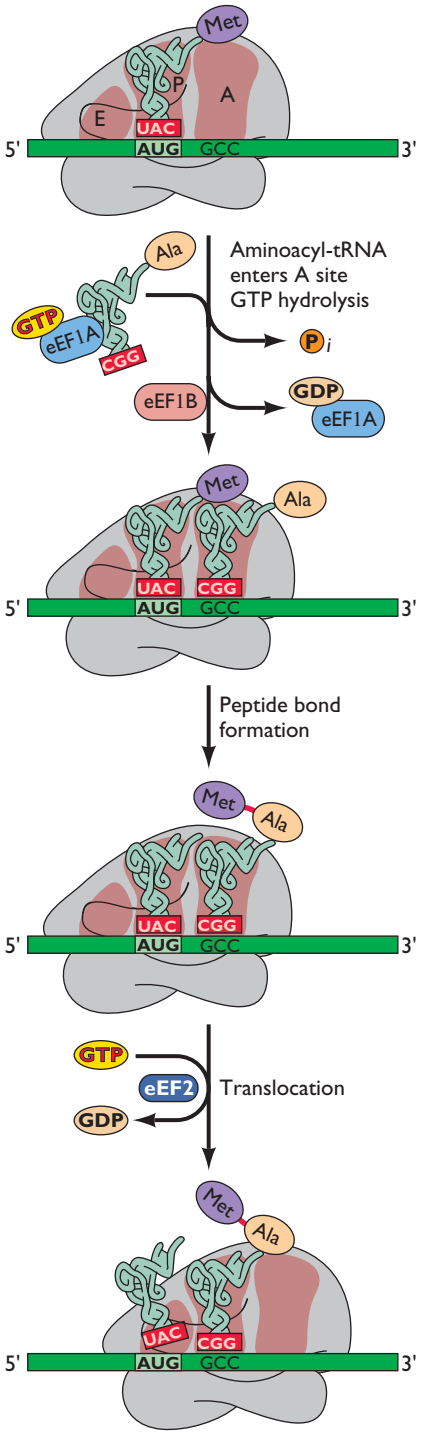


Figure 11.10 Translation elongation. There are three tRNA-binding sites on the ribosome, called peptidyl (P), aminoacyl (A), and exit (E). After the initiating Met-tRNA$_i$ is positioned in the P site, the second aminoacyl-tRNA (alanyl-tRNA is shown) is brought to the A site by eEF1A bound to GTP. After GTP hydrolysis, eEF1A is released. The guanine nucleotide exchange protein eEF1B exchanges GDP of eEF1A-GDP with GTP, allowing eEF1A to interact with a tRNA synthetase and bind a newly aminoacylated tRNA. The peptide bond is then formed; this is followed by movement of the ribosome three nucleotides along the mRNA, a reaction that requires GTP hydrolysis and eEF2. The peptidyl (Met-Ala) tRNA moves to the P site, and the uncharged tRNA moves to the E site. The A site is now empty, ready for another aminoacyl-tRNA. Adapted from G. M. Cooper, *The Cell: a Molecular Approach* (ASM Press, Washington, DC, and Sinauer Associates, Sunderland, MA, 1997), with permission.

proteins normally reside in the nucleus and become relocalized to the cytoplasm during infection. It has been suggested that La, Ptb, poly(rC)-binding proteins, and others act as RNA chaperones, maintaining the IRES in its appropriate three-dimensional structure for binding to ribosomes and translation initiation proteins. In support of this hypothesis is the observation that all are RNA-binding proteins that can form multimers that contact the IRES at multiple points.

Elongation and Termination

During elongation, the ribosome selects aminoacylated tRNA according to the sequence of the mRNA codon, and catalyzes the formation of a peptide bond between the nascent polypeptide and the incoming amino acid. The 40S ribosomal subunit is responsible for both decoding and selection of the cognate tRNA. The RNA of the 60S subunit catalyzes the peptidyl transferase reaction without any soluble nonribosomal proteins or nucleotides. Elongation is assisted by three proteins that maintain the speed and accuracy of translation. In the 80S initiation complex, the Met-tRNA$_i$ is bound to the peptidyl (P) site of the ribosome (Fig. 11.10). Elongation of the peptide chain begins with addition of the next amino acid encoded by the triplet that occupies the acceptor (A) site. An important component of this process is elongation factor eEF1A, which is bound to aminoacylated tRNA, a molecule of GTP, and the nucleotide exchange protein eEF1B. Interaction between the codon and the anticodon leads to a conformational change in the ribosome called **accommodation**, the hydrolysis of GTP and the release of eEF1A-GDP. Accommodation preserves the fidelity of translation, because it can occur only upon proper codon-anticodon base pairing, and is required for GTP hydrolysis. If an incorrect tRNA enters the A site, accommodation does not occur and the aminoacylated tRNA is rejected. The large ribosomal subunit catalyzes the formation of a peptide bond between the amino acids occupying the P and A sites. The 80S ribosome then moves 3 nucleotides along the mRNA. Translocation is dependent upon eEF2 and hydrolysis of GTP. This motion moves the uncharged tRNA to the exit (E) site and the peptidyl-tRNA to the P site, allowing a new aminoacylated tRNA to enter the A site and allowing release of the uncharged

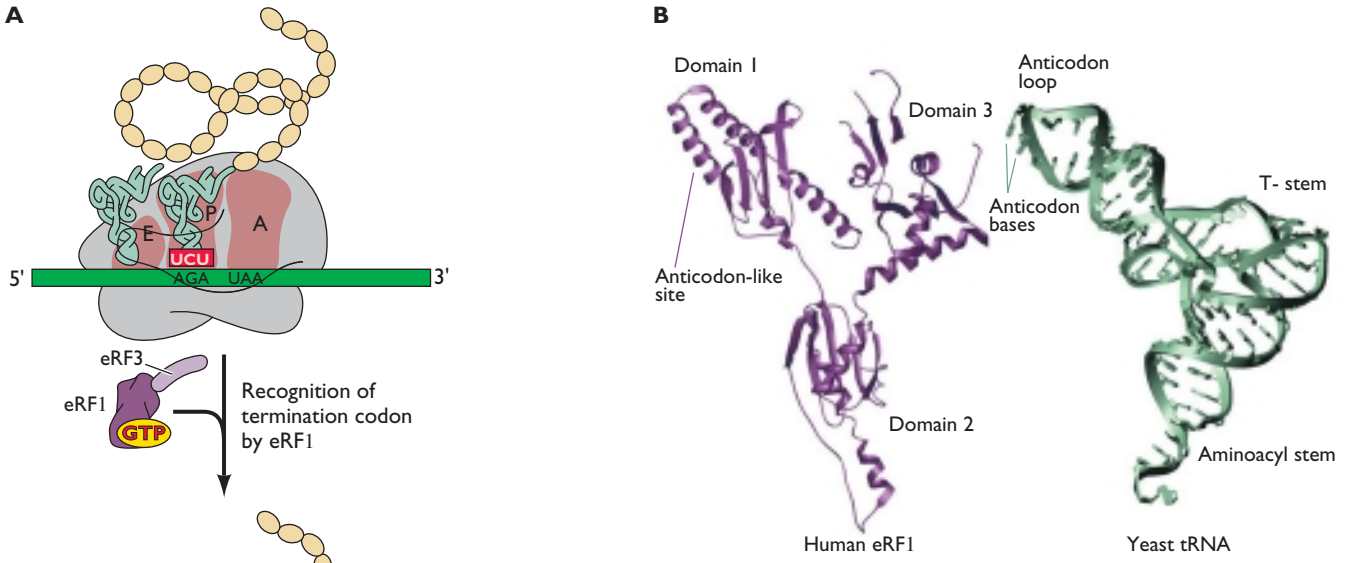


Figure 11.11 Translation termination. (A) Overview of termination. When a termination codon is encountered at the A site, it is usually recognized by a release factor (eRF) instead of a tRNA. The peptide chain is then released, followed by dissociation of tRNA and ribosome from the mRNA. eRF3, which is bound to the C terminus of eRF1, is a GTPase that is required for release of the protein. **(B)** Atomic structure of eRF1 and yeast $tRNA^{Phe}$. The structure of eRF1 mimics that of tRNA, providing a mechanism for recognition of termination codons. (A) Adapted from G. M. Cooper, *The Cell: a Molecular Approach* (ASM Press, Washington, DC, and Sinauer Associates, Sunderland, MA, 1997), with permission. (B) Adapted from H. Song, P. Mugnier, A. K. Das, H. M. Webb, D. R. Evans, M. F. Tuite, B. A. Hemmings, and D. Barford, *Cell* **100:**311–321, 1998, with permission.

tRNA. This cycle is repeated until the ribosome encounters a stop codon. mRNAs are usually bound by many ribosomes **(polysomes)**, with each ribosome separated from its neighbors by approximately 100 to 200 nucleotides, synthesizing a polypeptide chain.

Termination is a modification of the elongation process: once the stop codon enters the A site of the ribosome, it is recognized by the 40S subunit, and the 60S subunit cleaves the ester bond between the protein chain and the last tRNA. Recognition of the three stop codons (UAA, UAG, and UGA) by the 40S ribosomal subunit is facilitated by the **release proteins** eRF1 and eRF3 (Fig. 11.11). The structure of eRF1 mimics that of tRNA, allowing the release protein to occupy the A site of the ribosome. The N terminus of eRF1 recognizes all three stop codons. Once bound in the A site, eRF1 and eRF3 together induce a rearrangement of the 80S ribosome, translocation of the P site codon, and release of the polypeptide. The interaction between eRF1 and the ribosome stimulates the GTPase activity of eRF3, which is bound to the C terminus of eRF1. GTP hydrolysis is required for release of the nascent polypeptide.

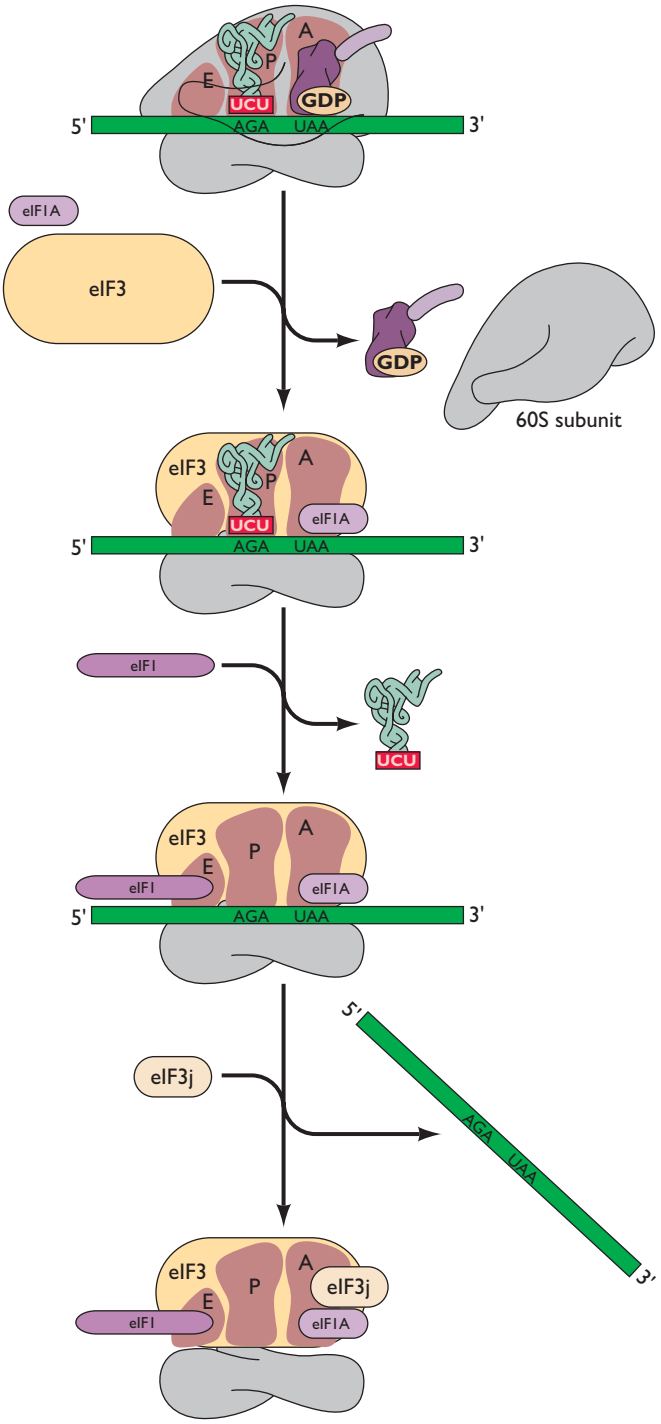


Figure 11.12 Ribosome recycling. After peptide release, eIF1A and eIF3 cause dissociation and release of the 60S ribosomal subunit. Release of the P-site deacylated tRNA is promoted by eIF1 and is followed by dissociation of mRNA mediated by eIF3j binding.

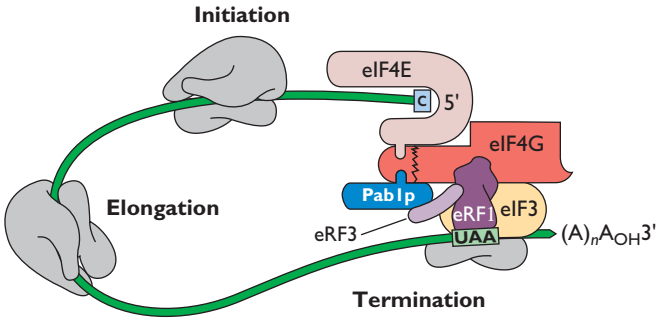


Figure 11.13 Juxtaposition of mRNA ends. Shown is a juxtaposition of mRNA ends by interactions of termination and initiation proteins, Pab1p, and the mRNA 5′ and 3′ ends. eRF3 binds both eRF1 and Pab1p. Adapted from N. Uchida, S. Hoshino, H. Imataka, N. Sonenberg, and T. Katada, *J. Biol. Chem.* **277:**50286–50292, 2002, with permission.

The E site, in addition to accommodation, is another important determinant of the fidelity of protein synthesis. When the E site is occupied by a deacylated tRNA, the affinity of the A site for aminoacyl-tRNA is low. Consequently, incorrect tRNAs are readily rejected. When the E site is empty, the affinity of the A site for aminoacyl-tRNA is significantly higher, making rejection of incorrect tRNAs less likely. An occupied E site also prevents tRNA slippage; when this site is empty, increased ribosomal frameshifting occurs.

Although stop codons are the major determinants of translation termination, other sequences can affect the efficiency of this process. The nucleotide immediately downstream of the stop codon can influence chain termination and ribosome dissociation. In eukaryotes, the preferred termination signals are UAA(A/G) and UGA(A/G).

After release of the polypeptide chain, the 60S ribosomal subunit, tRNA, and mRNA are released by the cooperation of eIF1, eIF1A, and eIF3 (Fig. 11.12). It has been suggested that 40S ribosomal subunits preferentially engage in new rounds of translation initiation on the same mRNA. This hypothesis is supported by the finding that eIF3, which remains bound to the 40S ribosomal subunit after termination, also binds eIF4G (Fig. 11.13). Other observations that are consistent with this model include the ability of eRF3 to bind Pab1p (Fig. 11.13) and the stimulation of 60S ribosomal subunit joining by Pabp1 at initiation. As a result, ribosomes may shuttle from the 3′ end of the mRNA back to the 5′ end, beginning the synthesis of another molecule of the protein.

The Diversity of Viral Translation Strategies

A variety of unusual translation mechanisms have evolved that expand the coding capacity of viral genomes, which are relatively small, and allow the synthesis of multiple polypeptides from a single RNA genome (Fig. 11.14).

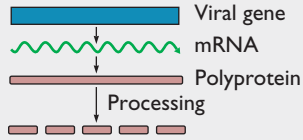
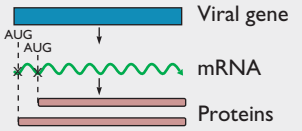
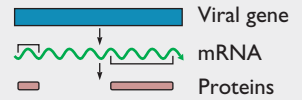
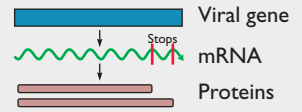
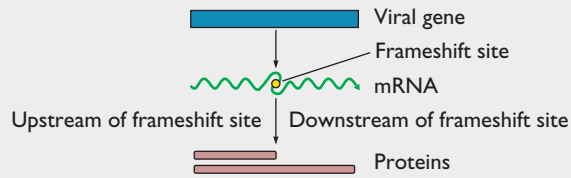
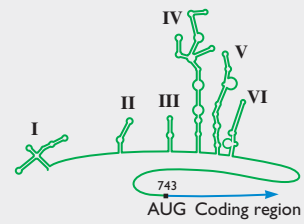
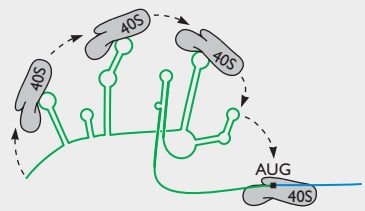
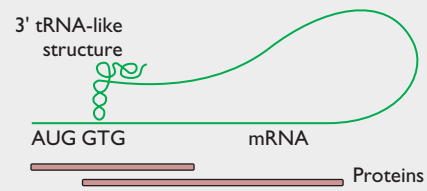
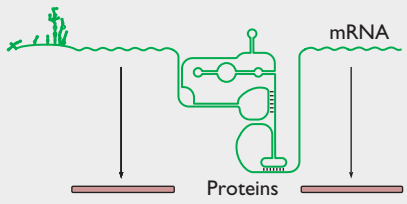
Mechanism of translation	Examples	
Polyprotein synthesis	Picornaviruses Flaviviruses Alphaviruses Retroviruses	
Leaky scanning	Sendai virus P/C mRNA Influenza B virus RNA 6 Human immunodeficiency virus type 1 Env/Vpu Human T-lymphotropic virus Tax, Rex Simian virus 40 VP2, VP3 Simian virus 40 agnoprotein	
Reinitiation	Influenza B virus RNA 7 Cytomegalovirus gp48 mRNA	
Suppression of termination	Alphavirus nsP4 Retrovirus Gag-Pol	
Ribosomal frameshifting	Coronavirus ORF1a-ORF1b Human astrovirus type 1 ORF1a-ORF1b Retrovirus Gag-Pol	
Internal ribosome entry	Picornaviruses Flaviviruses	
Ribosome shunting	Adenovirus Cauliflower mosaic virus	
Internal initiation mediated by tRNA-like structure in the 3' untranslated region	Turnip yellow mosaic virus	
Bicistronic mRNAs	Cricket paralysis virus *Rhopalosiphum padi* virus	

Figure 11.14 The diversity of viral translation strategies.

All were discovered in virus-infected cells and subsequently shown to operate during translation of cellular mRNAs. Nontranslational solutions for maximizing the number of proteins encoded in viral genomes are discussed in other chapters and include the synthesis of multiple subgenomic mRNAs, mRNA splicing, and RNA editing.

Polyprotein Synthesis

One strategy allowing the production of multiple proteins from a single RNA genome is to synthesize from a single mRNA a polyprotein precursor, which is then proteolytically processed to form functional viral proteins.

A dramatic example of protein processing occurs in picornavirus-infected cells: nearly the entire (+) strand RNA is translated into a single large polyprotein (Fig. 11.15A). Processing of this precursor is carried out by two virus-encoded proteases, $2A^{pro}$ and $3C^{pro}$, which cleave between Tyr and Gly and between Gln and Gly, respectively. In both cases, flanking amino acid residues control the efficiency of cleavage so that not all Tyr-Gly and Gln-Gly pairs in the polyprotein are processed. These two proteases are active in the nascent polypeptide and release themselves from the polyprotein by self-cleavage. Consequently, the polyprotein is not observed in infected cells because it is processed as soon as the protease coding sequences have been translated. After the proteases have been released, they cleave other polyprotein molecules.

Protein production can be controlled by the rate and extent of polyprotein processing. In addition, alternative utilization of cleavage sites can produce proteins with different activities. For example, the poliovirus protease $3C^{pro}$ does not process the capsid protein precursor P1 efficiently. Rather, the $3C^{pro}$ precursor, 3CD, is required for processing of P1. By regulating the amount of 3CD produced, the extent of capsid protein processing can be controlled. Because 3CD and $3C^{pro}$ process Gln-Gly pairs in the remainder of the polyprotein with the same efficiency, an interesting question is why 3CD, which also contains $3D^{pol}$ protein, is further processed to produce $3C^{pro}$ (Fig. 11.15A). The answer is that 3CD protein, while active as a protease, does not possess RNA polymerase activity and therefore some molecules must be cleaved to allow RNA replication.

Some viral precursor proteins are processed by cellular proteases. The genome of flaviviruses contains an open reading frame of more than 10,000 bases (Fig. 11.15B). This mRNA is translated into a polyprotein precursor that is processed by a viral serine protease and by host signal peptidase. The latter enzyme is located in the endoplasmic reticulum, where it removes the signal sequence from proteins translocated into the lumen (Chapter 12). The viral proteins processed by the cellular signal peptidase must therefore be inserted into the endoplasmic reticulum.

Leaky Scanning

Although the vast majority of eukaryotic mRNAs are monocistronic (Fig. 11.1), some viral mRNAs that encode two proteins in overlapping reading frames have been identified. The P/C gene of Sendai virus is the model for genes that encode mRNAs with such translational flexibility (Fig. 11.16). P protein is translated from an open reading frame beginning with an AUG codon at nucleotide 104. C proteins are produced from a different reading frame, which begins at nucleotide 81, and are completely different from P proteins. No less than four C proteins (called C′, C, Y1, and Y2) are produced by translation beginning at four in-frame initiation codons. The first start site is an unusual ACG codon, and the third, fourth, and fifth are at AUG codons; the result is a nested set of proteins with a common C terminus.

The first three initiation sites on P/C mRNA are likely to be arranged to permit translation by **leaky scanning**, when ribosomes skip the first AUG codon and initiate at subsequent AUG triplets. The first start site, $ACG^{81/C'}$, is surrounded by a good initiation context but is inefficient because of the unusual start codon. Some ribosomes bypass this initiator codon and initiate at the second, (CGCAUGG). Although the second is an AUG codon, the context is poor, and some ribosomes bypass it and find their way to the third initiation codon, which has a better context (AAGAUGC). Consistent with this hypothesis, mutagenesis of $ACG^{81/C'}$ to AUG abolishes initiation at $AUG^{104/P}$ and $AUG^{114/C}$. When successive initiation codons are used in leaky scanning, they are increasingly efficient as start sites.

The last two C-protein initiation codons, $AUG^{183/Y1}$ and $AUG^{201/Y2}$, are not likely to be translated by leaky scanning because they are in the poorest contexts of the five. Furthermore, mutagenesis of $ACG^{81/C'}$ to AUG has no effect on the synthesis of Y1 and Y2 proteins. Rather, translation of Y1 and Y2 proteins is believed to be initiated by ribosome shunting. An interesting question is how the different translation strategies of P/C mRNA are coordinated such that, for example, shunting does not dominate at the expense of translation of upstream AUG codons. The answer to this question is not known, but Y protein synthesis relative to that of the other C proteins varies in different cell lines. This result suggests that cellular proteins might regulate ribosome shunting on P/C mRNA, although no such protein has been identified.

Translation of overlapping reading frames also occurs in other viral mRNAs (Fig. 11.17). Influenza viruses are classified into three types, A, B, and C; most of the previous discussion in this textbook has concerned work on

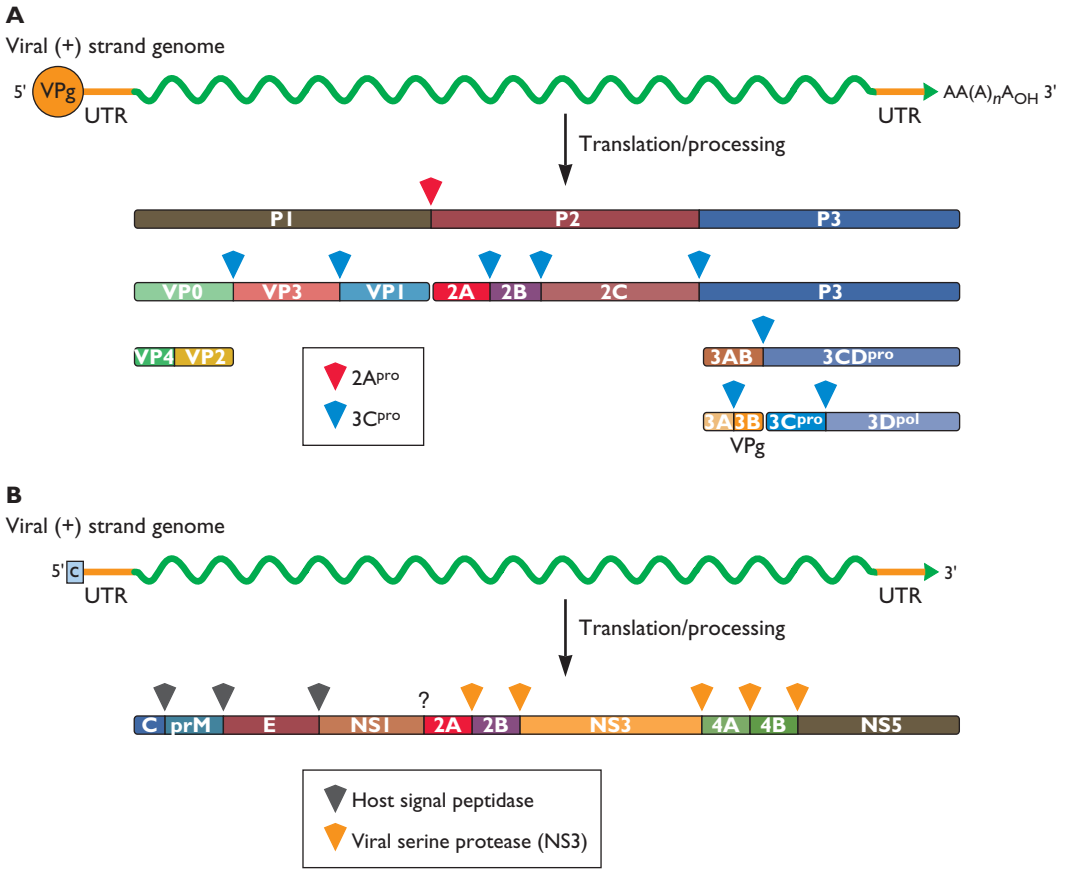
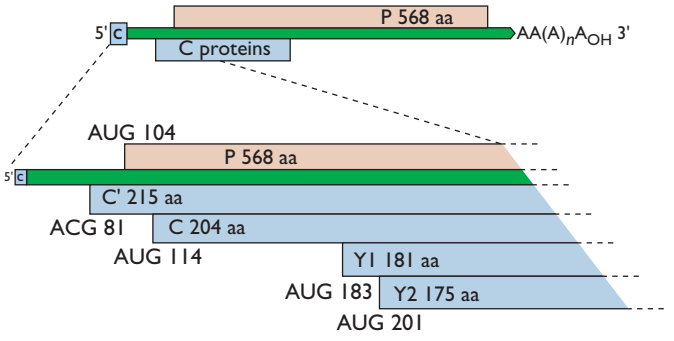


Figure 11.15 Polyprotein processing of picornaviruses and flaviviruses. (A) Processing map of protein encoded by the poliovirus genome. The viral RNA is translated into a long precursor polyprotein that is processed by two viral proteases, $2A^{pro}$ and $3C^{pro}$, to form viral proteins. Cleavage sites for each protease are shown. **(B)** Cleavage map of protein encoded in the flavivirus genome. Processing of the flavivirus precursor polyprotein is carried out either by the host signal peptidase or by the viral protease NS3.

Figure 11.16 Leaky scanning and mRNA editing in the Sendai virus P/C gene. P and C protein open reading frames are shown as brown and blue boxes, respectively. An enlargement of the 5′ end of the mRNA is shown below, indicating the different start sites for four of the C proteins. aa, amino acids. Adapted from J. Curran et al., *Semin. Virol.* **8:**351–357, 1997, with permission.



influenza A virus. The mRNA synthesized from influenza B virus RNA segment 6 encodes two different proteins, NB and NA, in overlapping reading frames. NB protein synthesis is initiated at the 5′-proximal AUG codon, while initiation at an AUG codon 4 nucleotides downstream produces the NA protein.

Reinitiation

Reinitiation is another strategy for producing two proteins from a single mRNA. About 10% of mRNAs contain an additional short open reading frame upstream of the main open reading frame (Fig. 11.17). These open reading frames may be translated, with reinitiation occurring at the downstream AUG. For example, a 22-amino-acid peptide is synthesized from an open reading frame in the 5′ region of human cytomegalovirus gp48 mRNA. Other examples

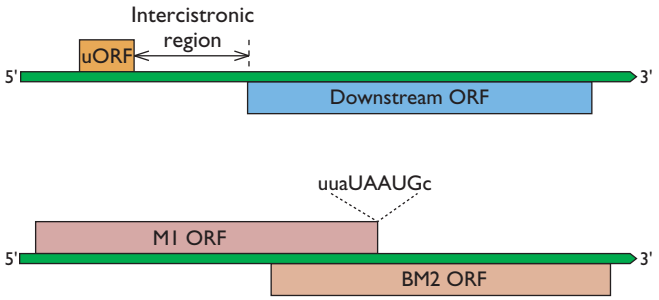


Figure 11.17 Reinitiation of translation. (Top) Some mRNAs contain one or more short upstream open reading frames (uORFs) that may be translated. Expression of the longer, downstream ORF depends on reinitiation. **(Bottom)** mRNA produced from influenza B virus RNA segment 7 encodes two proteins, M1 and BM2. The initiation AUG codon for BM2 overlaps the termination codon of M1. Synthesis of BM2 occurs by reinitiation.

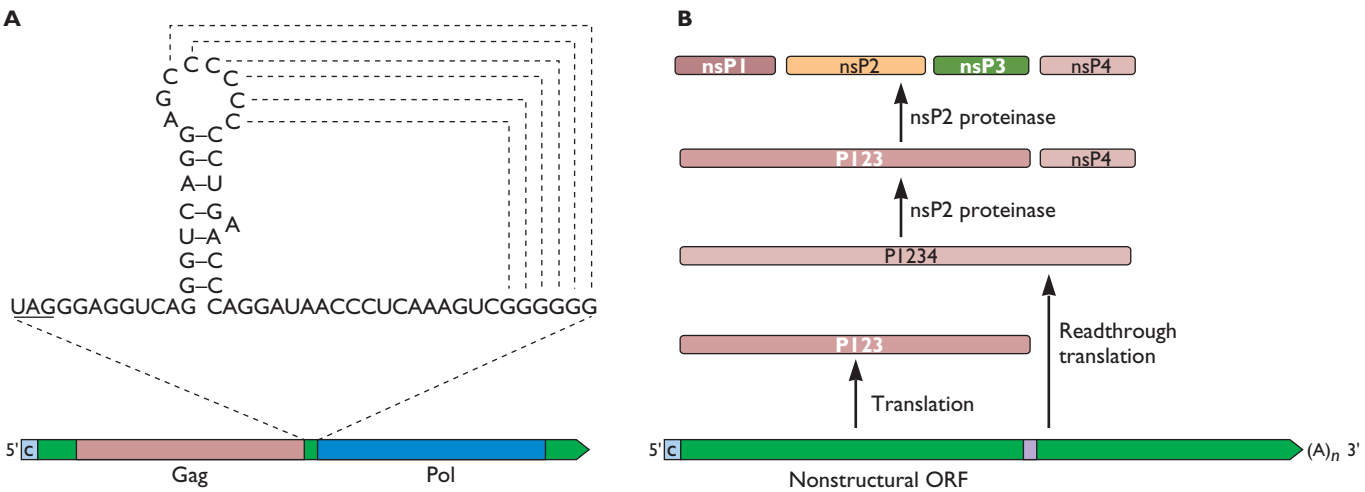
are found in mRNAs of retroviruses and in cellular mRNAs, such as those encoding *S*-adenosylmethionine decarboxylase and fibroblast growth factor 5. These short open reading frames can affect the translation of downstream open reading frames. The extent of regulation depends on many factors, such the as sequence context of the upstream open reading frame AUG initiation codon, the presence of RNA secondary structure, and the distance between the upstream and downstream open reading frames.

In most cases, translation reinitiation involves short upstream open reading frames that precede the main open reading frame. In contrast, reinitiation of translation of longer, overlapping reading frames occurs on mRNA of influenza B virus RNA 7, which encodes two proteins, M1 protein and BM2 protein (Fig. 11.17). M1 protein is translated from the 5′-proximal AUG codon, while the BM2 protein AUG initiation codon is part of the termination codon for M1 protein (UAAUG). Translation of the BM2 open reading frame is dependent on the synthesis of M1 protein, as deletion of the M1 AUG codon abrogates BM2 synthesis.

Suppression of Termination

Suppression of termination occurs during translation of many viral mRNAs as a means of producing a second protein with an extended C terminus. The Gag and Pol genes of Moloney murine leukemia virus are encoded in a single mRNA and separated by an amber termination codon, UAG (Fig. 11.18). Infected cells contain a polyprotein precursor called Gag-Pol. This precursor is synthesized by translational suppression of the amber termination codon. The efficiency of suppression is about 4 to 10%. The Gag-Pol precursor is subsequently processed proteolytically to liberate the Gag and Pol proteins. Without this suppression mechanism, the viral enzymes reverse transcriptase (RT) and integrase (IN) could not be produced. In a similar way, translational suppression of a different termination codon, UGA, is required for the synthesis of nsP4 of alphaviruses (Fig. 11.18). In this example, the efficiency of synthesis is about 10% of that of the normally terminated nsP3 protein. Because nsP4 encodes the RNA-dependent RNA

Figure 11.18 Suppression of termination codons of alphaviruses and retroviruses. (A) Structure of the termination site between Gag and Pol of Moloney murine leukemia virus. The stop codon that terminates synthesis of Gag is underlined; it is followed by a pseudoknot that is important for suppression of termination. Adapted from J. H. Strauss and E. G. Strauss, *Microbiol. Rev.* **58:**491–562, 1994, with permission. **(B)** Suppression of termination during the synthesis of alphavirus P123 to produce nsP4, the RNA-dependent RNA polymerase. The termination codon is shown on the RNA as a box.



polymerase, suppression is essential for viral RNA replication. Translational suppression in eukaryotic mRNAs is extremely rare.

Most translational suppression occurs when normal tRNAs misread termination codons. The misreading of the amber codon in Moloney murine leukemia virus Gag protein for a Gln codon is an example. More rare are suppressor tRNAs that can recognize termination codons and insert a specific amino acid. One example is a suppressor tRNA that inserts selenocysteine, the 21st amino acid, in place of a UGA codon.

The nucleotide sequence 3′ of the termination codon plays an important role in the efficiency of translational suppression. In bacteria, the nucleotide next to this codon is highly influential. In eukaryotes, the signals range from very simple to complex. In Sindbis virus, efficient suppression of the UGA codon requires only a single C residue 3′ of the termination codon. The effect of this nucleotide on Sindbis virus suppression may be explained in three ways. The 3′ nucleotide might influence the recognition of termination codons by release proteins; it might affect the interaction of misreading tRNAs with the codon by increasing the energy of base pairing; or the misreading tRNA and a tRNA recognizing the next codon might interact, affecting the efficiency of elongation and suppression.

In contrast, readthrough of the UAG codon in Moloney murine leukemia virus mRNA requires a purine-rich sequence 3′ of the termination codon, as well as a pseudoknot structure further downstream (see Chapter 6 for a description of pseudoknots). Because of these differences, it is likely that readthrough can be mediated by different mechanisms. It has been suggested that the pseudoknot of Moloney murine leukemia virus RNA causes the ribosome to pause and allow the suppressor tRNA to compete with eRF1 at the suppression site. The mechanism involved, and the role of the eight-nucleotide, purine-rich segment of the suppression signal, is not known. Maximal readthrough efficiency also requires the interaction of viral reverse transcriptase with eRF1.

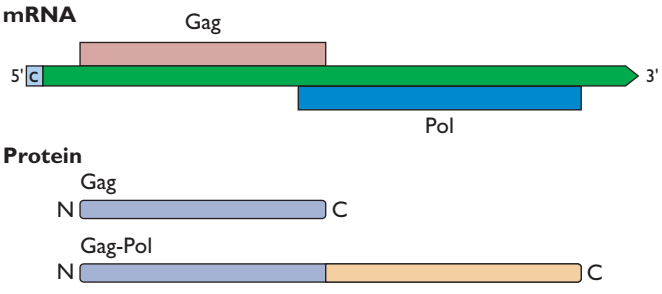
Suppression of termination is far more prevalent during translation of viral mRNAs than of cellular mRNAs. The RNA sequences and structures required for suppression are not found in most cellular mRNAs. For example, there is a strong bias against cytidine residues at the 3′ end of UGA termination codons in cellular mRNAs. Suppression by tRNAs charged with selenocysteine has been found in fewer than 50 eukaryotic mRNAs.

Ribosomal Frameshifting

Ribosomal frameshifting is a process by which, in response to signals in mRNA, ribosomes move into a different reading frame and continue translation in that new frame. It was discovered in cells infected with Rous sarcoma virus and has since been described for many other viruses, including additional retroviruses, eukaryotic (+) strand RNA viruses, and herpes simplex virus. There are several examples of frameshifting during translation of mammalian mRNAs. Frameshifting may occur by shifting the reading frame 1 base toward the 5′ end (–1 frameshifting) or the 3′ end (+1 frameshifting) of the mRNA.

In the genome of retroviruses, the *gag* and *pol* genes may be separated by a stop codon (Fig. 11.18), or they may be in different reading frames, with *pol* overlapping *gag* in the –1 direction (Fig. 11.19). During synthesis of Rous sarcoma virus Gag, ribosomes frameshift before reaching the Gag stop codon and continue translating Pol, such that a Gag-Pol fusion is produced at about 10% of the frequency of Gag. Studies on the requirements for frameshifting in retroviruses and coronaviruses have identified two essential components: a "slippery" homopolymeric sequence, which is a heptanucleotide stretch with two homopolymeric triplets of the form X-XXY-YYZ (e.g., in Rous sarcoma virus A-AAU-UUA), and an RNA secondary structure, usually a pseudoknot, five to eight nucleotides downstream. These observations led to the proposal of the tandem shift model for frameshifting, in which two tRNAs in the zero reading frame (X-XXY-YYZ) slip back by one nucleotide during the frameshift to the –1 phase (XXX-YYY). Each tRNA base pairs with the mRNA in the first two nucleotides of each codon (Fig. 11.20). The peptidyl-tRNA is transferred to the P site, the –1 frame *pol* codon is decoded, and translation continues to produce the fusion protein. In this model, slippage occurs before peptide transfer, with the peptidyl- and aminoacyl-tRNAs bound to the P and A sites. However, it is possible that the shift occurs after peptide transfer but before translocation of the tRNAs, or when the aminoacyl-tRNA occupies the A site. These models cannot

Figure 11.19 Frameshifting on a retroviral mRNA. The structure of open reading frames is illustrated. Rous sarcoma virus mRNA encodes Gag and Pol proteins in reading frames that overlap by –1. Normal translation and termination produce the Gag protein; ribosomal frameshifting to the –1 frame results in the synthesis of a Gag-Pol fusion protein.



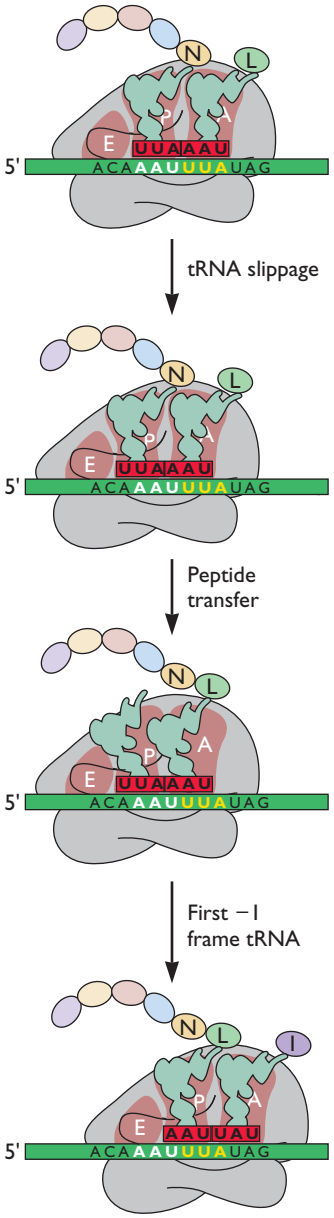


Figure 11.20 A model for –1 frameshifting. Slippage of the two tRNAs occurs after aminoacyl-tRNA enters the A site but before peptide transfer. Slippage allows the tRNAs to form only two base pairs with the mRNA. The site shown is that of Rous sarcoma virus. One-letter amino acid codes are used. Adapted from P. J. Farabaugh, *Microbiol. Rev.* **60:**103–134, 1996, with permission.

be distinguished by mutagenesis or by the sequence of the protein products.

The pseudoknot is thought to cause the ribosome to pause over the slippery sequence, increasing the probability that realignment to the –1 reading frame will occur. Some viral mRNAs contain simple stem-loop structures rather than pseudoknots at this position, but the highest frequencies of frameshifting are associated with the presence of the latter structure. However, when the pseudoknot of a coronavirus is replaced with a stem-loop structure, frameshifting is abolished but ribosomal pausing still occurs. Therefore, pausing is not sufficient for frameshifting; the pseudoknot might also interact with the ribosome to promote frameshifting. It has been suggested that cellular proteins might interact with the pseudoknot to influence frameshifting. For example, eEF1A, eEF2, and a ribosome-associated chaperone complex all have been shown to regulate frameshifting.

Bicistronic mRNAs

The mRNAs of members of the *Dicistroviridae*, including cricket paralysis virus and *Rhopalosiphum padi* virus, are bicistronic (Fig. 11.14). The upstream open reading frame begins with an AUG codon and is preceded by an IRES similar to those of picornaviruses. The downstream open reading frame, which encodes the viral capsid proteins, is translated independently from a completely different IRES. The 40S ribosomal subunit binds directly to the intergenic region that is partially folded to mimic a tRNA. The tRNA-like structure occupies the P site of the ribosome, and initiation occurs from the A site at a non-methionine codon. Translation of this cistron is therefore dependent on ribosomes and elongation and termination proteins. Because initiation proteins are not required, translation regulation may occur at the stages of elongation and termination.

Regulation of Translation during Viral Infection

Alterations in the cellular translation apparatus are commonplace in virus-infected cells. As part of the antiviral defense, or in response to stress caused by virus infection, the cell initiates measures designed to inhibit protein synthesis and limit virus production. Many viral genomes encode proteins or nucleic acids that neutralize this response, restore translation, and maximize virus replication. In addition, many viral gene products modify the host translation apparatus to favor synthesis of viral proteins over those of the cell. As a result, the entire synthetic capability of the cell can be turned to the production of new virus particles, which should enhance virus yield and perhaps accelerate replication. This supposition is supported by the growth defects of different viral mutants that cannot inhibit cellular translation. These cellular and viral modifications of the translation apparatus usually affect the initiation stages, which are rate-limiting.

Inhibition of Translation Initiation after Viral Infection

Phosphorylation of eIF2α

Members of a large family of secreted proteins, including interferons, are produced as part of the rapid innate immune response of vertebrates in response to viral infection (discussed in Volume II, Chapter 3). Interferons diffuse to neighboring cells, bind to cell surface receptors, and activate signal transduction pathways that result in transcription of hundreds of cellular genes and the establishment of an **antiviral state**. Several interferon-induced genes encode enzymes that effectively prevent association of mRNA with polysomes and hence translation, including RNA-activated protein kinase (Pkr) and RNase L. RNase L degrades RNA and is not considered further here, while Pkr phosphorylates eIF2α, thereby inhibiting translation initiation. Because the block to translation is global, the infected cell may die, but by slowing down viral replication the organism may be spared.

Pkr is a serine-threonine protein kinase composed of an N-terminal regulatory domain and a C-terminal catalytic domain (Fig. 11.21; see also Volume II, Chapter 3). Small quantities of an inactive form of Pkr are present in most uninfected mammalian tissues. Transcription of its gene is induced 5- to 10-fold by interferon. Pkr is activated by the binding of dsRNA to two dsRNA-binding motifs at the N terminus of the protein (Fig. 11.22). Such dsRNA is produced in cells infected by either DNA or RNA viruses. Activation is accompanied by autophosphorylation of Pkr. Low concentrations of dsRNA activate Pkr, but high concentrations are inhibitory, leading to the suggestion that one molecule of Pkr phosphorylates another while both are bound to the same molecule of dsRNA (Fig. 11.22). Phosphorylation is thought to induce conformational changes in the enzyme, thereby rendering the kinase active without further need for dsRNA. Pkr molecules may also be activated by a cell protein, Pact, independently of dsRNA (Fig. 11.22).

Two other eIF2α protein kinases regulate translation during virus infection. In mammalian cells, Gcn2p is activated during amino acid starvation when uncharged tRNA binds a histidyl-tRNA synthetase-like domain in the protein (Fig. 11.21). During infection with Sindbis virus, Gcn2p is activated upon binding of viral RNA, leading to phosphorylation of eIF2α and restriction of virus replication.

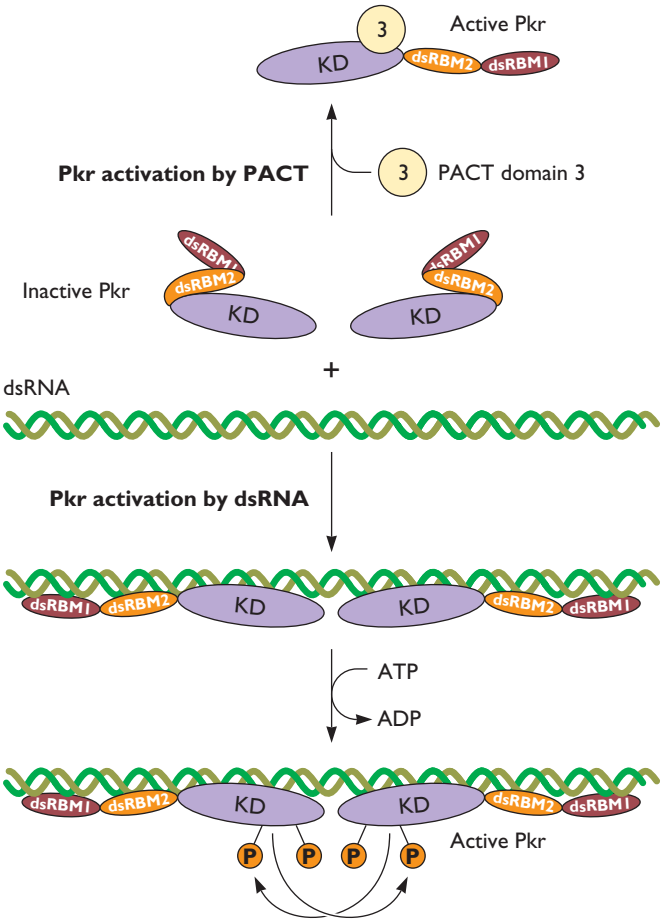
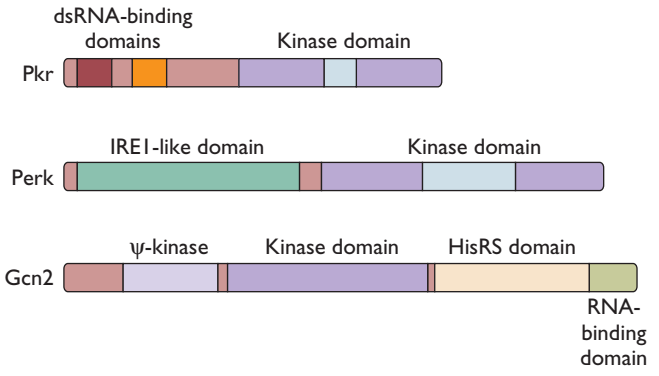


Figure 11.22 Model of activation of Pkr. Pkr is maintained in an inactive monomer by the interaction between a Pact domain 3-binding sequence in Pkr and dsRBM2. Pkr is activated when it binds Pact or dsRNA. When two or more molecules of inactive Pkr bind to one dsRNA molecule, cross-phosphorylation occurs because of the physical proximity of the molecules. Phosphorylation is thought to cause a conformational change in the kinase domain (KD) to allow phosphorylation of other substrates, including eIF2α. dsRBM, double-stranded RNA-binding motif. Adapted from J. W. B. Hershey et al. (ed.), *Translational Control* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1996), with permission.

Figure 11.21 Schematic structures of three eIF2α kinases. IRE1, inositol-requiring enzyme 1; ψ-kinase, pseudokinase domain; HisRS domain, histidyl-tRNA synthetase-like domain. Adapted from C. G. Proud, *Semin. Cell Dev. Biol.* **16**:3–12, 2005, with permission.



Consistent with a role in mediating antiviral responses, Sindbis virus replication is more efficient in cells lacking Gcn2p. Pkr-like ER kinase (Perk), a transmembrane protein of the endoplasmic reticulum, is a component of the unfolded protein response. Its lumenal domain senses the equilibrium between unfolded and misfolded proteins and chaperone proteins. Under conditions of intracellular stress (Box 11.5), such as occurs during virus infection, Perk oligomerizes within the membrane, is activated, and phosphorylates eIF2α in the cytoplasm.

Activated Pkr, Gcn2p, and Perk phosphorylate eIF2α on serine 51. This initiation protein is part of the complex that also contains GTP and Met-tRNA$_i$ (Fig. 11.3). After GTP hydrolysis, the bound GDP must be exchanged for GTP to permit the binding of another Met-tRNA$_i$. This exchange is carried out by eIF2B (Fig. 11.23). When the alpha subunit of eIF2 is phosphorylated, eIF2-GDP binds eIF2B with such high affinity that it is effectively trapped; recycling of eIF2 stops, and ternary complexes are depleted. eIF2B is less abundant than eIF2, and phosphorylation of about 10 to 40% of eIF2 (depending on the cell type and the relative concentrations of eIF2 and eIF2B) results in the complete sequestration of eIF2B, leading to a block in protein synthesis. As viral translation is also impaired, the production of new virus particles is diminished.

Viral Regulation of Pkr

Most viral infections induce activation of eIF2α kinases and consequent phosphorylation of eIF2α. Global inhibition of translation is clearly a threat to successful viral replication. At least five different viral mechanisms to block Pkr activation or to prevent activated Pkr from inhibiting translation can be distinguished (Table 11.2).

Inhibition of dsRNA binding. The 166-nucleotide adenovirus VA-RNA I, which accumulates to massive concentrations (up to 10^9 copies per cell) late in infection following transcription of the viral gene by RNA polymerase III, is a potent inhibitor of Pkr. An adenovirus mutant that cannot express the VA-RNA I gene grows poorly. In cells infected with this mutant virus, eIF2α becomes extensively phosphorylated, causing global translational inhibition. VA-RNA I binds the dsRNA-binding region of Pkr and blocks activation. It has been suggested that binding of VA-RNA I to Pkr blocks interaction with authentic dsRNA and hence prevents activation of the kinase. Epstein-Barr virus and human immunodeficiency virus type 1 genomes encode small RNAs that inhibit Pkr activation *in vitro*, but whether they function in a similar manner in infected cells is not clear.

While adenovirus VA-RNA I binds Pkr and blocks activation by dsRNA, the vaccinia virus genome encodes a protein that sequesters dsRNA. The viral E3L protein contains the same dsRNA-binding motif as Pkr; it binds dsRNA and prevents it from activating the kinase. Deletion of the gene encoding the E3L protein renders the virus more sensitive to interferon and results in larger quantities of active Pkr in infected cells. The influenza virus NS1 protein and the reovirus σ3 protein also sequester dsRNA.

Inhibition of kinase function. The genomes of several viruses encode proteins that directly inhibit the kinase activity of Pkr, and some do so by acting as pseudosubstrates. For example, vaccinia virus K3L protein has amino acid homology to the N terminus of eIF2α. The protein binds tightly to Pkr within the catalytic cleft and blocks autophosphorylation. The growth of vaccinia virus mutants lacking the K3L gene is severely impaired by interferon. Human immunodeficiency virus type 1 Tat protein may inhibit Pkr by a similar mechanism.

In uninfected cells, Pkr is associated with the chaperone proteins Hsp40 and Hsp70. Influenza virus infection induces the release of Hsp40. Such dissociation may allow aberrant refolding of Pkr by Hsp70, leading to inactivation of the enzyme. Virus infection may also activate a cellular protein, p58^{IPK}, that binds Pkr and prevents autophosphorylation. Activated p58^{IPK} can also block phosphorylation of eIF2α by Perk. In cells infected with herpes simplex virus type 1, acute endoplasmic reticulum stress occurs, but Perk is not activated. The viral glycoprotein gB associates with the lumenal domain of Perk and prevents its activation and subsequent phosphorylation of eIF2α. Another herpes simplex virus protein, Us11, binds to both Pkr and Pact and blocks Pkr activation.

Dephosphorylation of eIF2α. Another mechanism for reversing the consequences of Pkr activation is dephosphorylation of its target. In herpes simplex virus-infected cells, Pkr is activated but eIF2α is not phosphorylated. During infection with viruses lacking the viral ICP34.5 gene, Pkr is activated and eIF2α becomes phosphorylated, causing global inhibition of protein synthesis. This viral protein product associates with a type 1a protein phosphatase and acts as a regulatory subunit, redirecting the enzyme to dephosphorylate eIF2α. The effects of activated Pkr are reversed, ensuring continued protein synthesis. In a similar fashion, the E6 protein of human papillomavirus activates a phosphatase, leading to dephosphorylation of eIF2α.

Beneficial Effects of eIF2α Phosphorylation on Viral Replication

Phosphorylation of eIF2α is not always detrimental to virus replication. Reovirus replication is more efficient in the presence of phosphorylated eIF2α. Such conditions promote the synthesis of a transcriptional regulator (Atf4)

BOX 11.5 BACKGROUND

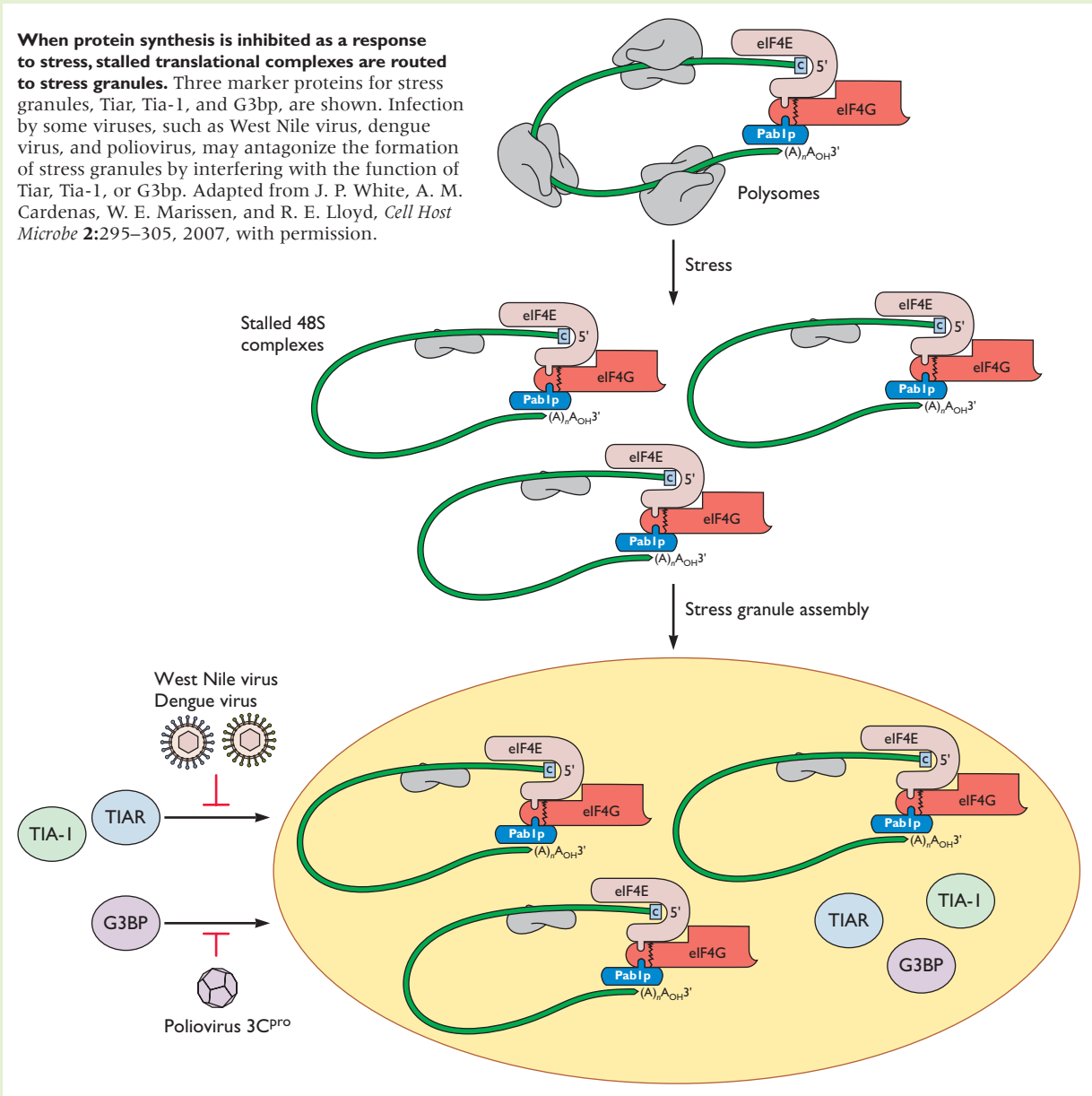
Viruses and cellular stress

In response to environmental stresses such as heat, starvation, or oxidation, mammalian cells produce cytoplasmic granular RNA structures called stress granules. These structures are aggregates of stalled translational complexes containing intact mRNAs, 40S ribosomal subunits, and a variety of translation initiation proteins such as eIF3 and eIF4G. They are thought to form when protein synthesis is interrupted, specifically by phosphorylation of eIF2α, or inhibition of eIF4A helicase activity. During conditions of stress, mRNAs are protected from degradation within the stress granule. Depending on the condition of the cell, the mRNAs may be subsequently routed to RNA processing bodies (P bodies) for degradation, or to the polysome pool for translation.

Viral infections also induce the formation of stress granules, but different mechanisms have evolved to block or reverse their formation and allow the production of viral proteins. For example, during rotavirus infection, eIF2α is phosphorylated, but stress granule components are relocated and these structures are not formed. Early in cells infected with poliovirus, stress granules form, but they are subsequently dispersed. One of the key proteins for nucleation of stress granule formation, Ras-Gap SH3 domain-binding protein, is cleaved by the poliovirus protease $3C^{pro}$. Viral interference with stress granule formation demonstrates a critical role for this cellular response in limiting virus infection.

Schütz, S., and P. Sarnow. 2007. How viruses avoid stress. *Cell Host Microbe* **2:**284–285.

White, J. P., A. M. Cardenas, W. E. Marissen, and R. E. Lloyd. 2007. Inhibition of cytoplasmic mRNA stress granule formation by a viral proteinase. *Cell Host Microbe* **2:**295–305.



When protein synthesis is inhibited as a response to stress, stalled translational complexes are routed to stress granules. Three marker proteins for stress granules, Tiar, Tia-1, and G3bp, are shown. Infection by some viruses, such as West Nile virus, dengue virus, and poliovirus, may antagonize the formation of stress granules by interfering with the function of Tiar, Tia-1, or G3bp. Adapted from J. P. White, A. M. Cardenas, W. E. Marissen, and R. E. Lloyd, *Cell Host Microbe* **2:**295–305, 2007, with permission.

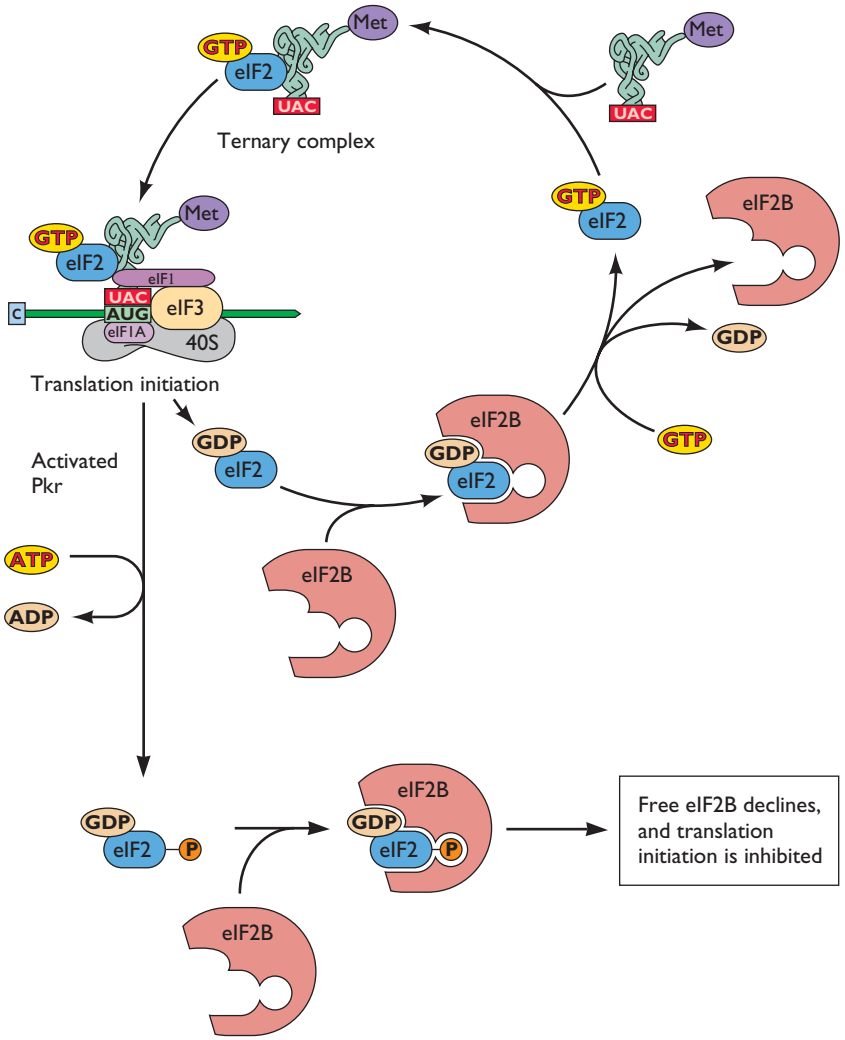


Figure 11.23 Effect of eIF2α phosphorylation on catalytic recycling. eIF2-GTP and tRNA-Met_i form the ternary complex required for translation initiation. During initiation, GTP is hydrolyzed to GDP, and in order for initiation to continue, eIF2 must be recharged with GTP. This recycling is accomplished by eIF2B, which exchanges GTP for GDP on eIF2. When eIF2 is phosphorylated on the alpha subunit, it binds irreversibly to eIF2B, preventing the latter from carrying out its role in recycling active eIF2. As a result, the concentration of eIF2-GTP declines and translation initiation is inhibited.

that controls the synthesis of a variety of proteins important for cellular recovery from stress. Furthermore, inhibition of cellular translation favors translation of the more abundant viral mRNAs. Atf4 is thought to induce the synthesis of mRNAs whose protein products benefit viral replication in cells infected with human cytomegalovirus. The latent membrane protein 1 oncogene of Epstein-Barr virus also induces synthesis of Atf4 by activating Perk. This transcriptional activator in turn increases the synthesis of latent membrane protein 1 mRNA. The increased levels of the protein lead to signaling required for B-cell proliferation.

Regulation of eIF4F

The eIF4F protein plays several important roles during 5′-end-dependent initiation, including recognition of the cap, recruitment of the 40S ribosomal subunit, and unwinding of RNA secondary structure. It is not surprising, therefore, that several viral proteins modify the activity of this protein. The cap-binding subunit eIF4E is frequently a target, probably because its activity can be modulated in at least two ways and because it is present in limiting quantities in cells. The cap-binding complex can also be inactivated by cleavage of eIF4G.

Table 11.2 Targets of viral inhibitors of eIF2α phosphorylation[a]

Target	Virus	Inhibitor	Mechanism
dsRNA	Herpes simplex virus	US11	Binds and sequesters dsRNA
	Influenza virus	NS1	Binds and sequesters dsRNA
	Reovirus	σ3	Binds and sequesters dsRNA
	Vaccinia virus	E3L	Binds and sequesters dsRNA
Pkr	Adenovirus	VA-RNA I	Blocks activation by dsRNA
	Epstein-Barr virus	EBER	Blocks activation by dsRNA
	Human immunodeficiency virus type 1	TAR RNA	Blocks activation by dsRNA
	Herpes simplex virus	US11	Binds Pkr
	Kaposi sarcoma herpesvirus	vIRF-2	Binds Pkr
	Baculovirus	PK2	Inhibits dimerization
	Hepatitis C virus	NS5A	Inhibits dimerization
	Human immunodeficiency virus type 1	Tat	Reduces Pkr expression
eIF2α	Hepatitis C virus	E2	Pseudosubstrate, blocks Pkr-eIF2α interaction
	Human immunodeficiency virus type 1	Tat	Pseudosubstrate, blocks Pkr-eIF2α interaction
	Vaccinia virus	K3L	Pseudosubstrate, blocks Pkr-eIF2α interaction
Phosphatase	Herpes simplex virus	γ34.5	Binds phosphatase, directs to eIF2α
	Simian virus 40	T antigen	Downstream of eIF2α?
Pact	Herpes simplex virus	US11	Binds Pact

[a]RBM, RNA-binding motif.

Cleavage of eIF4G

Poliovirus infection of mammalian cells in culture results in a dramatic inhibition of cellular protein synthesis. By 2 h after infection, polyribosomes are disrupted and translation of nearly all cellular mRNAs dramatically declines (Fig. 11.24). Translationally competent extracts from infected cells can readily translate poliovirus mRNA but not capped mRNAs. Studies of these extracts have demonstrated that they lack functional eIF4F. In poliovirus-infected cells, both isoforms of eIF4G are proteolytically cleaved. As the N-terminal domain of eIF4G binds eIF4E, which in turn binds the 5′ cap of cellular mRNAs, such cleavage prevents eIF4F from recruiting 40S ribosomal subunits to capped mRNAs (Fig. 11.25). Poliovirus mRNA is uncapped and is translated by internal ribosome binding, a process that does not require intact eIF4G. In fact, IRES-mediated initiation function appears to require the C-terminal fragment of eIF4G, which, as discussed above, is necessary to recruit 40S ribosomal subunits to the IRES. Consequently, cleavage of eIF4G not only inhibits translation of cellular mRNAs but also is a strategy for stimulating IRES-dependent translation. Although both eIF4GI and eIF4GII are cleaved in poliovirus- and rhinovirus-infected cells, the kinetics of shutoff of host translation correlates only with cleavage of eIF4GII.

Cleavage of eIF4G is carried out by viral proteases such as $2A^{pro}$ of poliovirus, rhinovirus, and coxsackievirus and the L protease of foot-and-mouth disease virus. Purified $2A^{pro}$ of rhinovirus cleaves eIF4G directly *in vitro*, although very inefficiently unless this protein is bound to eIF4E; that is, eIF4F appears to be the target of $2A^{pro}$ cleavage. The binding of eIF4E to eIF4G might induce conformational changes in the latter protein that make it a more efficient substrate for the protease. Poliovirus $2A^{pro}$ efficiently cleaves eIF4GI but not eIF4GII *in vitro*, consistent with the differential processing of these proteins during viral infection.

Modulation of eIF4E Activity by Phosphorylation

The regulated phosphorylation of eIF4E at Ser209 has been recognized for many years. Inhibition of cellular translation during mitosis and heat shock correlates with reduced phosphorylation of eIF4E. Two protein kinases, Mnk1 and Mnk2, that are associated with eIF4G phosphorylate Ser209 of eIF4E. However, the effect of phosphorylation on the function of eIF4E is unclear. It has been suggested that phosphorylation of eIF4E allows tighter binding to the 5′-terminal cap.

A decrease in eIF4E phosphorylation may be responsible for the inhibition of mRNA translation in cells infected with some viruses. For example, cellular protein synthesis is inhibited at late times in cells infected with adenovirus, a result of virus-induced underphosphorylation of eIF4E. The viral L4 100-kDa protein binds to the C terminus of eIF4G, preventing binding of Mnk1, and hence presumably

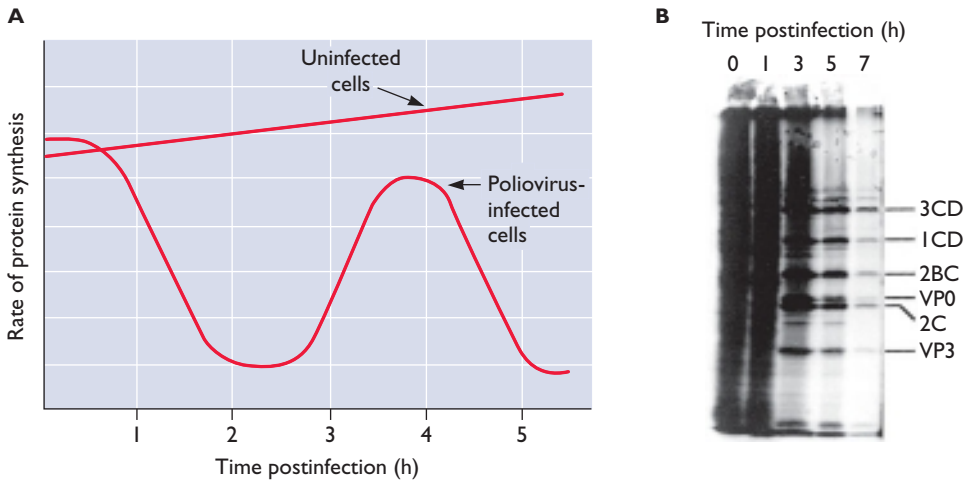
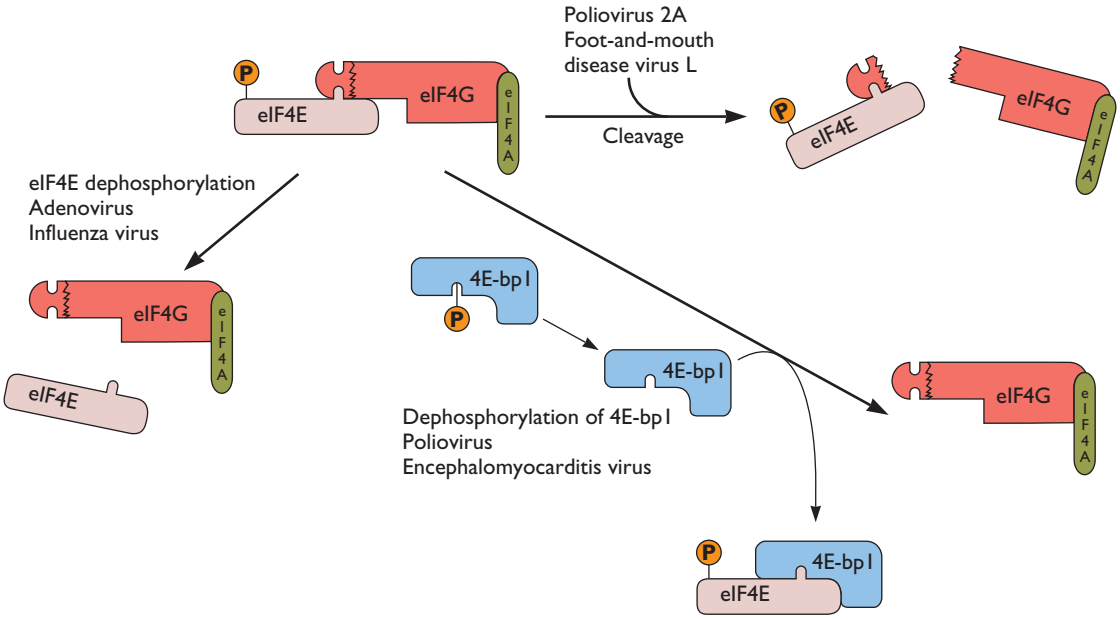


Figure 11.24 Inhibition of cellular translation in poliovirus-infected cells. (A) Rate of protein synthesis in poliovirus-infected and uninfected cells. During poliovirus infection, host cell translation is inhibited by 2 h after infection and is replaced by translation of viral proteins. Adapted from H. Fraenkel-Conrat and R. R. Wagner (ed.), *Comprehensive Virology* (Plenum Press, New York, NY, 1984), with permission. **(B)** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled proteins at different times after poliovirus infection. In this experiment, host translation was shut off by 5 h postinfection and was replaced by the synthesis of viral proteins, some of which are labeled at the right.

blocks phosphorylation of eIF4E. Adenoviral late mRNAs continue to be translated because they possess a reduced requirement for eIF4E. The majority of these viral mRNAs contain the tripartite leader (Fig. 10.12), a common 5′ noncoding region that mediates translation by ribosome shunting. Initiation by this mechanism is less dependent on eIF4F, presumably because the shunting of part of the 5′ untranslated region reduces the requirement for RNA-unwinding (helicase) activity associated with initiation by cap binding and scanning. Furthermore, adenovirus late

Figure 11.25 Regulation of eIF4F activity. The illustration shows regulation of eIF4F activity, and inhibition of translation, by dephosphorylation of eIF4E, interaction with two eIF4E-binding proteins, and proteolytic cleavage of eIF4G.



mRNAs efficiently recruit the small quantities of phosphorylated eIF4E present late in infection, a feature of mRNAs with little RNA secondary structure near the 5′ cap. The tripartite leader therefore confers selective translation of viral over cellular mRNAs under conditions in which eIF4E is underphosphorylated. Adenovirus-induced translation inhibition not only boosts viral late mRNA translation but also enhances cytopathic effects and consequently release of virus from cells.

Despite the correlation between reduced phosphorylation of eIF4E and translation inhibition in virus-infected cells, it has also been reported that phosphorylation of this protein may have little or no effect on its affinity for the 5′-terminal cap structure and the rate of protein synthesis. The role of eIF4E phosphorylation during translation remains controversial.

Modulation of eIF4E Activity by Specific Binding Proteins

Three related low-molecular-weight cellular proteins, 4E-bp1, 4E-bp2, and 4E-bp3, can bind to eIF4E and inhibit translation following 5′-end-dependent scanning, but not by internal ribosome entry (Fig. 11.25). The first was found to be identical to a previously described protein, called phosphorylated heat- and acid-stable protein regulated by insulin (Phas-I). This protein was known to be an important substrate for phosphorylation in cells treated with insulin and growth factors. Phosphorylation of 4E-bp *in vitro* prevents it from associating with eIF4E. It was subsequently shown that when bound to 4E-bp, eIF4E cannot bind to eIF4G. As a result, active eIF4F is not formed. eIF4G and 4E-bp proteins carry a common sequence motif that binds eIF4E. Treatment of cells with hormones and growth factors leads, through signal transduction pathways, to the phosphorylation of 4E-bp and its release from eIF4E. Translation of mRNAs with extensive secondary structure in the 5′ untranslated region is preferentially sensitive to the phosphorylation state of 4E-bp.

Infection with several viruses results in alteration of the phosphorylation state of 4E-bp (Fig. 11.25). In contrast to the shutoff that occurs in poliovirus-infected cells, inhibition of cellular protein synthesis in encephalomyocarditis virus-infected cells occurs late in infection and is not mediated by cleavage of eIF4G. Rather, encephalomyocarditis virus infection induces dephosphorylation of 4E-bp1. As a result, translation of cellular mRNAs is inhibited, but, because the viral mRNA contains an IRES, its translation is unaffected. Phosphorylation of 4E-bp1 is also observed in cells infected with vesicular stomatitis virus, poxviruses, and herpes simplex virus type 1, but how this modification favors translation of viral mRNAs is not known.

Phosphorylation of 4E-bp is carried out by the mammalian target of rapamycin kinase (mTor). Rapamycin is an immunosuppressant that binds to an immunophilin protein (Fkbp). The latter binds to mTor and blocks phosphorylation of 4E-bp. As expected, treatment of cells with this drug inhibits translation initiation by 5′-end-dependent scanning but not by internal ribosome entry. The mTor complex regulates protein synthesis in response to a variety of signals, such as amino acid concentrations, energy state, and growth factors. As would be expected, mTor is activated during infection by a wide variety of viruses, leading to increased protein synthesis under conditions (e.g., virus-induced stress) that would otherwise limit translation.

Regulation of Poly(A)-Binding Protein Activity

The poly(A)-binding protein Pab1p plays a crucial role in mRNA translation, bringing together the ends of the mRNA (Fig. 11.4). In cells infected with rotaviruses, inhibition of host translation is a consequence of blocking the function of Pab1p (Fig. 11.26). The 3′ ends of rotaviral mRNAs are not polyadenylated and therefore cannot interact with this protein. Instead, these 3′ untranslated regions contain a conserved sequence that binds the viral protein nsP3. This viral protein associates with eIF4G, bringing together the viral mRNA ends. Therefore, nsP3 assumes the function of Pab1p in translation of rotavirus mRNAs. The nsP3 protein occupies the Pab1p-binding site of eIF4G, thereby evicting Pab1p and preventing juxtaposition of the mRNA ends. The binding of nsP3 to eIF4G is the molecular basis for rotavirus inhibition of host cell translation.

Regulation of eIF3

Three interferon-induced human genes, *ISG54*, *ISG56*, and *ISG60*, encode proteins (P54, P56, and P60) that bind subunits of eIF3 and block translation. The P56 protein binds the e subunit of eIF3, while P54 binds to the c and e subunits. Both P54 and P56 interfere with stabilization of the ternary complex (eIF2-GTP-tRNAi-Met), and P54 also blocks formation of the 48S initiation complex (Fig. 11.3). Both 5′-end-dependent and internal initiation are inhibited by P56. The inhibition of internal initiation by the hepatitis C virus IRES by P56 is probably one of the main reasons for the antiviral effect of interferon on replication of this virus.

Another mechanism for regulation of the activity of eIF3 is binding of one of the subunits, eIF3f, by the spike glycoprotein of severe acute respiratory syndrome (SARS) coronavirus. This interaction leads to reduced translation of cellular genes. It is not known how viral mRNAs are translated. The eIF3a and eIF3b subunits are cleaved by the

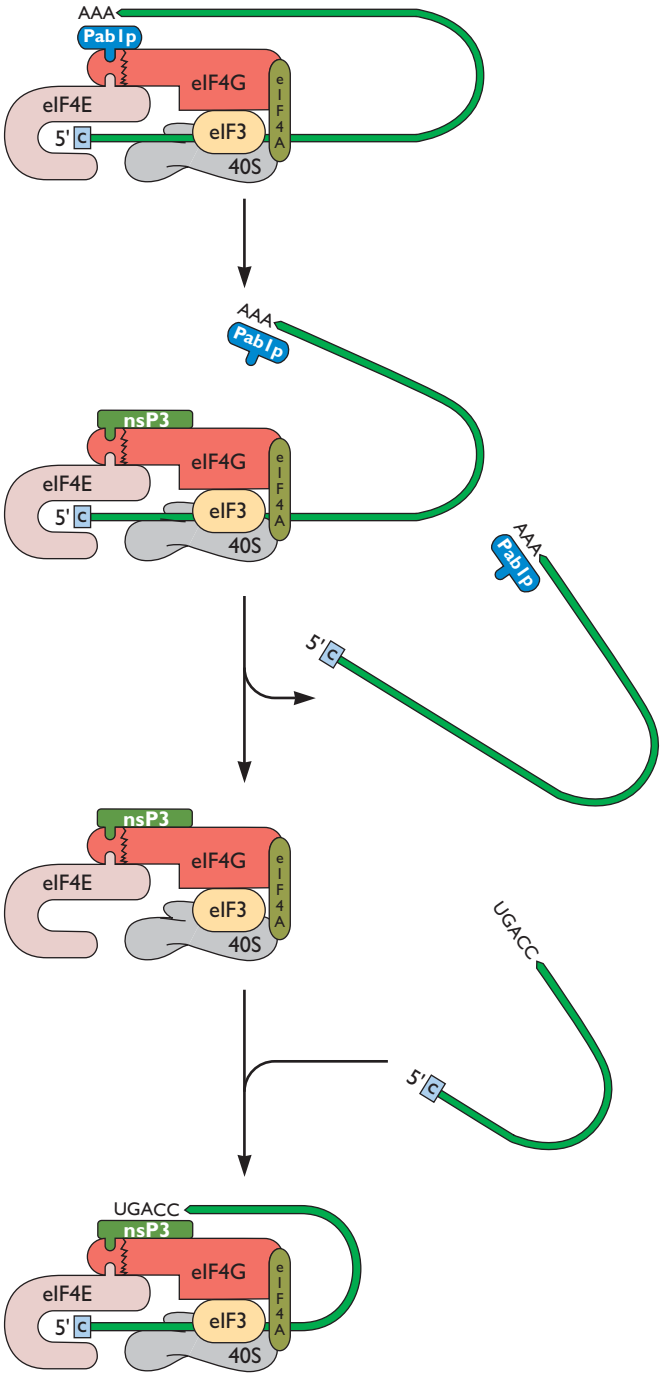


Figure 11.26 Eviction of Pab1p from eIF4G by rotavirus nsP3. The ends of host cell mRNA brought together by the interaction of Pab1p with poly(A) and eIF4G are shown at the top. Rotavirus nsP3 associates with eIF4G at the Pab1p-binding site, and also binds the 3′ untranslated region of the viral mRNA. As a result, host mRNAs are replaced by viral mRNAs in initiation complexes, and translation occurs in a 5′-end-dependent manner. Adapted from M. Piron, P. Vende, J. Cohen, and D. Poncet, *EMBO J.* **17:**5811–5821, 1998.

viral protease in cells infected with the picornavirus foot-and-mouth disease virus, further contributing to inhibition of host protein synthesis caused by cleavage of eIF4G.

Regulation by miRNA

Micro-RNAs (miRNAs) (Chapter 10) regulate the expression of ~30% of all mammalian protein-coding genes by influencing either mRNA stability or translation. They function as part of a ribonucleoprotein (miRNP or RNA-induced silencing complex [Risc]) which includes members of the Argonaute family of proteins. The target site on an mRNA is most often within the 3′ noncoding region and is present in multiple copies (Fig. 11.27). There is evidence that binding of miRNPs to the 3′ noncoding region inhibits its translation at the initiation step. In one study, miRNA blocked cap-dependent but not IRES-dependent initiation. It has been suggested that the Argonaute 2 protein competes with eIF4E for binding to the 5′ cap. Translational repression depends not only on the presence of a 5′ cap but also on a poly(A) tail. Alternative mechanisms of action of miRNAs include inhibition of joining of the 60S ribosomal subunit, and interference with elongation and termination. The mechanisms have not yet been established.

miRNAs clearly play important roles in virus infections. They may modulate inflammatory responses to virus infections. As might be expected, viral gene products, such as adenovirus VA-RNA I, that block production of miRNAs have been identified. miRNAs may also influence cell susceptibility: a liver-specific miRNA has been identified which markedly enhances hepatitis C virus replication. The genomes of some mammalian viruses have been shown to encode miRNAs. One miRNA encoded in the genome of simian virus 40 reduces viral gene expression, thus reducing elimination of infected cells by cytotoxic T lymphocytes. miRNAs will probably be found to have enormous influence on viral pathogenesis, and may also present new opportunities for interfering with viral growth (Volume II, Chapter 9).

Perspectives

The study of protein synthesis in virus-infected cells has revealed a great deal about how proteins are made and how the process is regulated. Very early in infection, intrinsic defense responses are mounted, and protein synthesis is inhibited in an attempt to limit viral replication. Should infection proceed, cellular stress responses, which cause further reduction in translation, are activated. As viral proteins and RNAs are produced, modifications to the cellular translation apparatus take place to favor the production of viral proteins. The interplay of cellular and viral modifications is an important determinant of the outcome of infection.

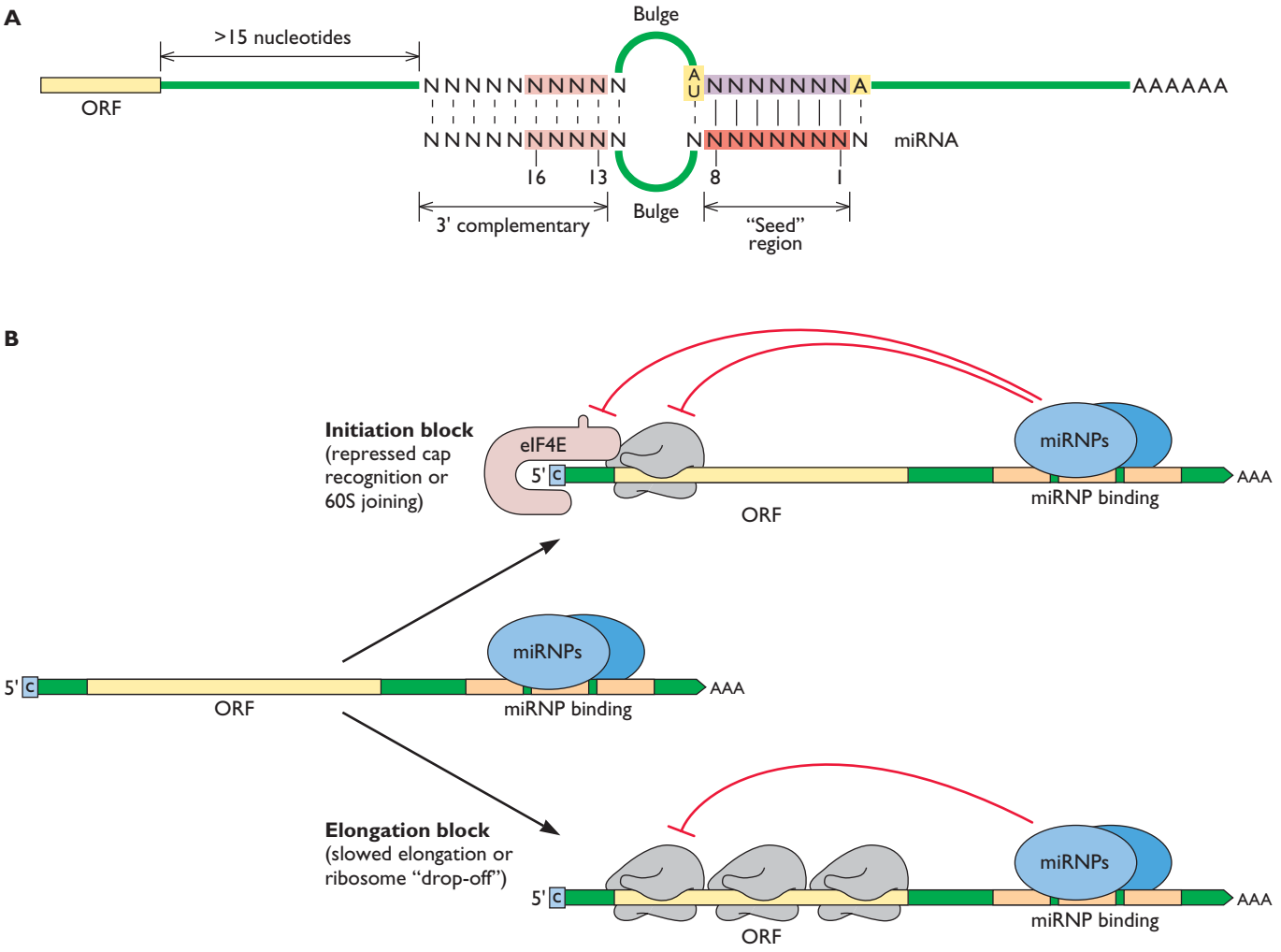


Figure 11.27 Models for miRNA inhibition of translation. (A) Base pairing of miRNA in the 3′ untranslated region of mRNA. Perfect base pairing of nucleotides 2 to 8 (purple/red) in the seed region nucleates the association of the two RNAs. Mismatches in this region decrease repression, with the exception of the two bases shown in yellow. If the central bulge is not present, endonucleolytic cleavage by Argonaute occurs. Good base pairing is required in the 3′ complementarity region, especially in the bases shown in pink. **(B)** Two possible mechanisms of translational repression mediated by miRNA. Binding of miRNPs can impair initiation (top) or elongation (bottom).

Despite years of work in many laboratories, many aspects of translational regulation during virus infection remain unclear. Unanswered questions include the role of phosphorylation in activity of eIF4E, why translation is made more efficient when mRNA ends are juxtaposed, and how viral mRNAs are preferentially translated. What are the signals that lead to phosphorylation of eIF4E-binding proteins through mTor? How are stress responses counteracted so that virus infection can proceed? How do eIF2α kinases benefit viral replication?

The recent discovery of miRNA in mammalian cells has led to the realization that these small RNAs may have significant impact on the outcome of viral infection. An important question is whether miRNAs block protein synthesis at one or more steps. Although results from many laboratories suggest that miRNAs interfere at many steps in translation, it is too early to make general conclusions, because so many different experimental systems have been examined. It is possible that translation initiation is the main target for inhibition, which then leads to alterations in elongation, termination, mRNA, and protein stability. Understanding how miRNAs inhibit translation is likely to improve our understanding of this essential process, and reveal new ways to limit viral infections.

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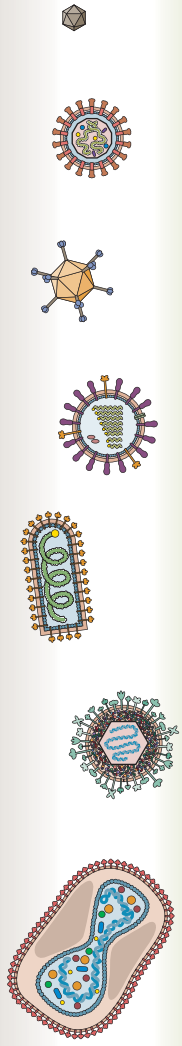


12

Intracellular Trafficking

Anatomy is destiny.
SIGMUND FREUD

Introduction

Successful viral reproduction requires the intracellular assembly of progeny virions from their protein, nucleic acid, and, in many cases, membrane components. In preceding chapters, we have considered molecular mechanisms that ensure the synthesis of the macromolecules from which virions are constructed in the host cell. Because of the structural and functional compartmentalization of eukaryotic cells, virion components are generally produced at multiple intracellular locations, and must be brought together for assembly. Intracellular trafficking, or sorting, of viral nucleic acids, proteins, and glycoproteins to the appropriate sites is therefore an essential prelude to the assembly of all animal viruses.

From our point of view, animal cells are very small, with typical diameters of 10 to 30 μm. However, in the microscopic world inhabited by viruses, an animal cell is large: the distances over which virion components must be transported within a cell are roughly equivalent to up to a mile on the macroscopic, human scale. The properties of the intracellular milieu indicate that viral particles, genomes, or subassemblies could not reach the appropriate intracellular destinations during entry or egress within reasonable periods simply by diffusion (Boxes 5.2 and 12.1). Their movement therefore requires transport systems and a considerable expenditure of energy, supplied by the host cell. The cellular highways most commonly used for movement of viral components for assembly are those formed by microtubules (as is also true during entry). These filaments are polarized and highly organized within the cell, with (−) ends at the centrosome (near the nucleus) and (+) ends at the cell periphery. They are traveled by cellular (−) end- and (+) end-directed motor proteins that convert the chemical energy of ATP into kinetic energy, and carry cargo.

The intracellular trafficking of viral macromolecules must be appropriately directed, so that individual virion components are delivered to the correct assembly site. Virion assembly can occur at any one of several intracellular addresses, depending on whether the particles are enveloped or naked,

BOX 12.1 DISCUSSION

Getting from point A to point B in heavy traffic

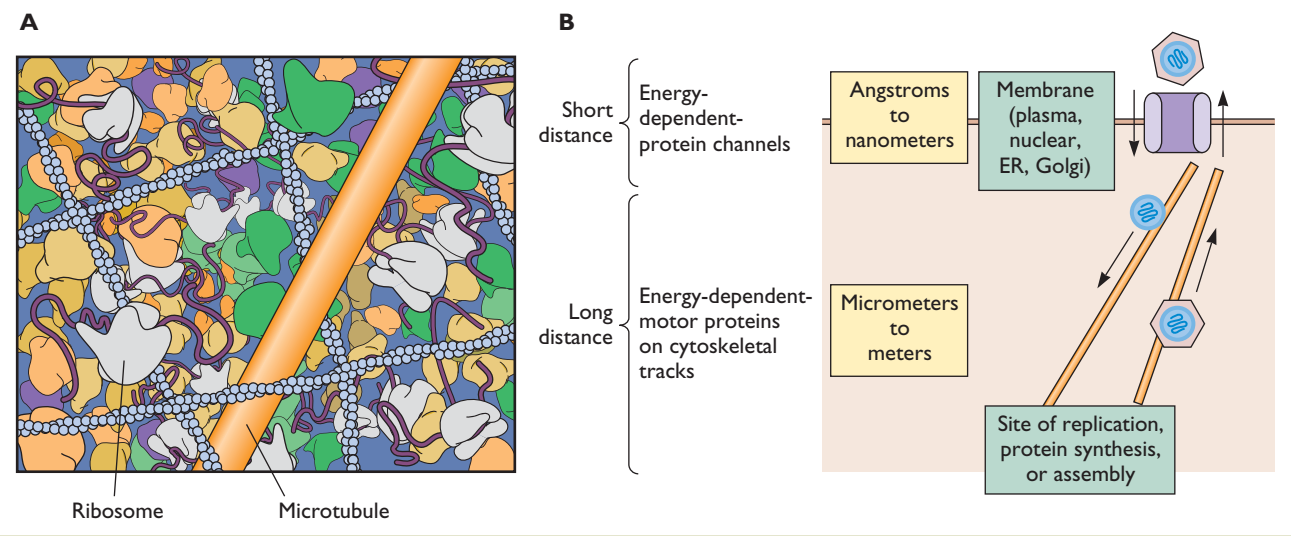
Major problems in cell biology are directional movement and coordination of such movements in space and time. Concentrations of high-molecular-weight reactants and products are rarely controlled by diffusion, as they are *in vitro*. Indeed, the inside of a cell is so tightly packed with organelles and cytoskeletal structures (panel A in the figure) that it is simply inappropriate to think of the contents of the cytoplasm, the nucleus, or organelle lumens as a "gel" or a "suspension."

Directional movement in cells is achieved by two general processes (panel B). Short-distance movement across membranes, or in and out of capsids, is measured in angstroms to nanometers and is accomplished primarily via protein channels.

- Common channels are transporters, translocons, pores, and portals.
- Movement generally requires energy.
- Diffusion in the classical sense contributes little to the process.

Long-distance movement of proteins, viral particles or their components, and organelles inside cells is measured in micrometers to meters. It

- invariably requires energy
- is mediated by molecular motors moving on cytoskeletal tracks; myosin motors move cargo on actin fibers, while dynein and kinesin motors move cargo on microtubules.



and on the site of and mechanism of genome replication (Table 12.1). All viral envelopes are derived from one of the host cell's membranes, which are modified by insertion of viral proteins. Many virus particles assemble at the plasma membrane, but some envelopes are derived from membranes of internal compartments. Assembly of enveloped viruses therefore requires delivery of some viral proteins to the appropriate membrane, as well as transport of other virion proteins and the nucleic acid genome to the modified membrane. Other common assembly sites are the cell nucleus and within the cytoplasm, where all virion components are also made. These strategies impose less complex trafficking problems than does assembly of enveloped viruses at membrane sites, but additional mechanisms may be required for egress of progeny virions from the cell. In some cases, genome-containing nucleocapsids are formed in infected cell nuclei, but virion assembly is completed at a cellular membrane. Such spatial and temporal separation of assembly reactions depends on appropriate coordination among multiple transport processes.

The need for movement of proteins and nucleic acids from one cellular compartment to another, or for insertion of proteins into specific membranes, is not unique to viruses. The majority of cellular proteins are made by translation of messenger RNAs (mRNAs) by cytoplasmic polyribosomes and must then be transported to their sites of operation. Similarly, most cellular RNA species are exported from the nucleus, in which transcription takes place. Eukaryotic cells are therefore constantly engaged in transport of macromolecules among their compartments via intracellular trafficking systems. The cellular systems that sort macromolecules to each of many possible intracellular sites are just as indispensable for viral replication

Table 12.1 Intracellular trafficking requirements for virus assembly

Assembly site(s)	Virus(es)	Trafficking requirements
Within the nucleus	Adenovirus, papovavirus	Transport of structural proteins from cytoplasm to nucleus
Within the cytoplasm	Picornavirus	Transport of structural proteins to specialized vesicles in which genome replication and assembly take place
At the plasma membrane	Alphavirus, retrovirus, rhabdovirus	Transport of viral glycoproteins to the plasma membrane; transport of other virion proteins made in the cytoplasm to the plasma membrane; transport of viral RNA genomes from nuclear or cytoplasmic sites of synthesis to the plasma membrane
At an internal cellular membrane	Bunyavirus, coronavirus, poxvirus	Transport and sorting of viral glycoproteins to the appropriate internal membrane; transport of other virion proteins and genomes to the internal membrane
Within the nucleus and at a cellular membrane	Herpesvirus, orthomyxovirus	Transport of structural proteins to the nucleus for assembly of the nucleocapsid; transport of the nucleocapsid from the nucleus to the membrane site of assembly; transport of internal virion proteins from cytoplasmic sites of synthesis to the membrane assembly site; transport and sorting of viral glycoproteins to the membrane assembly site

as the cellular biosynthetic machineries responsible for transcription, DNA synthesis, or translation. Indeed, the advances in our understanding of cellular trafficking mechanisms can be traced to initial studies with viral membrane or nuclear proteins. In the following sections, the cellular transport pathways required during viral reproduction are described in the context of the site at which virion assembly takes place.

Assembly within the Nucleus

Assembly of the majority of viruses with DNA genomes, including adenoviruses, papillomaviruses and polyomaviruses, takes place within infected cell nuclei, the site of viral DNA synthesis. All structural proteins of these nonenveloped viruses are imported into the nucleus following synthesis in the infected cell cytoplasm (Fig. 12.1), allowing complete assembly within this organelle (Table 12.1). In contrast, assembly of the more complex herpesviruses, which contain a DNA-containing nucleocapsid assembled within the nucleus, is completed at extranuclear sites (Table 12.1), as is that of some enveloped RNA viruses, such as orthomyxoviruses. In these cases, a subset of virion proteins must be imported into the nucleus.

As far as we know, all viral structural proteins that enter the nucleus do so via the normal cellular pathway of nuclear protein import. As discussed in Chapter 5, these same pathways are responsible for import of both viral genomes (or nucleoproteins) and viral nonstructural proteins that function in the nucleus early in the infectious cycle. Proteins destined for the nucleus are so labeled by the presence of nuclear localization signals (see Fig. 5.21). These signals are recognized by components of the cellular nuclear import machinery for subsequent transport into the nucleus.

Import of Viral Proteins for Assembly

Viral proteins that are known to be localized to the nucleus generally contain specific sequences that target them for import (see Fig. 5.21). The nuclear localization signals of viral proteins cannot be distinguished from those of cellular proteins. Indeed, many of the efforts to identify the cellular receptors that initiate nuclear import employed the well-characterized nuclear localization signal of simian virus 40 large T antigen. It is therefore thought that viral proteins that enter the nucleus contain typical nuclear localization signals. Nevertheless, such signals have been verified for only a small fraction of the many viral proteins that are imported into the nucleus. Furthermore, there are a large number of cellular, nuclear localization signal receptors, some of which are expressed in tissue-specific fashion. These receptors may recognize different types of viral (and cellular) nuclear localization signals.

The cellular components that mediate import of viral proteins into the nucleus are present at finite concentrations. A typical mammalian cell contains on the order of 3,000 to 4,000 nuclear pore complexes, each with a very high translocation capacity (a mass flow of up to 80 MDa/s with 10^3 translocation events/s). However, nuclear import also depends on the limited supply of soluble transport proteins. As large quantities of viral structural proteins must enter the nucleus prior to assembly, there is potential for competition among viral and cellular proteins for access to receptors, the nuclear pore complex, or transport proteins. Such competition is minimized in cells infected by the larger DNA viruses, such as adenoviruses and herpesviruses: by the time structural proteins are made during the late phase of infection, cellular protein synthesis is inhibited severely. The proteins of viruses that do not induce inhibition of cellular protein synthesis, such as those of the

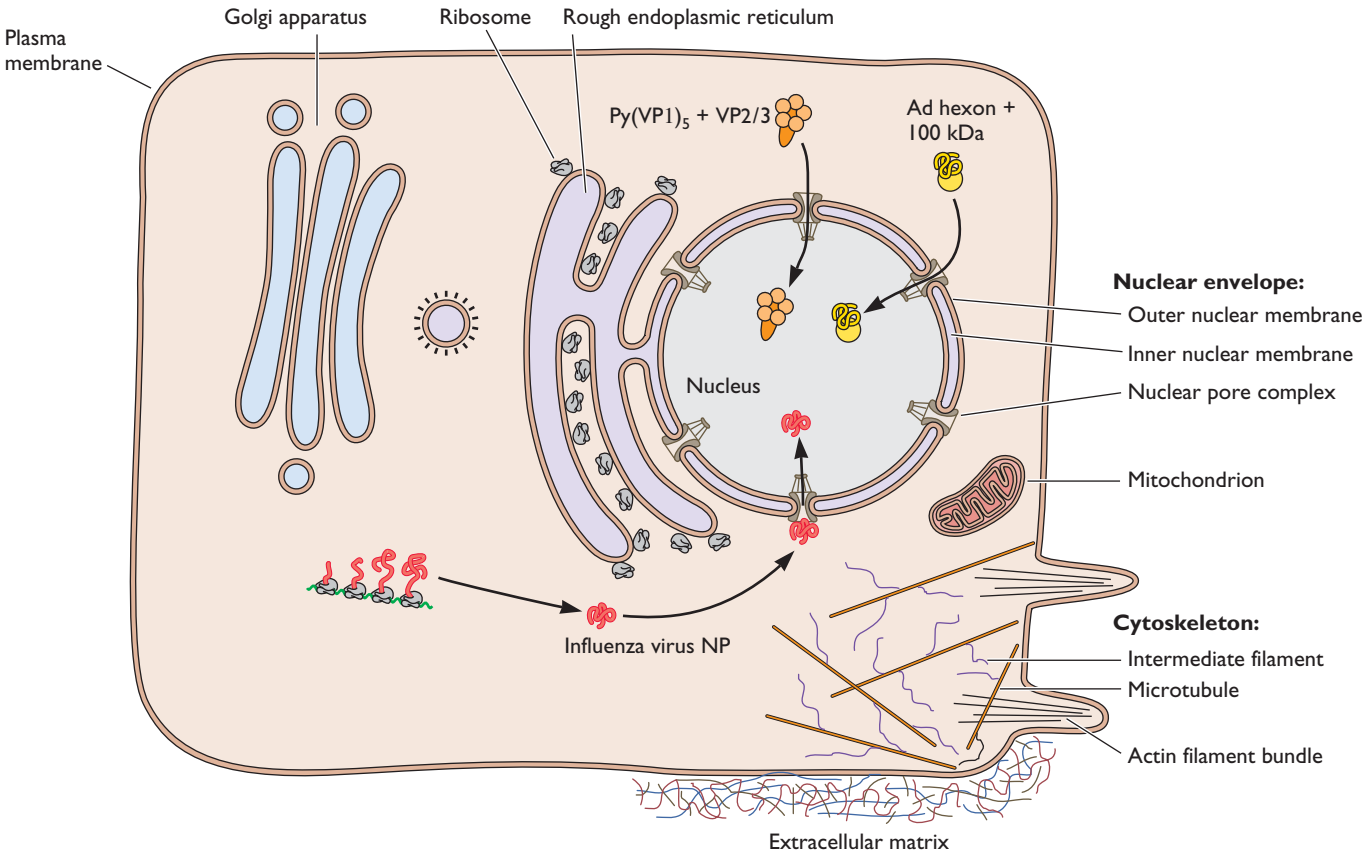


Figure 12.1 Localization of viral proteins to the nucleus. The nucleus and major membrane-bound compartments of the cytoplasm, as well as components of the cytoskeleton, are illustrated schematically and not to scale. Viral proteins destined for the nucleus are synthesized by cytoplasmic polyribosomes, as illustrated for the influenza virus NP protein. They engage with the cytoplasmic face of the nuclear pore complex and are translocated into the nucleus by the protein import machinery of the host cell. Some viral structural proteins enter the nucleus as preassembled structural units (polyomaviral [Py] VP1 pentamers associated with one molecule of either VP2 or VP3) or in association with a viral chaperone (adenoviral [Ad] hexon monomers bound to the L4 100-kDa protein).

polyomaviruses, must enter the nucleus despite continual transport of cellular proteins. Whether import of viral proteins is favored in such circumstances, for example, by the presence of particularly effective nuclear localization signals, is not known.

Many viral structural proteins that enter infected cell nuclei form multimeric capsid components. In some cases, structural units of the virion are assembled in the cytoplasm prior to import into the nucleus. Pentamers of the major capsid protein (VP1) of simian virus 40 and polyomavirus specifically bind a monomer of either VP2 or VP3, the minor virion proteins, which share C-terminal sequences (Appendix, Fig. 15B). Such heteromeric assemblies are the substrates for import into the nucleus. Indeed, efficient nuclear localization of polyomavirus VP2 and VP3 proteins occurs only in cells in which VP1 is also made. Assembly of the heteromeric complex facilitates import of the minor structural proteins, even though each contains a nuclear localization signal. The increased density of these signals may allow more effective competition for essential components of the import pathway, or the nuclear localization signals may be more accessible in the complex.

Despite such potential advantages as increased efficiency of import of viral proteins and transport of the structural proteins in the appropriate stoichiometry, import of preassembled capsid components is not universal. For example, adenoviral hexons, which are trimers of viral protein II, are found only in the nucleus of the infected cell. Association of newly synthesized hexon monomers with a late, nonstructural protein, the L4 100-kDa protein, is essential for both the entry of hexon subunits into the nucleus and their assembly into trimers. The monomeric

hexon-L4 protein complex must be the import substrate. The viral L4 100-kDa protein might supply the nuclear localization signal for hexon import, or promote nuclear retention, by facilitating assembly of hexon trimers within the nucleus.

Assembly at the Plasma Membrane

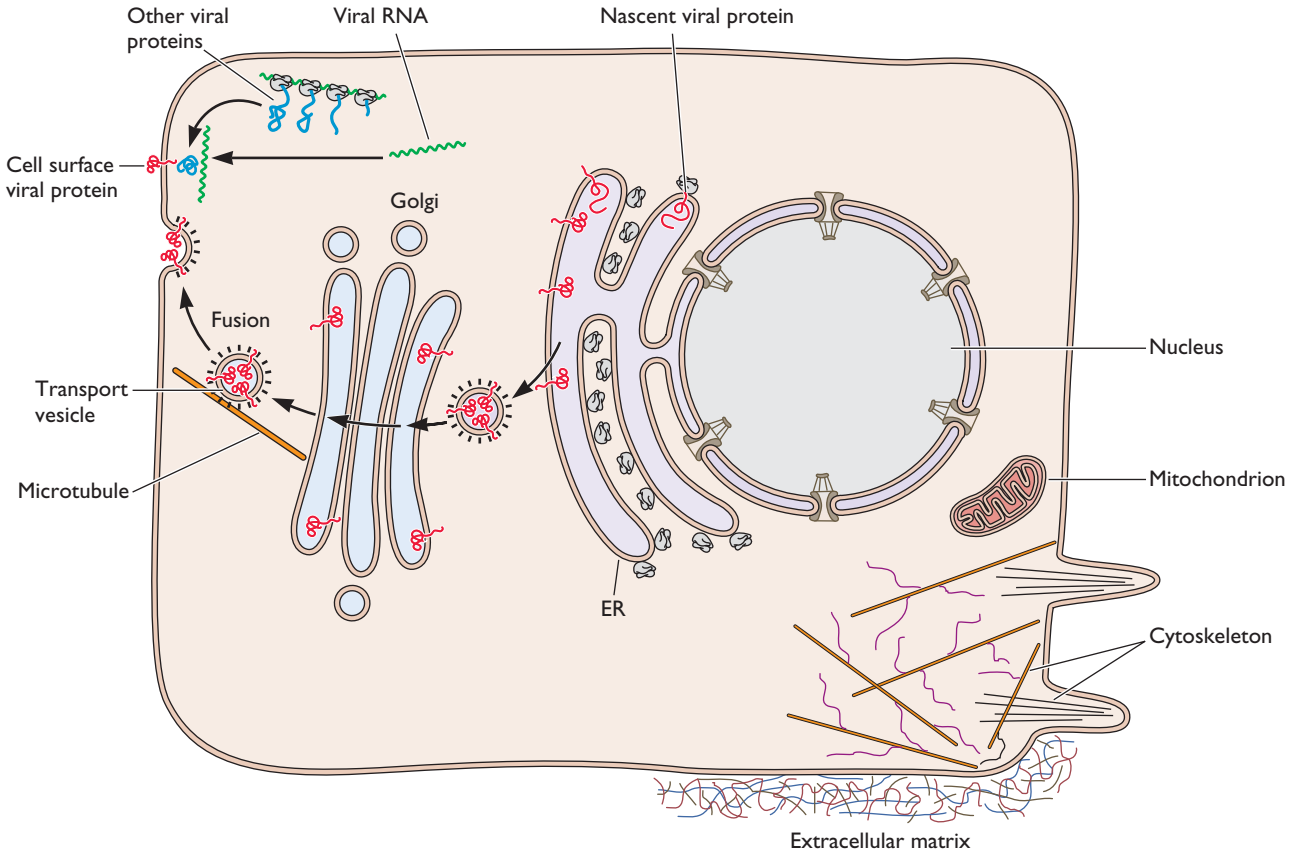
Assembly of enveloped viruses frequently takes place at the plasma membrane of infected cells (Table 12.1). Before such virions can form, viral integral membrane proteins must be transported to this cellular membrane (Fig. 12.2). The first stages of the pathway by which viral and cellular proteins are delivered to the plasma membrane were identified more than 30 years ago, and the process is now quite well understood. Viruses with envelopes derived from the plasma membrane also contain internal proteins, which may be membrane associated, and, of course, nucleic acid genomes. These internal components must also be sorted to appropriate plasma membrane sites for assembly (Fig. 12.2).

Transport of Viral Membrane Proteins to the Plasma Membrane

Viral membrane proteins reach their destinations by the highly conserved, cellular **secretory pathway**. Many of the steps in the pathway have been studied by using viral membrane glycoproteins, such as the vesicular stomatitis virus G and influenza virus hemagglutinin (HA) proteins. These viral proteins offer several experimental advantages: they are frequently synthesized in large quantities, their synthesis is initiated in a controlled fashion following infection, and their transport can be studied readily by genetic, biochemical, and imaging methods.

Entry into the first staging post of the secretory pathway, the endoplasmic reticulum (ER), is accompanied by membrane insertion of integral membrane proteins. Viral envelope proteins generally span the cellular membrane into which they are inserted only once, and therefore contain a single transmembrane domain. In viral proteins, transmembrane segments (described in Chapter 5) usually

Figure 12.2 Localization of viral proteins to the plasma membrane. Viral envelope glycoproteins (red) are cotranslationally translocated into the ER lumen and folded and assembled within that compartment. They travel via transport vesicles to and through the Golgi apparatus and from the Golgi apparatus to the plasma membrane. The internal proteins of the particle (purple) and the genome (green) are also directed to plasma membrane sites of assembly.



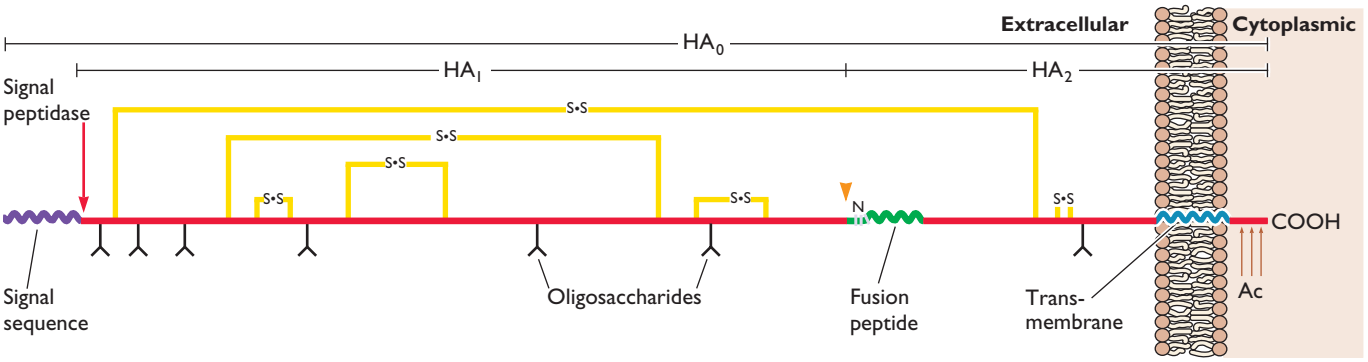


Figure 12.3 Primary sequence features and covalent modifications of the influenza virus HA protein. The primary sequence of the HA0 protein is depicted by the red line in the center, with the orange arrowhead indicating the site of the proteolytic cleavages that produce the HA1 and HA2 subunits. The fusion peptide, the N-terminal signal sequence which is removed by signal peptidase in the ER, and the C-terminal transmembrane domain are hydrophobic. Disulfide bonds, one of which maintains covalent linkage between the HA1 and HA2 proteins following HA0 cleavage, are indicated, as are sites of N-linked glycosylation (oligosaccharides) and palmitoylation (Ac).

separate large extracellular from smaller cytoplasmic domains (Fig. 12.3). The former include the binding sites for cellular receptors, crucial for initiation of the infectious cycle, whereas the latter are important in virus assembly. As discussed in Chapter 4, viral membrane proteins are usually oligomers. Most interactions among the subunits of viral membrane proteins are noncovalent, but some examples of association via covalent interchain disulfide bonds are known. Oligomer assembly takes place during transit from the cytoplasm to the cell surface, as does the proteolytic processing necessary to produce some mature (functional) envelope glycoproteins from the precursors that enter the secretory pathway. For example, the influenza virus HA0 precursor is cleaved into HA1 and HA2 subunits (Fig. 12.3). Viral (and cellular) proteins that travel the secretory pathway also possess distinctive structural features, including disulfide bonds and covalently linked oligosaccharide chains (Fig. 12.3). As illustrated in Figure 12.4 for the influenza A virus HA0 protein, these characteristic covalent modifications (as well as oligomerization) take place as proteins travel through a series of specialized compartments that provide the chemical environments and enzymatic machinery necessary for their maturation. The first such compartment, the ER, is encountered by viral membrane proteins as they are synthesized.

Translocation of Viral Membrane Proteins into the Endoplasmic Reticulum

All proteins destined for insertion into the plasma membrane, or the membranes of such intracellular organelles as the Golgi apparatus, enter the ER as they are translated (Fig. 12.2). This membranous structure appears as a basketwork of tubules and sacs extending throughout the cytoplasm (Fig. 12.5A). The ER membrane demarcates a geometrically convoluted but continuous internal space, the **ER lumen**, from the remainder of the cytoplasm. The ER lumen has a chemically distinctive environment, topologically equivalent to the outside of the cell. Proteins that enter the ER during their synthesis are therefore sequestered from the cytoplasmic environment as they are made.

Most proteins, regardless of their final ports of call, are synthesized on polyribosomes in the cytoplasm. However, polyribosomes engaged in synthesis of proteins that will enter the secretory pathway become associated with the cytoplasmic face of the ER membrane soon after translation begins. Areas of the ER to which polyribosomes are bound form the **rough ER** (Fig. 12.5B). The association of polyribosomes with the ER membrane is directed by a short sequence in the nascent protein termed the **signal peptide**. It is now taken for granted that the primary sequences of proteins include "zip codes" specifying the cellular addresses at which the proteins must reside to fulfill their functions, such as the nuclear localization signals discussed in the previous section. The signal peptides of proteins that enter the ER lumen were the first such zip codes to be identified, and established this paradigm some 30 years ago. Signal peptides are commonly found at the N termini of proteins destined for the secretory pathway. They are usually about 20 amino acids in length and contain a core of 15 hydrophobic residues. Signal peptides are often transient structures that are removed enzymatically during protein translocation into the ER by a protease located in the lumen, signal peptidase.

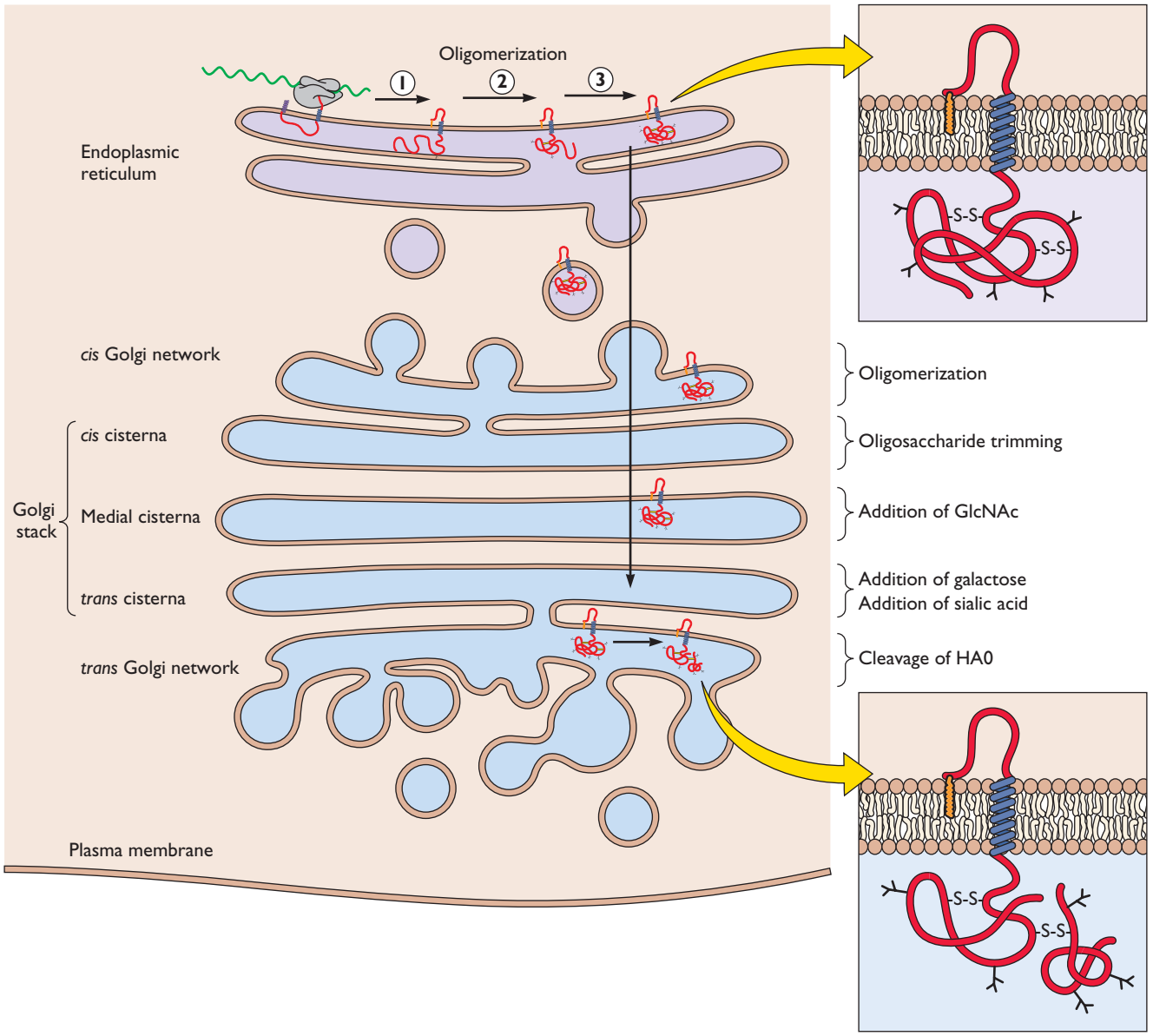


Figure 12.4 Maturation of influenza virus HA0 protein during transit along the secretory pathway. The modifications that occur during transit of the influenza virus HA0 protein through the various compartments of the secretory pathway are illustrated. In the ER, these are translocation and signal peptide cleavage (1), disulfide bond formation, and addition of N-linked core oligosaccharides (2), as the protein folds (3). The cytoplasmic domain acquires palmitate (orange) (see insert, top) while the protein travels to the plasma membrane, but it has not been established when this modification takes place. For simplicity, the protein is depicted as a monomer, although oligomerization also takes place in the ER lumen. Note that the protein domain initially introduced into the ER lumen, in this case the N-terminal portion of the protein (type I orientation), corresponds to the extracellular domain of the cell surface protein.

Translation of a protein that will enter the ER begins in the normal fashion and continues until the signal peptide emerges from the ribosome (Fig. 12.6). This signal then directs binding of the translation machinery to the ER membrane by means of two components: the signal peptide is recognized by the **signal recognition particle (SRP)**, which in turn binds to the cytoplasmic domain of an integral ER membrane protein termed the **SRP receptor**. Binding of the signal recognition particle to the ribosome temporarily halts translation, to allow the

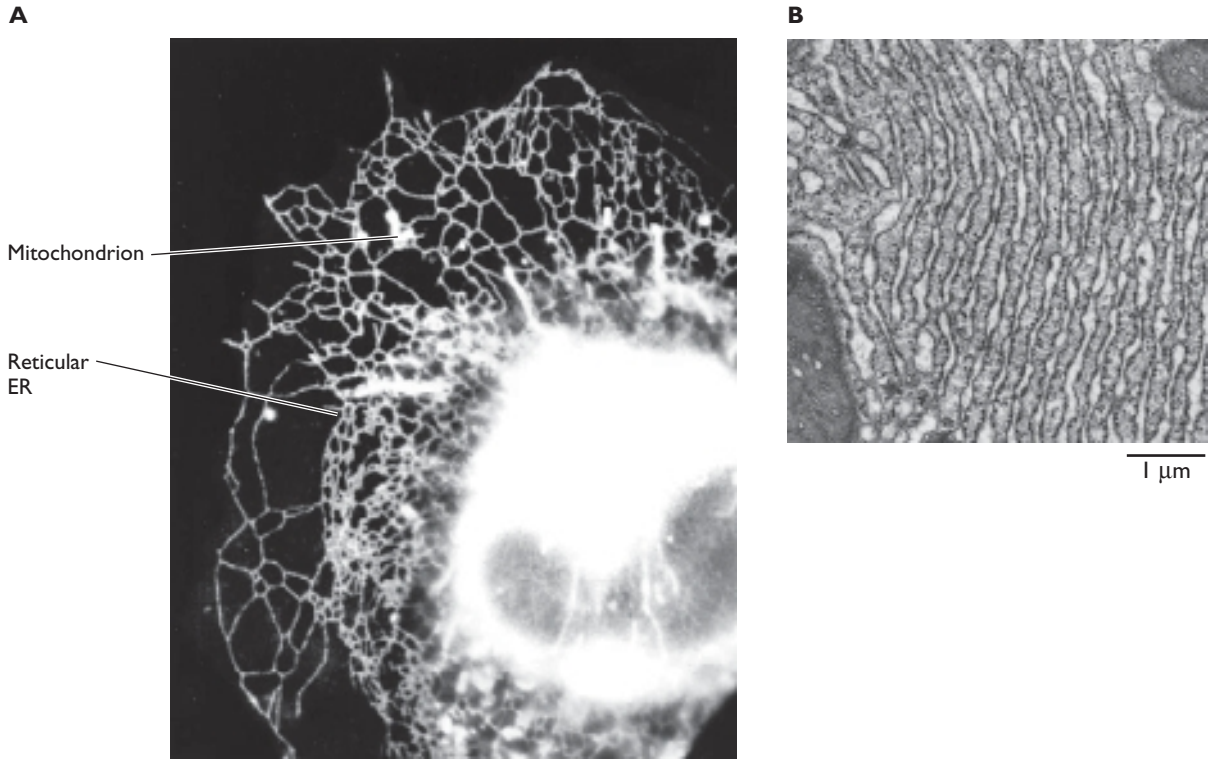


Figure 12.5 The endoplasmic reticulum. (A) The ER of a mammalian cell in culture. The reticular ER, which extends throughout the cytoplasm, was visualized by fluorescence microscopy of fixed African green monkey kidney epithelial cells stained with the lipophilic fluorescent dye 3,3′-dihexyloxacarbocyanine iodide. This dye also stains mitochondria. The ER membrane accounts for over half of the total membrane of a typical animal cell and possesses a characteristic lipid composition. Courtesy of M. Terasaki, University of Connecticut Health Center. **(B)** Electron micrograph of the rough ER in rat hepatocytes. Note the many ribosomes associated with the cytoplasmic surface of the membrane. From R. A. Rodewald, Biological Photo Service.

stalled translation complex to bind to the ER membrane via the SRP receptor. Both the signal recognition particle and its receptor contain subunits that bind guanosine triphosphate (GTP), and efficient targeting requires the presence of this nucleotide. Following the initial docking of the complex at the membrane, the ribosome becomes tightly bound to the membrane, and engaged with a protein translocation channel, which forms a gated, aqueous pore through the ER membrane. This interaction is coordinated with release of the signal recognition particle, association of the signal peptide with the translocation channel, and resumption of translation. Because the ribosome remains bound to the membrane upon release of signal recognition particle, continued translation facilitates movement of the growing polypeptide chain through the membrane. Such coupling of translation and translocation ensures that the protein crosses the membrane as an unfolded chain that can be accommodated within the translocation channel. Movement of the growing polypeptide through the membrane channel is facilitated by binding of the lumenal chaperone Grp78 (Bip) to the nascent protein.

When the protein entering the ER is destined for secretion from the cell, translocation continues until the entire polypeptide chain enters the lumen. During translocation, the signal peptide is proteolytically removed by signal peptidase, releasing the soluble protein into the ER. In contrast, translocation of integral membrane proteins, such as viral envelope proteins, halts when a hydrophobic **stop transfer signal** is encountered in the nascent protein. This sequence may be the signal peptide itself or a second, internal hydrophobic sequence. The number, location, and orientation of such sequences within a protein determine the topology with which it is organized in the ER membrane (Box 12.2). The programming of insertion of proteins into the ER membrane by signals built into their primary sequences ensures that every molecule of a particular protein adopts the identical topology in the

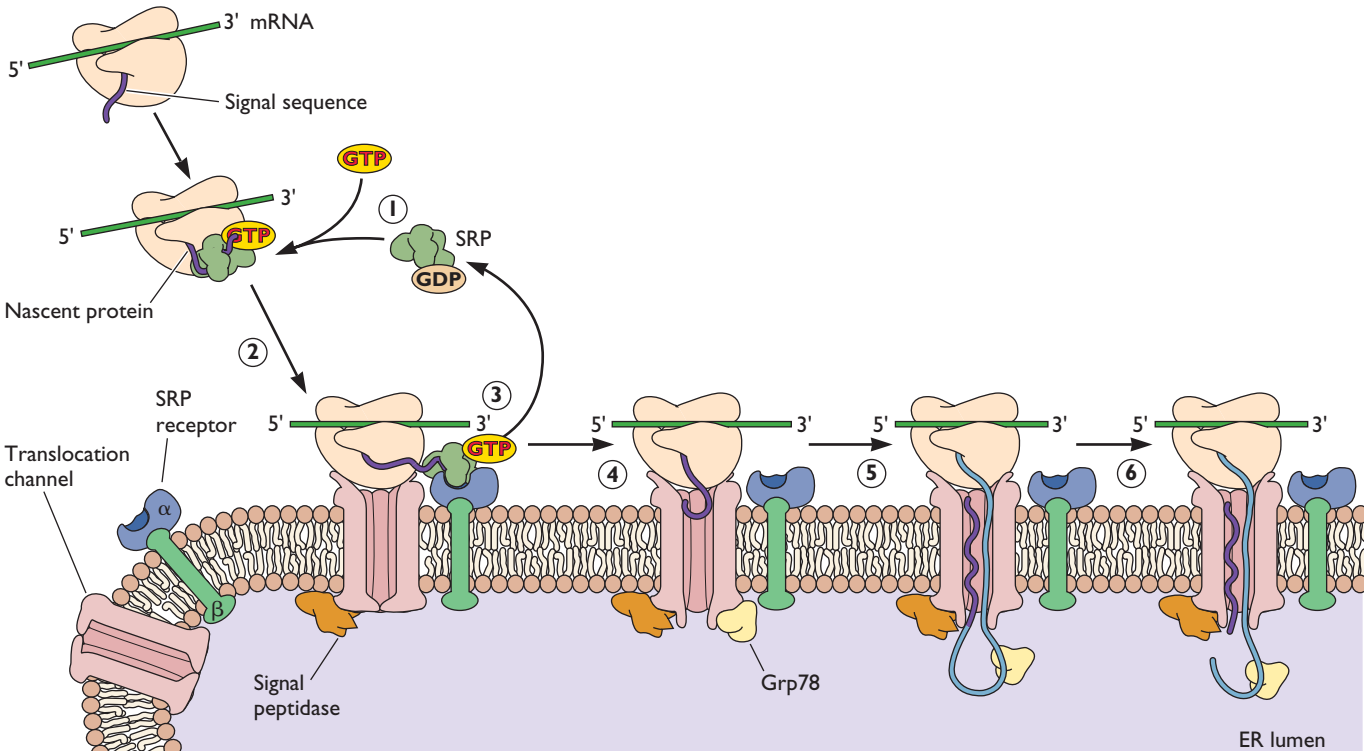


Figure 12.6 Targeting of a nascent protein to the ER membrane. Translation of an mRNA encoding a protein that will enter the ER lumen proceeds until the signal peptide (purple) emerges from the ribosome. The signal recognition particle (SRP), which contains a small RNA molecule and several proteins, binds to both the signal peptide and the ribosome to halt or pause translation, upon binding of GTP to one of the protein subunits (step 1). The nascent polypeptide-SRP-ribosome complex then binds to the SRP receptor in the ER membrane (step 2). This interaction triggers hydrolysis of GTP bound to both the SRP and its receptor, release of SRP (step 3), and close association of the ribosome with, and binding of the hydrophobic signal peptide to, the heterotrimeric protein translocation channel (step 4). These interactions trigger opening of the cytosolic end of the channel. The lumenal end of the translocation channel is also initially closed. Translation is then resumed, and the seal maintained at the lumenal end of the channel early in translocation is reversed by binding of the chaperone Grp78. The growing polypeptide chain is transferred through the membrane as its translation continues (step 5). In some cases, signal peptidase removes the signal peptide cotranslationally (step 6). A lateral gate in the channel opens within the membrane for transfer of the transmembrane domain(s) of translocated proteins into the ER membrane.

membrane. As this topology is maintained during the several membrane budding and fusion reactions by which proteins reach the cell surface, the way in which a protein is inserted into the ER membrane determines its orientation in the plasma membrane.

Reactions within the ER

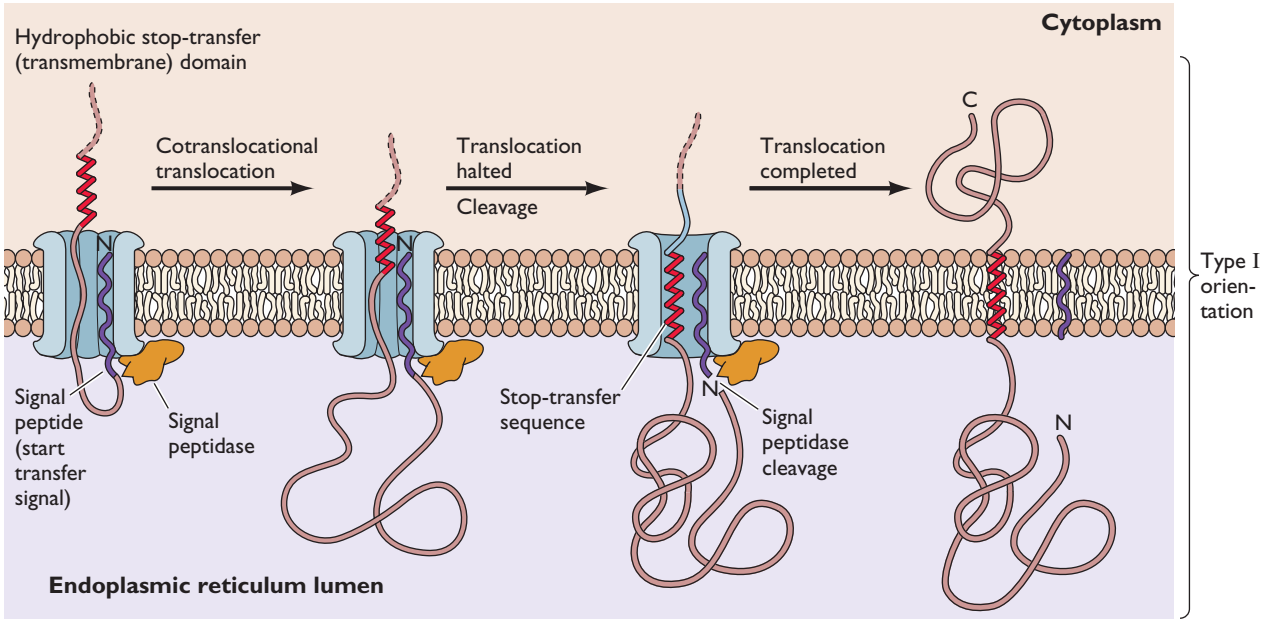
The folding and initial posttranslational modification of proteins that enter the secretory pathway take place within the ER. The lumen contains many enzymes that catalyze chemical modifications, such as disulfide bond formation and glycosylation, or that promote folding and oligomerization (Table 12.2).

Glycosylation. Viral envelope proteins, like cellular proteins that travel the secretory pathway, are generally modified by the addition of oligosaccharides to either asparagine (N-linked glycosylation) or serine or threonine (O-linked glycosylation). The presence of oligosaccharides on a protein can be detected, as changes in the protein's electrophoretic mobility, following exposure of cells to inhibitors of glycosylation, or of the protein to enzymes that cleave the oligosaccharide (Fig. 12.7A). The first steps in N-linked glycosylation take place as a polypeptide chain emerges into the ER lumen. Oligosaccharides rich in mannose preassembled on a lipid carrier are added to asparagine residues by an oligosaccharyltransferase (Fig. 12.7B).

BOX 12.2 BACKGROUND

Establishing the topology of integral membrane proteins that contain a single transmembrane domain

Many proteins with a single transmembrane domain, including the influenza virus HA0 protein, contain not only an N-terminal signal peptide, but also a second hydrophobic sequence. The signal peptide initiates cotranslational translocation of the nascent polypeptide through the protein translocation channel, as shown in more detail in Fig. 12.6. As shown in the figure, the resident ER enzyme signal peptidase (tan) cleaves off the signal peptide, forming the N terminus of the mature protein. Translocation continues until the second hydrophobic sequence is encountered. This sequence acts as a stop transfer signal and halts translocation, with lateral discharge of the protein into the ER membrane. This mechanism results in type I orientation (i.e., N terminus within the lumen), as in the influenza virus HA0 protein (Fig. 12.4). The position of the stop transfer sequence within the polypeptide determines the sizes of its lumenal (extracellular) and cytoplasmic domains. In proteins that contain multiple hydrophobic sequences of this kind, the first one encountered by the signal recognition particle starts the transfer, the second is a stop-transfer sequence, and so on.



Subsequently, several sugar residues are trimmed from N-linked core oligosaccharides in preparation for additional modifications that take place as the protein travels from the ER to the plasma membrane.

Sites of N-linked glycosylation are characterized by the sequence NXS/T (where X is any amino acid except proline), but not every potential glycosylation site is modified. Even a single specific site within a protein is not necessarily modified with 100% efficiency. Each glycoprotein population therefore comprises a heterogeneous mixture of **glycoforms**, varying in whether a particular site is glycosylated, as well as in the composition and structure of the oligosaccharide present at each site. As many viral and cellular proteins contain a large number of potential N-linked glycosylation sites, particular proteins can exist in an extremely large number of glycoforms. This property complicates investigation of the physiological functions of oligosaccharide chains present on glycoproteins. Nevertheless, this modification has been assigned a wide variety of roles.

As essential components of receptors and ligands, oligosaccharides participate in many molecular recognition reactions. These processes include binding of certain hormones to their cell surface receptors, interactions of cells with one another, binding of virions, such as influenza A virus and herpesviruses, to their host cells, and later steps in virus entry. Some sugar units serve as signals, targeting proteins to specific locations, in particular to lysosomes.

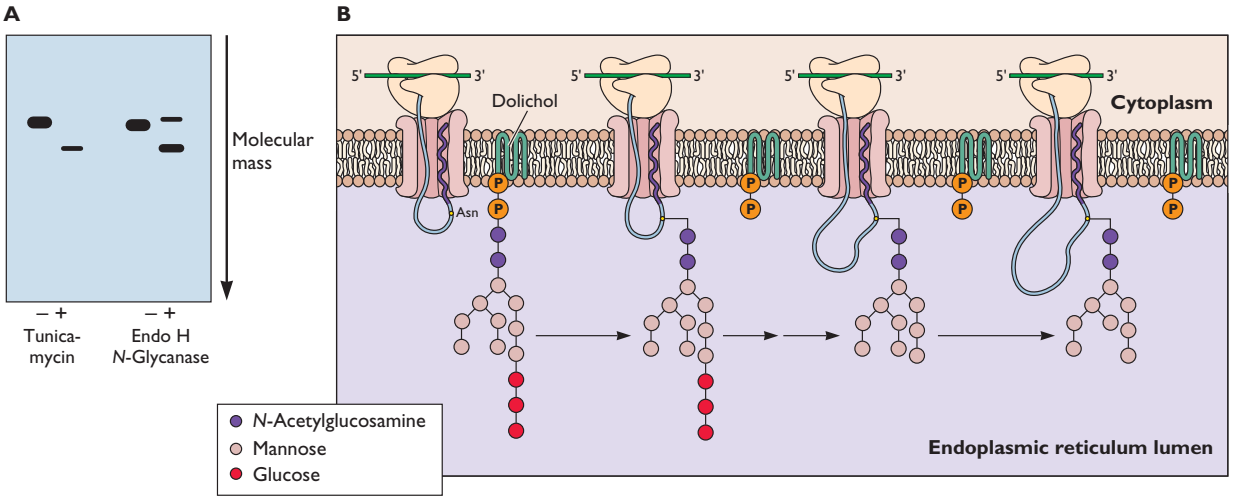
Table 12.2 Some ER enzymes and chaperones that participate in quality control

Protein	Properties and functions
Calnexin (Cnx)	Integral membrane lectin that binds to almost all glycoproteins in the ER; promotes proper folding; retains incompletely folded glycoproteins in the ER
Erp57	Soluble thioloxidoreductase; as cochaperone, binds to and functions with calnexin/calreticulin
Glucosidases I and II	Catalyze removal of terminal glucose residues from high-mannose, N-linked oligosaccharides; glucosidase II promotes release of substrates from Cnx
Grp78 (Bip)	Lumenal protein; binds to exposed hydrophobic surfaces on incompletely folded proteins; retains such proteins in the ER
Protein disulfide isomerase	Catalyzes formation and isomerization of disulfide bonds; retains incompletely folded or assembled proteins in the ER; appears to operate with Grp78/Bip
UDP-glucose-glycoprotein transferase	Lumenal ER enzyme that specifically recognizes unfolded proteins; adds glucose residues to their N-linked oligosaccharides; promotes recognition by Cnx and thus ER retention

Glycosylation has also been suggested to fulfill more general functions, such as protecting proteins (and virus particles) that circulate in body fluids from degradation and host immune defenses. Many proteins contain such a large number of glycosylation sites that carbohydrate can contribute more than 50% of the mass of the mature protein, for example, the poliovirus receptor and the respiratory syncytial virus G protein. The hydrophilic oligosaccharides are present on the surface of such proteins, where they can form a sugar "shell," masking much of the proteins' surfaces, and epitopes recognized by antiviral antibodies (Box 12.3).

Studies of viral glycoproteins have established that glycosylation is absolutely required for the proper folding of some of these proteins. For example, elimination (by mutagenesis) of all sites at which the vesicular stomatitis virus G or influenza virus HA proteins are glycosylated blocks the folding of these proteins and their exit from the ER (see "Protein folding and quality control" below). Before a protein folds, its hydrophobic amino acids, which are ultimately buried in the interior, are exposed. Such exposed hydrophobic patches on individual unfolded polypeptide chains tend to interact with one another nonspecifically, leading to aggregation. The hydrophilic oligosaccharide chains are thought to counter this tendency.

Figure 12.7 Detection and structure of N-linked oligosaccharides. (A) Detection of N-linked oligosaccharides using inhibitors or specific enzymes. Addition to cells of tunicamycin, an inhibitor of the first step in synthesis of the oligosaccharide precursor, prevents N-linked glycosylation, so that the mobility of glycoproteins is altered (left). *In vitro* treatment of glycoproteins with enzymes that cleave within the oligosaccharide, such as endoglycosidase H (Endo H) or *N*-glycanase, can also alter glycoprotein mobility (right). Glycosylation of a protein can also be assayed by incorporation of radioactively labeled monosaccharides. **(B)** The branched, mannose-rich oligosaccharide added via an N-glycosidic bond to asparagine residues of proteins is shown at the left. This common precursor is transferred to N-linked glycosylation sites as proteins are translocated into the ER, from the lipid carrier dolichol phosphate upon which the oligosaccharide is assembled. While within the ER, three glucose residues and one mannose residue are trimmed from the core oligosaccharide.

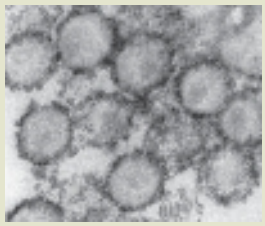


BOX 12.3 DISCUSSION

The evolving sugar "shield" of human immunodeficiency virus type 1

Mutational studies have implicated N-linked glycosylation at specific sites in the envelope proteins of several viruses in protection against host neutralizing antibodies. The Env protein of human immunodeficiency virus type 1 (HIV-1) provides a dramatic example of this phenomenon.

The SU (gp120) subunit of the HIV-1 Env protein carries a large number of oligosaccharide chains, which form a dense shell that masks much of the protein's surface (see the figure). These oligosaccharides govern several properties of HIV-1. For example, the tropism of the virus for CCr5 or CXCr4 coreceptors correlates with specific patterns of glycosylation in the variable loops of the SU subunit. However, a major function of such modification is to block access of host anti-HIV-1 antibodies to SU protein epitopes: high-resolution structural studies of the SU protein core have confirmed that N-linked oligosaccharides cover much of the protein's surface. Furthermore, the sugar chains are highly ordered, forming the outer surface of the Env spike. As predicted from this arrangement, N-linked glycosylation at specific sites blocks binding of monoclonal antibodies that recognize nearby sequences in the protein.



Electron micrograph of HIV-1 particles, showing carbohydrates stained with ruthenium red (dark). Courtesy of Edwin P. Ewing, Jr., Centers for Disease Control and Prevention (CDC), Atlanta, GA (CDC Public Health Image Library).

Several observations have led to the hypothesis that HIV-1 carries an evolving carbohydrate "shield" that enhances immune evasion. For example, the number of N-linked oligosaccharides added to SU tends to increase during the course of an HIV-1 infection, and the sites of N-linked glycosylation also change.

Chen, B., E. M. Vogan, H. Gong, J. J. Skehel, D. C. Wiley, and S. C. Harrison. 2005. Structure of an unliganded simian immunodeficiency virus gp120 core. *Nature* **433:**834–841.

Scanlan, C. N., J. Offer, N. Zitman, and R. A. Derek. 2007. Exploiting the defensive sugars of HIV-1 for drug and vaccine design. *Nature* **446:**1038–1045.

Disulfide bond formation. A second chemical modification that generally is restricted to proteins entering the secretory pathway, and essential for the correct folding of many, is the formation of intramolecular disulfide bonds between pairs of cysteine residues (Fig. 12.3). These bonds can make important contributions to the stability of a folded protein. However, they rarely form in the reducing environment of the cytoplasm. The more oxidizing ER lumen provides an appropriate chemical environment for disulfide bond formation. This compartment contains high concentrations of protein disulfide isomerase and other thioloxidoreductases, enzymes that catalyze the formation, reshuffling, or even breakage of disulfide bonds under appropriate redox conditions (Table 12.2). As formation of the full and correct complement of disulfide bonds in a protein is often the rate-limiting step in its folding, these enzymes are important catalysts of this process.

The cellular enzymes that promote formation of disulfide bonds are present in the ER lumen. Consequently, this modification typically is limited to proteins that enter, or protein domains exposed to, this compartment. Remarkably, however, several viral membrane proteins present in mature virions of the poxvirus vaccinia virus have stable disulfide bonds in their **cytoplasmic** domains: the genome of this virus encodes all the enzymes necessary to catalyze the formation of disulfide bonds in the cytoplasm (Box 12.4).

Protein folding and quality control. A number of other cellular proteins assist the folding of the extracellular domains of viral membrane glycoproteins as they enter the lumen of the ER (Table 12.2). In contrast to the enzymes described above, these proteins do not alter the covalent structures of proteins. Rather, their primary function is to facilitate folding, largely by preventing improper associations among unfolded, or incompletely folded, polypeptide chains, such as the nonspecific, hydrophobic interactions described above. Such **molecular chaperones** play essential roles in the folding of individual polypeptides and in the oligomerization of proteins. The ER chaperones, which include Grp78 and calnexin, are also crucial for **quality control** processes that determine the sorting and fate of newly synthesized proteins translocated into the ER.

Grp78 is a member of the Hsp70 family of stress response proteins. It associates transiently with incompletely folded viral and cellular proteins. Binding of this chaperone, generally at multiple sites in a single nascent protein molecule, is thought to protect against misfolding and aggregation by sequestering sequences prone to nonspecific interaction, such as hydrophobic patches. The release of unfolded proteins from Grp78 is controlled by the hydrolysis of ATP bound to the chaperone. Multiple cycles of association with, and dissociation from, Grp78 probably take place as a protein folds. Once the sequences

BOX 12.4 EXPERIMENTS

A viral thiol oxidoreductase system that operates in the cytoplasm

The intracellular mature virion of the poxvirus vaccinia virus is the first of two infectious particles assembled in infected cells. This particle carries an envelope containing viral membrane proteins surrounding an internal core in which the DNA genome is packaged. In 1999, it was reported that some viral core proteins synthesized in the cytosol, as well as the cytoplasmic domains of some membrane proteins, contain stable disulfide bonds. This property explained the previously reported sensitivity of vaccinia virions to disruption by reducing agents. In addition, it raised the intriguing question of how disulfide bonds could be introduced into viral proteins or domains that are **never** exposed to the cellular site of thiol oxidation, the ER lumen. Within a few years, viral genes were shown to encode all the components necessary to catalyze formation of disulfide bonds.

This viral thioloxidoreductase system comprise three components, and the final substrates, which include the mature virions proteins L1R and F9L. The order in which the three viral enzymes act, summarized in the figure, was deduced from a variety of experimental observations. For example,

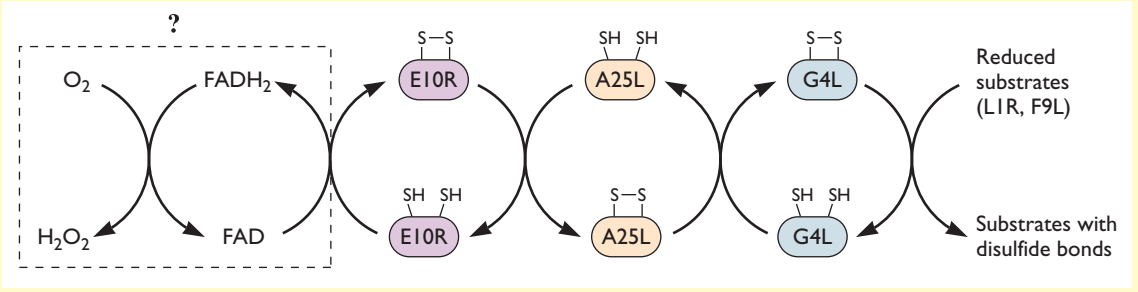
- when expression of the E10R gene was repressed in infected cells, only reduced A2.5L was detected
- conversely, inhibition of synthesis of G4L did not prevent oxidation of A2.5L
- E10R and A2.5 were shown form stable, disulfide-linked heterodimers when synthesized in the absence of other viral proteins
- covalent interactions between A2.5L and G4L or between G4L and substrates, were also identified, but only when thiol-disulfide exchange was prevented
- formation of the A2.5 and G4L heterodimer required synthesis of the E10R protein, as predicted by the pathway shown in the figure

The proteins that comprise the viral thiol oxidoreductase pathway are conserved among all poxvirus, and all three vaccinia virus proteins are essential for assembly of intracellular mature virions.

Lockner, J. K., and G. Griffiths. 1999. An unconventional role for cytoplasmic disulfide bonds in vaccinia virus proteins. *J. Cell Biol.* **144:**267–279.

Senkevich, T. G., C. L. White, E. V. Koonin, and B. Moss. 2002. Complete pathway for disulfide bond formation encoded by poxviruses. *Proc. Natl. Acad. Sci. USA* **99:**6667–6672.

The coupled oxidation-reduction (thiol-exchange) reactions among the proteins of the vaccinia virus disulfide bond formation are depicted in order (left to right). The transfer of electrons to oxygen via flavin adenine dinucleotide FAD (left) is based on homology of E10R with members of a family of flavin adenine dinucleotide (FAD)-containing sulfhydryl oxidases, and has not been demonstrated experimentally. Adapted from T. G. Senkevich et al., *Proc. Natl. Acad. Sci. USA* **99:**6667–6672, 2002, with permission.



to which the chaperone binds are buried in the interior of the protein, such interactions cease. For example, molecules of vesicular stomatitis virus G, Semliki Forest virus E1, or influenza virus HA0 proteins that have acquired the full complement of correct disulfide bonds can no longer associate with Grp78.

Calnexin is an integral membrane protein of the ER that also binds transiently to immature proteins, as does its soluble relative, calreticulin, present in the ER lumen. In contrast to Grp78, which recognizes protein sequences directly, calnexin and calrecticulin distinguish newly synthesized glycoproteins by binding to immature oligosaccharide chains. For example, the vesicular stomatitis virus G and influenza virus HA0 proteins bind to calnexin only when their oligosaccharide chains retain terminal glucose residues (Fig. 12.7B). In fact, formation of the mature oligosaccharide is intimately coupled with folding of glycoproteins and their retention within the ER (Fig. 12.8). Proteins with monoglucosylated sugars are recognized by calnexin (or calreticulin), which forms a complex with an ER thiol-oxidoreductase, but they are released upon removal of the glucose by the enzyme glucosidase II (Table 12.2). An enzyme that re-adds terminal glucose (uridine diphosphate [UDP]-glucose-glycoprotein transferase [Table 12.3]) appears to be the "sensor" of the folded state of the glycoprotein: it recognizes incompletely folded

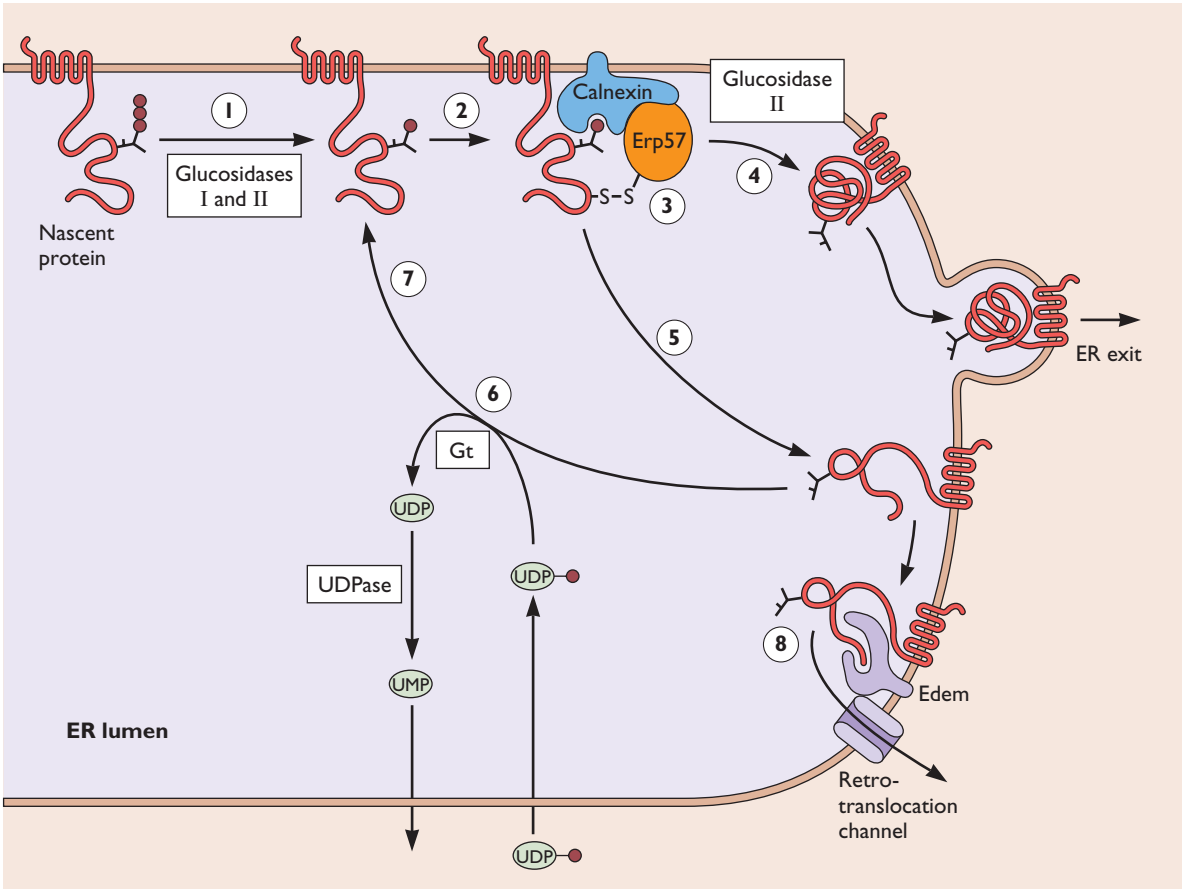


Figure 12.8 Integration of folding and glycosylation in the ER. The model illustrates the coordination of ER retention by calnexin with glycosylation and folding of a newly synthesized glycoprotein (red) containing an N-linked oligosaccharide, depicted as in Fig. 12.3. Trimming of terminal glucose residues by glucosidases I and II (1) yields a monoglucosylated chain, to which calnexin (or calreticulin) binds (2). Because the thioloxidoreductase Erp57 forms a complex with calnexin, the newly synthesized protein is brought into contact with Erp57, with which transient intermolecular disulfide bonds (-S-S-) can form. When the remaining glucose is removed by glucosidase II (3), the protein dissociates from the calnexin-Erp57 complex. If it has attained its native structure, the protein can leave the ER (4). However, if it is incompletely (or incorrectly) folded (5), the protein is specifically recognized by UDP-glucose glycoprotein transferase (Gt) (6), which re-adds terminal glucose residues to the oligosaccharide (7) and therefore allows rebinding to calnexin. Cycles of binding and modification are repeated until the protein is either folded properly or targeted for degradation (8), via binding to Edem and retrotranslocation to the cytosol. Adapted from L. Ellgaard et al., *Science* **286:**1882, 1999, with permission.

proteins by virtue of exposed hydrophobic amino acids and specifically reglucosylates the proteins, controlling cycles of substrate binding and release from calnexin or calreticulin (Fig. 12.8). This specificity ensures that only fully folded proteins can escape these chaperones and travel along the secretory pathway.

The ER contains numerous other folding catalysts and chaperones, many specific for particular proteins. Relatively little is known about the parameters that determine the chaperone(s) to which a newly synthesized protein binds, and the order in which chaperones operate. However, studies of specific viral glycoproteins in living cells indicate that the positions of oligosaccharides within the protein chain are one important determinant of chaperone selection (Box 12.5).

Proteins that are misfolded or not modified correctly cannot escape covalent or noncovalent associations with ER enzymes or molecular chaperones. For example, a temperature-sensitive vesicular stomatitis virus G protein remains bound to calnexin, and hence to the ER membrane, at a restrictive temperature. Consequently, egress of nonfunctional proteins from the ER to subsequent compartments

Table 12.3 Viral envelope glycoprotein precursors processed by signal peptidase or furin-family proteases

Virus family	Precursor glycoprotein	Membrane-associated cleavage products
Signal peptidase		
Alphavirus	Envelope polyprotein precursor	E1, pE2
Bunyavirus	Translation product of M mRNA	G1, G2
Furin family proteases		
Alphavirus	pE2	E2
Flavivirus	PrM	M
Hepadnavirus	preC	C antigen[a,b]
Herpesvirus[b,c]	pre-gB	gB
Orthomyxovirus[d]	HA0	HA_1, HA_2
Paramyxovirus	F0	F1, F2
Retrovirus	Env	TM, SU

[a]This cleavage product is largely secreted into the extracellular medium, but is also associated with plasma membrane of infected cells.

[b]Cleavage is not necessary for production of infectious virus particles in cells in tissue culture.

[c]Some alphaherpesviruses (e.g., varicella-zoster virus), and all known betaherperviruses.

[d]Virulent strains of avian influenza A virus.

in the secretory pathway is prevented. These interactions also target misfolded (and misassembled) proteins for degradation. The first step in this process is translocation of such proteins from the ER into the cytoplasm **(retrotranslocation)**. The mechanisms responsible for specific recognition of misfolded or incompletely folded proteins, and induction of transport from ER to cytosol, are not yet fully understood. However, the stress-inducible ER membrane protein Edem (ER degradation-enhancing 1,2-mannosidase-like protein) has been implicated in diversion of misfolded glycoproteins for retrotranslocation. Furthermore, studies of herpesviral proteins that induce translocation of major histocompatibility complex (MHC) class I molecules from the ER to the cytosol have identified components of a membrane channel that appear to be dedicated to removal of misfolded proteins from the ER (Box 12.6). Once the proteins reenter the cytoplasm, oligosaccharides are removed and multiple copies of the protein ubiquitin (polyubiquitin) are added. Such modification targets the proteins for degradation by the proteasome. The quality control functions of resident ER chaperones therefore ensure that nonfunctional proteins are cleared from the secretory pathway at an early step.

Oligomerization. Most viral membrane proteins are oligomers that must assemble as their constituent protein chains are folded and covalently modified. Such assembly generally begins in the ER, as the surfaces that mediate interactions among protein subunits adopt the correct conformation. For many proteins, these reactions are completed within the ER. For example, influenza virus HA0 protein monomers are restricted to the ER lumen, whereas trimers are found in this and all subsequent compartments of the secretory pathway. Indeed, several viral and some cellular heteromeric membrane proteins must oligomerize to exit the ER, for folding of one subunit depends on association with the other(s). This requirement has been characterized in some detail for the glycoproteins of alphaviruses, such as Sindbis virus. These proteins are translated from the 26S subgenomic mRNA (Appendix, Fig. 25B) as a precursor that contains the E1 and E2 protein sequences, but are liberated from the precursor by signal peptidase cleavage as they enter the ER. The E1 protein associates with Grp78 and folds via three disulfide-bonded intermediates. Folding beyond the second intermediate, and release from chaperone association, requires dimerization of the E1 with the E2 protein. Furthermore, the E2 protein misfolds when synthesized in the absence of E1 protein. The association of these two envelope proteins within the ER therefore is essential for the productive folding, and exit, of both. Similarly, the herpes simplex virus type 1 envelope glycoproteins gH and gL must interact with one another for the transport of either from the ER, and in the absence of gL, gH cannot fold correctly.

Assembly of other viral membrane proteins is completed following exit from the ER: disulfide-linked dimers of the hepatitis B virus surface antigen form higher-order complexes in the next compartment in the pathway, and oligomers of the human immunodeficiency virus type 1 Env protein can be detected only in the Golgi apparatus.

BOX 12.5 EXPERIMENTS

Selectivity of chaperones for viral glycoproteins entering the ER

The parameters that determine which of the many ER chaperones operate on individual proteins are not fully understood. However, analysis of the folding and chaperone association of viral glycoproteins suggests that the position of glycosylation sites can determine chaperone selection. This conclusion is based on the following observations.

The Semliki Forest virus E1 and p62 (pre-E2) glycoproteins enter the ER cotranslationally, and are cleaved from a precursor by signal peptidase. However, E1, which folds via three intermediates differing in their disulfide bonding, initially associates with Grp78, whereas nascent p62 molecules bind to calnexin.

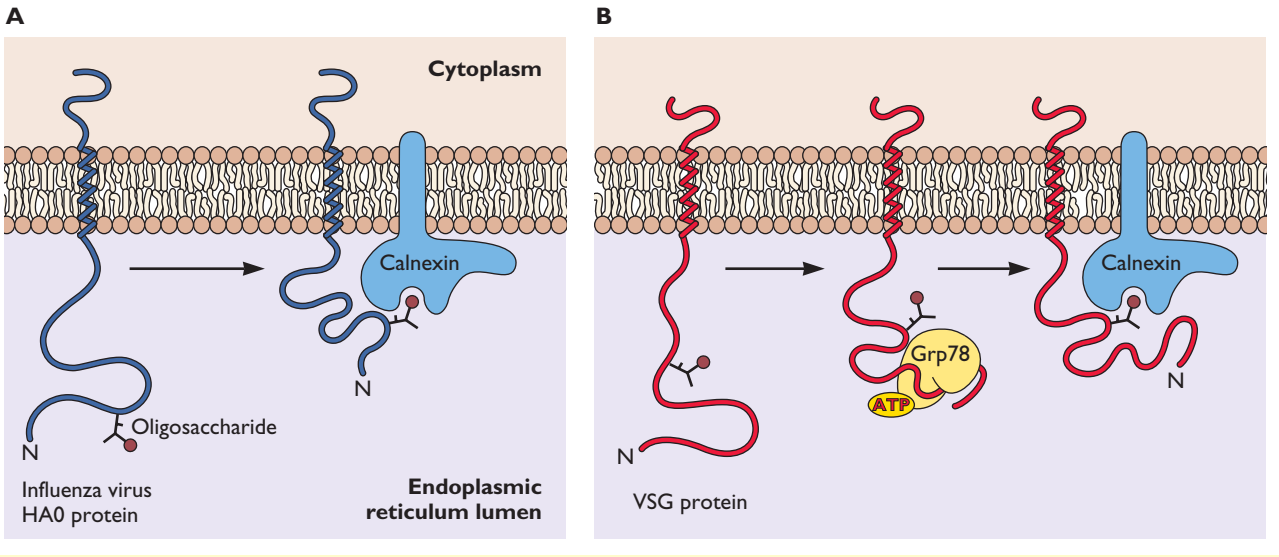
This difference is not a trivial result of a lack of Grp78-binding sites in p62: when access to calnexin was blocked, this protein did bind to Grp78.

One major difference between E1 and p62 is that the latter contains glycosylation sites close to the N terminus. Addition of oligosaccharides at such sites could allow recognition of nascent p62 by calnexin and preclude association with Grp78.

This hypothesis was tested by elimination of N-linked glycosylation sites of the influenza virus HA0 protein, either close to the N terminus or in more C-terminal positions. The wild-type protein does not bind to Grp78, but inhibition of glycosylation at N-terminal sites (positions 8, 22, and 38) led to association with this chaperone.

As summarized in the figure, these observations suggest that nascent proteins that carry N-linked glycosylation sites close to the N terminus (p62, HA0) enter the calnexin folding pathway directly **(A).** Others, such as the Semliki Forest virus E1, and the vesicular stomatitis virus G, proteins associate initially with Grp78 and protein disulfide isomerase and are transferred to the calnexin pathway as they mature **(B).**

Molinari, M., and A. Helenius. 2000. Chaperone selection during glycoprotein translocation into the endoplasmic reticulum. *Science* **288:**331–333.



At present, we can discern no simple rules describing the relationship of oligomer assembly and transport of membrane proteins from the ER. Nevertheless, oligomerization begins, and in some cases must be completed, within the ER, where it can be facilitated by the folding catalysts and chaperones characteristic of this compartment.

Vesicular Transport to the Cell Surface

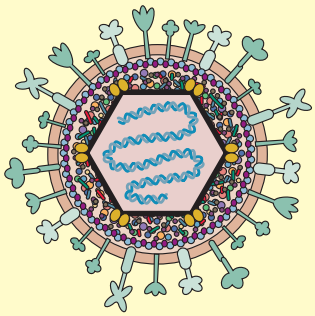
Mechanism of vesicular transport. Viral membrane proteins, like their cellular counterparts, travel to the cell surface through a series of membrane-bound compartments and vesicles. The first step in this pathway, illustrated schematically in Fig. 12.9, is transport of the folded protein from the ER to the Golgi apparatus. Within the Golgi apparatus (Fig. 12.10), proteins are sorted according to the addresses specified in their primary sequences or covalent modifications. **Transport vesicles**, and larger vesicular structures (Box 12.7), which bud from one compartment and move to the next, carry cargo proteins between compartments of the secretory pathway. Fusion of the vesicle membrane with that of the target compartment releases the cargo into the lumen of that compartment. Consequently, proteins that enter the secretory pathway upon translocation into the ER (and are correctly folded) are never again

BOX 12.6 EXPERIMENTS

How a herpesviral glycoprotein was exploited in indentification of an ER retrotranslocation channel

Two human cytomegalovirus (a betaherpesvirus) membrane glycoproteins, US2 and US11, were known to insert into the ER membrane and induce rapid transfer of MHC class I heavy chains from the ER to the cytosol (retro-translocation or dislocation). These cellular proteins then become polyubiquitmated and degraded by the cytosolic proteasome. In the case of the viral US11 protein, a glutamine residue (Glu192) in the transmembrane domain is essential for retrotranslocation of MHC class I proteins.

In one set of experiments, this property was exploited to purify human proteins that bound specifically to wild-type US11, but not to the viral protein carrying a Glu192 → Leu substitution. Several ER proteins bound to both US11 proteins, but one associated only with wild-type US11. This protein, identified by mass spectrometry, showed some similarity to the yeast Der1p protein that is known to participate in degradation of misfolded ER proteins, and was named derlin-1. Overproduction of a dominant-negative derivative of derlin-1 inhibited US11-mediated retrotranslocation of MHC class I proteins.



In an alternative approach, components of a canine ER retrotranslocation channel were identified by virtue of their interaction with a cytoplasmic ATPase (ATPase p97) known to be essential for degradation of both misfolded ER proteins in yeast and MHC class I molecules in US11-producing human cells. The protein complex identified in this way contained derlin-1 and a second ER membrane protein. These ER proteins were shown by immunoprecipitation to interact with both US11 and MHC class I proteins.

The US2-mediated degradation of MHC class I proteins was not inhibited by the dominant negative form of derlin that blocked degradation induced by the US11 protein. This observation indicates that US2 must function with a distinct retrotranslocation channel, perhaps one of the other two human derlin proteins or the channel through which nascent polypeptides enter the ER. The results of genetic experiments in yeast support the view that this channel is required for degradation of several ER proteins.

Lilley, B. W., and H. Ploegh. 2004. A membrane protein required for dislocation of misfolded membrane proteins from the ER. *Nature* **429:** 834–840.

Ye, Y., Y. Shibata, C. Yun, D. Ron, and T. A. Rapoport. 2004. A membrane protein complex mediates retro-translocation from the ER lumen to the cytosol. *Nature* **429:**841–847.

exposed to the cytosol of the cell. This strategy effectively sequesters proteins that might be detrimental, such as secreted or lysosomal proteases, and avoids exposure of disulfide-bonded proteins to a reducing environment.

Many soluble and membrane proteins that participate in vesicular transport have been identified and characterized by biochemical, molecular, and genetic methods. The properties of these proteins suggest that similar mechanisms control the budding and fusion of different types of transport vesicle, despite the stringent specificity requirements of vesicular transport. The general mechanism of vesicular transport is quite well understood (Fig. 12.11). Budding of transport vesicles from the membranes of compartments of the secretory pathway requires proteins that form external coats of the vesicles, such as the protein complex called CopII, which mediates ER-to-Golgi transport, and small GTPases (Fig. 12.11A). The coat proteins induce membrane curvature and vesicle budding, and are subsequently removed by various mechanisms. The vesicle then moves to the next compartment, by either passive diffusion or active transport via microtubule-associated motor proteins.

When a transport vesicle encounters its target membrane, it docks as a result of specific interactions among Snare proteins present in the vesicle and target membranes. Docking requires a member of each of the very large Rab and Arf families of small GTPases, which are important determinants of fusion specificity. A complex containing proteins necessary to prepare for membrane fusion then assembles in a regulated manner and juxtaposes the membranes that will fuse (Fig. 12.11B). One component of this complex binds to the **Snares** present in the membranes of the transport vesicle (v-Snare) and of the compartment with which it will fuse (t-Snare). The final reaction of vesicular transport, membrane fusion, requires the Snare proteins, which promote membrane fusion. A v-Snare and an appropriate t-Snare are sufficient to induce membrane fusion, but there is evidence that additional proteins may facilitate or accelerate this reaction.

The high density of intracellular protein traffic requires considerable specificity during vesicle formation and fusion. For example, vesicles that transport proteins from the ER to the first compartment of the Golgi apparatus, the *cis*-Golgi network (Fig. 12.9), must take up only the appropriate proteins when budding from the ER, and must fuse only with the membrane of the *cis*-Golgi network. Specificity of cargo loading during formation of these (and other) transport vesicles is achieved

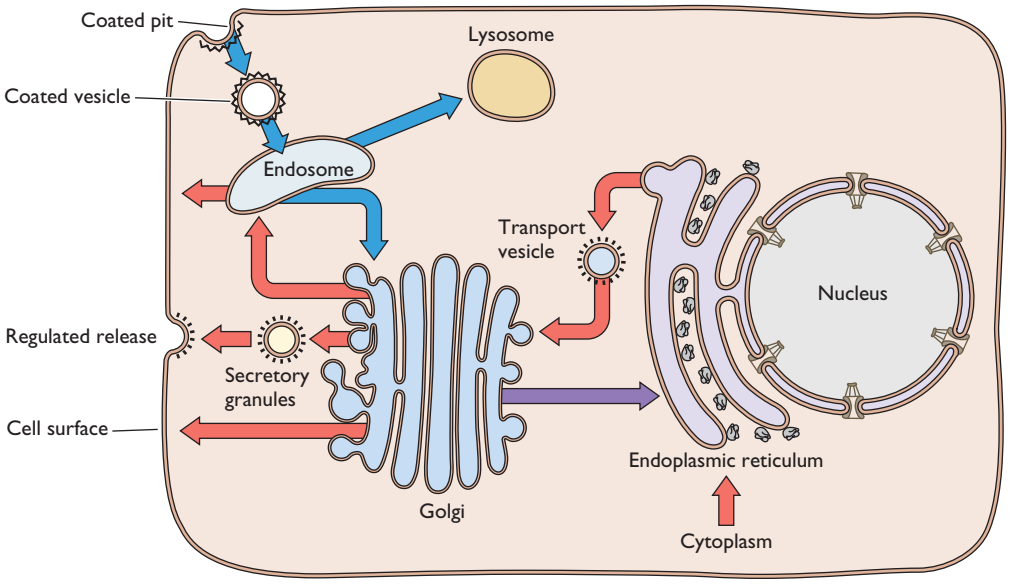


Figure 12.9 Compartments in the secretory pathway. The lumen of each membrane-bound compartment shown is topologically equivalent to the exterior of the cell. Proteins destined for secretion or for the plasma membrane travel from the ER to the cell surface via the Golgi apparatus, as indicated by the red arrows. However, proteins can be diverted from this pathway to lysosomes, or to secretory granules that carry proteins to the cell surface for regulated release. The return of proteins from the Golgi apparatus to the ER is indicated (purple arrow). The endocytic pathway (blue arrows), discussed in Chapter 5, and the secretory pathway intersect in endosomes and the Golgi apparatus.

by both direct association of cargo proteins with proteins of the Cop II vesicular coat and indirect interactions via transmembrane export receptors. Several types of protein establish the specificity of vesicular transport, that is, fusion of particular transport vesicles with the appropriate compartment, including the Snare proteins resident in the vesicle and target compartment membranes. Small GTP-binding proteins of the Rab and Arf families, each of which is associated with a specific organelle, provide, in the GTP-bound form, an "identity signal" recognized by proteins that participate in vesicle budding or fusion. Specific phosphoinositides (lipids) are also important determinants of the identity of some organelles, for example of Golgi compartments.

Reactions within the Golgi apparatus. One of the most important staging posts in the secretory pathway is the Golgi apparatus, which is composed of a series of membrane-bound compartments located near the cell nucleus. Proteins enter the Golgi apparatus from the ER via the *cis*-Golgi network, which is composed of connected tubules and sacs (Fig. 12.9 and 12.10). A similar structure, the *trans*-Golgi network, forms the exit face of this organelle. The *cis*- and *trans*-Golgi networks are separated by a variable number of cisternae termed the *cis*, medial, and *trans* compartments. Each of the compartments of the Golgi apparatus, which can comprise multiple cisternae, is the site of specific processing reactions (Fig. 12.12).

A number of viral envelope glycoproteins are also processed proteolytically by cellular enzymes resident in late Golgi compartments. Retroviral Env glycoproteins are cleaved in the *trans*-Golgi network to produce the TM (transmembrane) and SU (surface unit) subunits from the Env polyprotein precursor (Fig. 12.13A). The larger SU protein associates with TM noncovalently and in many simple retroviruses also via a disulfide bond (Fig. 12.13B). Similarly, the HA0 protein of certain avian influenza A viruses is cleaved into the HA1 and HA2 chains in the *trans*-Golgi network (Fig. 12.4). These and other viral membrane proteins (Table 12.3) are processed by members of a family of resident Golgi proteases that cleave after pairs of basic amino acids. The members of this family, which in mammalian cells includes furins found in the *trans*-Golgi network, are serine proteases related to the bacterial enzyme subtilisin. Various furin family members have been shown by genetic and molecular methods to process viral glycoproteins; their normal function is to process cellular polyproteins, such as certain hormone precursors.

These proteolytic cleavages typically are essential for formation of infectious particles, although they are not

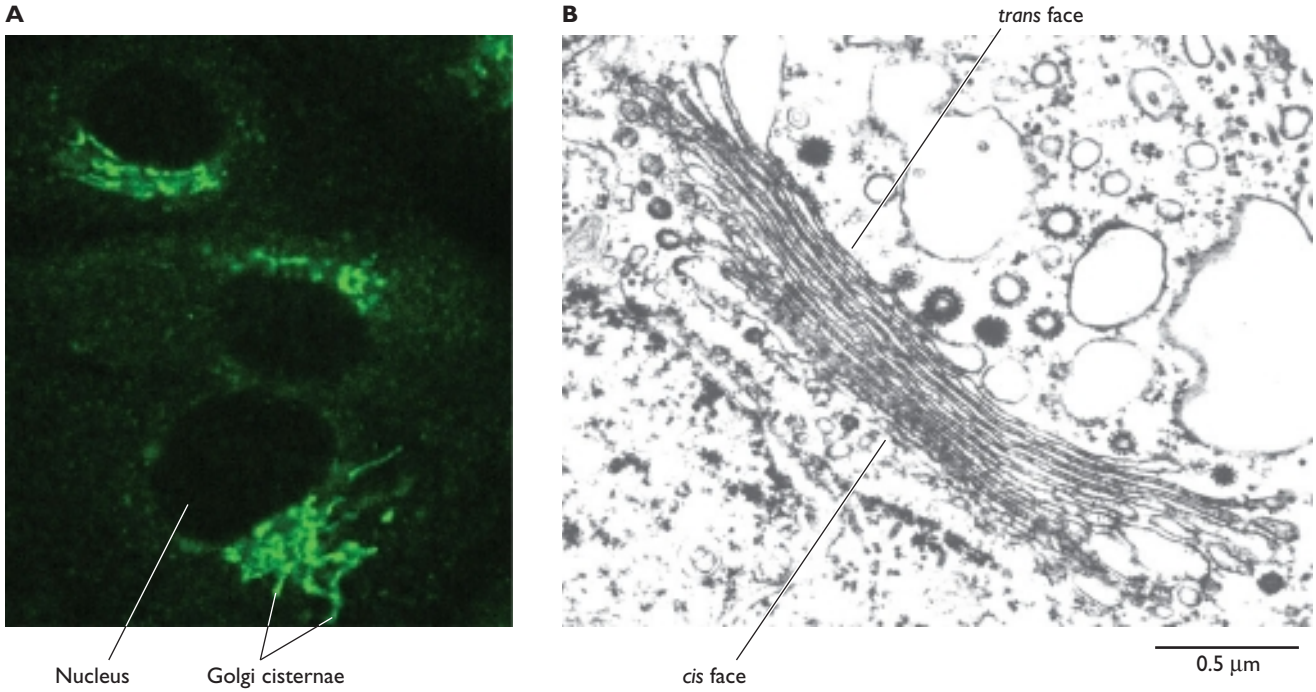


Figure 12.10 The Golgi apparatus. (A) Immunofluorescence of pig kidney epithelial cells following staining with antibody against a Golgi protein (p115). Courtesy of A. Brideau, Princeton University. **(B)** Electron micrograph of negatively stained sections of the alga *Ochromonas danica*, showing the flattened membranous sacs, termed cisternae. These are bordered on either side by more convoluted structures of connected tubules and sacs, the *cis*- and *trans*-Golgi networks. Because the cisternae lie on top of one another, they are often called Golgi stacks. The number of cisternae varies considerably from one cell type to another and under different physiological conditions. From G. T. Cole, Biological Photo Service.

necessary for assembly. For example, proteolytic processing of envelope proteins of retroviruses and alphaviruses is necessary for infectivity, probably because sequences important for fusion and entry become accessible. As discussed in more detail in Volume II, Chapter 1, virulent strains of avian influenza A virus encode HA0 proteins that can be processed by the ubiquitous furin family proteases, such that virus particles carrying fusion-active HA protein are released. It seems likely that the common dependence on furin family proteases (Table 12.3), which act on proteins relatively late in the secretory pathway, helps minimize complications that would arise if viral glycoproteins were initially synthesized with their fusion peptides in an active conformation. Furthermore, exposure to the low pH environment of *trans*-Golgi network compartments (pH ~6.0) can be a prerequisite for processing of viral envelope proteins. This requirement is exemplified by the envelope proteins of flaviviruses, such as dengue virus. The auxillary membrane protein (M) of this virus is synthesized as a precursor (prM) that forms heterodimers with the envelope (E) protein in the ER membrane. Dengue virus particles bud into the ER and travel the secretory pathway. When they reach the *trans*-Golgi network, the prM-E heterodimers undergo extensive conformational rearrangement that renders prM accessible to proteolysis by furin.

For at least one influenza A virus, the fowl plague virus, the ion channel activity of the viral M2 protein helps to maintain HA in a fusion-incompetent conformation following cleavage. This HA protein switches to the fusion-competent conformation at a pH higher than that required by HA proteins of human influenza A viruses. The M2 protein, which forms a proton channel, is found in infected cells at quite high concentrations in the membranes of secretory pathway compartments. By increasing the pH of normally acidic compartments, such as those of the *trans*-Golgi network, this protein prevents premature switching of proteolytically processed HA to the fusion-active conformation described in Chapter 5.

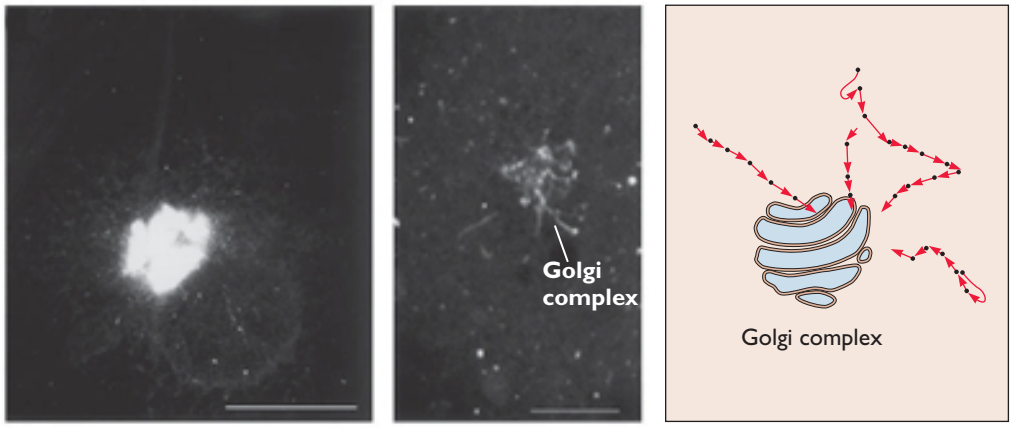
Although all the envelope proteins of viruses that assemble at the plasma membrane travel via the cellular secretory pathway, there is considerable variation in the rate and efficiency of their transport. A champion is the influenza virus HA protein, which folds and assembles

BOX 12.7 EXPERIMENTS

ER-to-Golgi transport in living cells

The vesicular stomatitis virus G protein made in cells infected with the mutant virus *ts*045 misfolds and is retained in the ER at high temperature (40°C). It refolds and is transported to the Golgi apparatus when the temperature is reduced to 32°C. This temperature-sensitive protein has therefore been used extensively to study transport through the secretory pathway. To allow examination of this process in living cells, the green fluorescent protein was attached to the cytoplasmic tail of the viral G protein. Control experiments established that this modification did not alter the temperature-sensitive folding or transport of the G protein. Time-lapse fluorescence microscopy of cells shifted from high to low temperature (see the figure) demonstrated that the chimeric G protein rapidly left the ER at multiple peripheral sites. The protein appeared in membranous structures, which were often larger than typical transport vesicles. These structures moved rapidly toward the Golgi in a stop-start manner, with maximal velocities of 1.4 μm/s. Such transport, but not formation of post-ER structures, was blocked when microtubules were depolymerized by treatment with nocodazole, or when the (–) end-directed microtubule motor dynein was inhibited. It was therefore concluded that vesicles and other membrane-bound structures that emerge from the ER at peripheral sites are actively transported to the Golgi complex.

Presley, J. F., N. B. Cole, T. A. Schroer, K. Hirschberg, K. J. M. Zaal, and J. Lippincott-Schwartz. 1997. ER-to-Golgi transport visualized in living cells. *Nature* **389:**81–85.



Shown are images of the vesicular stomatitis virus ts045 G protein-green fluorescent protein observed in cells 5 min after a shift to 32°C from 40°C **(A)** and 396 s after the transfer to 32°C **(B)**. Fluorescence associated with the Golgi complex was photobleached upon the shift to 32°C to allow visualization of delivery of the fluorescent protein from intermediates to the Golgi. **(C)** The path of such movement is shown schematically.

with a half time of only 7 min, with more than 90% of the newly synthesized molecules reaching the cell surface. Many other viral proteins do considerably less well. Parameters determining the rate and efficiency of transport may include the complexity of the protein and the inherent asynchrony of protein folding. With some exceptions (see "Inhibition of Transport of Particular Cellular Proteins" below), cellular proteins continue to enter and traverse the secretory pathway as enveloped viruses assemble at the plasma membrane. Competition among viral and cellular proteins, which may vary with the nature and physiological state of the host cell, is also likely to affect the transport of viral proteins to the cell surface.

We have focused our discussion of viral envelope proteins that travel the cellular secretory pathway on the well-understood maturation of their extracellular domains. However, the cytoplasmic portions of these proteins are also frequently modified. Many are **acylated** by the covalent linkage of the fatty acid palmitate to cysteine residues in their cytoplasmic domains (Table 12.4). This modification can be necessary for optimal production of progeny virions. For example, inhibition of palmitoylation of the Sindbis virus E2 glycoprotein or of the human immunodeficiency virus type 1 Env protein impairs virus assembly and budding. The bulky fatty acid chains attached to the short cytoplasmic tails may regulate envelope protein conformation or association with specific membrane domains (see "Signal Sequence-Independent Transport of Viral Proteins to the Plasma Membrane" below).

Sorting of Viral Proteins in Polarized Cells

Proteins that are not specifically targeted to an intracellular address travel from the Golgi apparatus to the plasma membrane. However, the plasma membrane is not uniform in all animal cells: differentiated cells often devote different parts of their surfaces to specialized functions, and the

plasma membranes of such **polarized cells** are divided into correspondingly distinct regions. During infection by many enveloped viruses, the asymmetric surfaces of such cells are distinguished during entry, and by the sorting of virion components to a specific plasma membrane region. In this section, we describe the final steps in the transport of proteins to specialized plasma membrane regions in two types of polarized cell in which animal viruses often replicate, epithelial cells and neurons (Fig. 12.14).

Epithelial Cells

As discussed in Chapter 2, epithelial cells, which cover the external surfaces of vertebrates and line all their internal cavities (such as the respiratory and gastrointestinal tracts), are primary targets of virus infection. The cells of an epithelium are organized into close-knit sheets, by both the tight contacts they make with one another and their interactions with the underlying basal lamina, a thin layer of extracellular matrix (Fig. 2.3). Within the best-characterized epithelia, such as those that line the intestine, each cell is divided into a highly folded **apical domain** exposed to the outside world and a **basolateral domain** (Fig. 12.14). The former performs more specialized functions, whereas the latter is associated with cellular housekeeping. These two domains differ in their protein and lipid content, in part because they are separated by specialized cell-cell junctions (tight junctions) (Fig. 2.3), which prevent free diffusion and mixing of components in the outer leaflet of the lipid bilayer. However, such physical separation does not explain how the polarized distribution of plasma membrane proteins is established and maintained.

Viruses have been important tools in efforts to elucidate the molecular mechanisms responsible for the polarity of typical epithelial cells, because certain enveloped viruses bud asymmetrically. For example, in the epithelial cells of all organs studied, influenza A virus buds apically and vesicular stomatitis virus buds basolaterally. As discussed in Volume II, Chapter 1, polarized assembly and release of virus particles can facilitate virus spread within or among host organisms. The polarity of virus budding is generally the result of accumulation of envelope proteins, such as HA and G, in the apical and basolateral domains, respectively. The most common mechanism for selective localization appears to be signal-dependent sorting of proteins in the *trans*-Golgi network, for packaging into appropriately targeted transport vesicles. Signals necessary for basolateral targeting comprise short amino acid sequences located in the cytoplasmic domains of membrane proteins. Many basolateral targeting signals overlap with those that direct proteins to clathrin-coated pits, the first staging post of the endocytic pathway. Indeed, in polarized epithelial cells, certain proteins are transferred through endosomes from one membrane domain to the other, a process termed transcytosis (see Volume II, Fig. 4.18). The sorting of viral glycoproteins to basolateral membrane domains can also be governed by additional viral proteins. The two envelope proteins of measles virus (F and H) possess signals that direct them to the basolateral membrane. However, when the viral matrix protein binds to the cytoplasmic tails of F and H, these proteins are redirected from the default basolateral sorting pathway, and accumulate at the apical surface of epithelial cells.

The signals that direct proteins to the apical membrane are currently a matter of debate, but both N-glycosylation and association of proteins with specific lipids may be important. The apical membranes of epithelial cells are enriched in sphingolipids and cholesterol, which form microdomains called **lipid rafts**. Such rafts are dynamic assemblies that can incorporate specific proteins selectively, and were initially shown to mediate apical transport of glycosylphosphatidylinositol-anchored proteins. The influenza virus HA and NA proteins associate specifically with lipid rafts via their transmembrane domains, which determine apical sorting. Cellular proteins known to participate in apical trafficking of viral glycoproteins, such as caveolin-1, annexin XIIIb, myelin, and lymphocyte-binding protein, are also associated with these rafts: inhibition of the activity or synthesis of these proteins disrupts transport of the influenza virus HA protein (and other proteins) from the Golgi complex to the apical membrane. Lipid rafts seem likely to be more generally important in targeting of viral membrane proteins and assembly: measles virus glycoproteins are selectively enriched in lipid rafts in nonpolarized cells, and association of the human immunodeficiency virus type 1 Gag polyprotein with these membrane domains promotes production of virus particles.

Neurons

Neurons are probably the most dramatically specialized of the many polarized cells of vertebrates. The axon is typically long and unbranched, whereas the dendrites form an extensive branched network of projections (Fig. 12.14). Axons are specialized for the transmission of nerve impulses, ultimately via the formation of synaptic vesicles and release of their contents. In contrast, dendrites provide a large surface area for the receipt of signals from other neurons. The nucleus, the rough ER, and the Golgi apparatus are also located in the dendritic region and the cell body of a neuron. Although axonal and dendritic surfaces are not separated by tight junctions, proteins must be distributed asymmetrically in neurons. Several mechanisms contribute to the establishment and maintenance of neuronal polarity, including transport of vesicles in specific directions along the highly organized

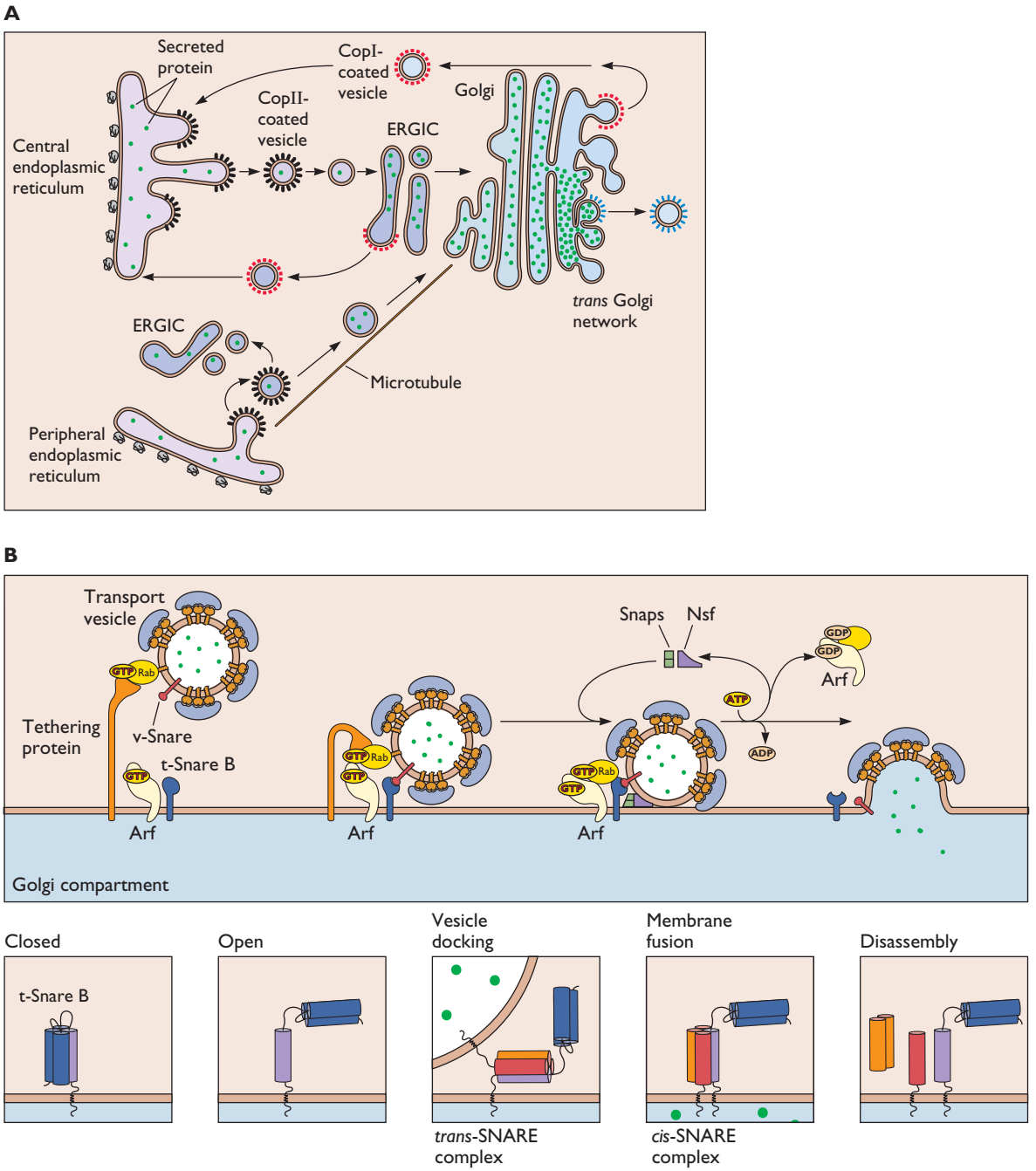


Figure 12.11 Protein transport from the ER to the Golgi. (A) Proteins leave the ER in transport vesicles at regions free of ribosomes in both the center of the cell (that is, near the Golgi) and peripheral regions. Vesicle formation is initiated by binding of cytoplasmic coat protein complex II (CopII), which contains a small GTPase (Sar1) and several other proteins, to the membrane. The vesicle membranes also carry proteins that direct them to appropriate destinations, such as the v-Snares syntaxin 5 and membrin. Cargo is loaded by interactions with proteins of the CopII coat and export receptors. The CopII coat induces budding and pinching off of vesicles, which then lose their coats and fuse to form vesicular tubular clusters. These clusters are also known as the ER-Golgi intermediate compartment (ERGIC). When formed at sites far from the Golgi, vesicles and vesicular-tubular compartments move to this organelle along microtubules (Box 12.7). Vesicles coated by the COP I coat bud off from these compartments and mediate transport of proteins back to the ER. This process is thought to make an important contribution to sorting proteins for forward transport. Whether vesicular-tubular compartments fuse with a fixed *cis*-Golgi network (stable

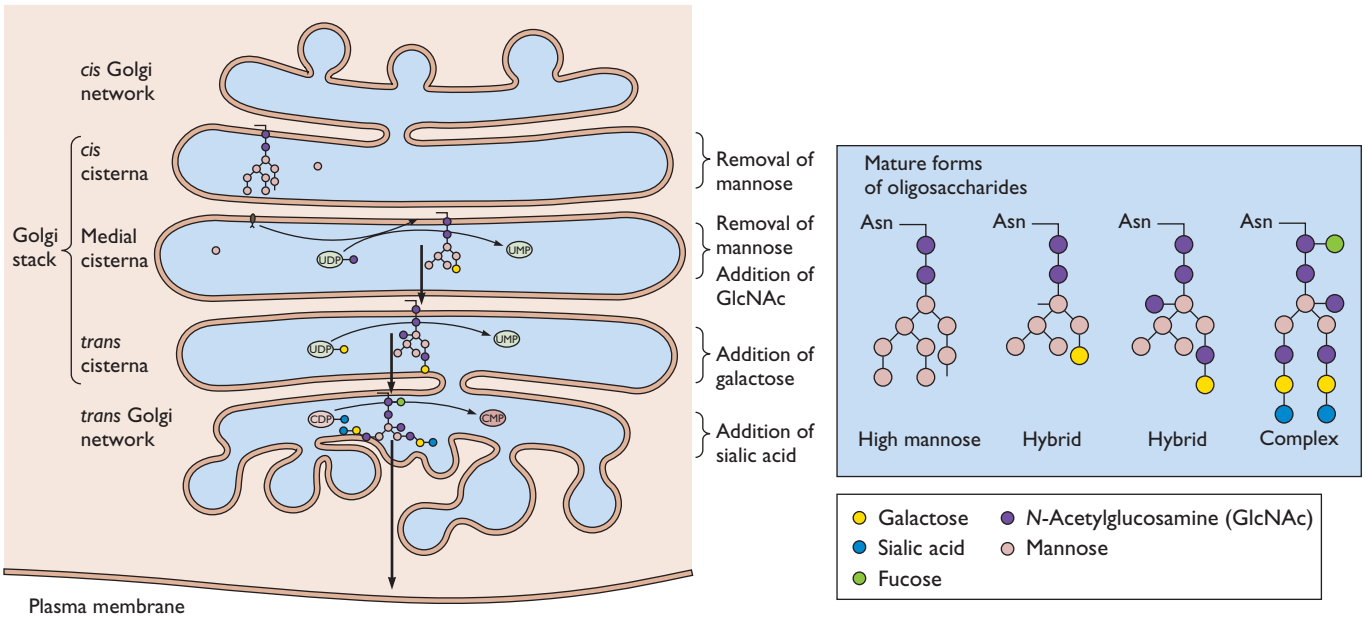


Figure 12.12 Compartmentalization of processing of N-linked oligosaccharides. The reactions by which mature N-linked oligosaccharide chains are produced from the high-mannose core precursor added in the ER (Fig. 12.7B) are shown in the Golgi compartment in which they take place. Trimming of terminal glucose and mannose residues of the common core precedes stepwise addition of the sugars found in the mature chains. The enzymes responsible for early reactions in maturation of oligosaccharides are located in *cis* cisternae, whereas those that carry out later reactions are present in the medial and *trans* compartments. Such spatial separation ensures that oligosaccharide processing follows a strict sequence as proteins pass through the compartments of the Golgi apparatus. Synthesis of O-linked oligosaccharides by glycosyltransferases, which add one sugar unit at a time to certain serine or threonine residues, also takes place in the Golgi apparatus.

microtubules of the axon **(axonal transport)** and transport of mRNA to dendrites. Nevertheless, it is thought that sorting and targeting of membrane proteins for delivery to axonal or dendritic surfaces is also essential for polarization.

The directional movement of vesicles and many cellular organelles in neurons is dependent on polarized microtubules and motor molecules that travel toward either their (−) or (+) ends. Such motors therefore mediate transport both toward the cell body from axons and dendrites and away from the cell body (Fig. 12.15). Infection, assembly, and egress of particles of viruses that infect neurons depend on these mechanisms. An important example is provided by the neurotropic alphaherpesviruses, a group

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Golgi compartment model) or form *cis* cisternae upon fusion (cisternal maturation model) and whether forward transport through the Golgi cisternae is mediated by transport vescles or maturation of Golgi cisternae have been questions of intense debate. Clear evidence for both vesicle transport and cisternal maturation has been obtained. It is generally accepted that both mechanisms operate, but that one or the other may predominate in specific cell types, or under some conditions. **(B)** Fusion of transport vesicle and target compartment membranes. Both vesicle (v-Snare and specific Rabs bound to GTP) and target compartment (t-Snare and specific Arfs and Rabs bound to GTP) proteins govern the specificity of membrane fusion. The first step is tethering of an uncoated vesicle by interaction of a tethering protein with the GTP-bound Rab. Tethering proteins (e.g., p119 and giantin) generally are extended α-helical proteins that form coiled coils. Docking then takes place by formation of specific v-Snare–t-Snare complexes. As shown below, this process is initiated by a t-Snare adopting an open conformation, which allows formation of a trans-Snare α-helical bundle with helices also contributed by the v-Snare and a second t-Snare. Docking is followed by recruitment of the soluble proteins *N*-ethylmaleimide-sensitive factor (Nsf) (an ATPase) and soluble Nsf-attachment proteins (Snaps) to form a membrane fusion complex. Fusion is accompanied by ATP hydrolysis and disassembly of the fusion complex.

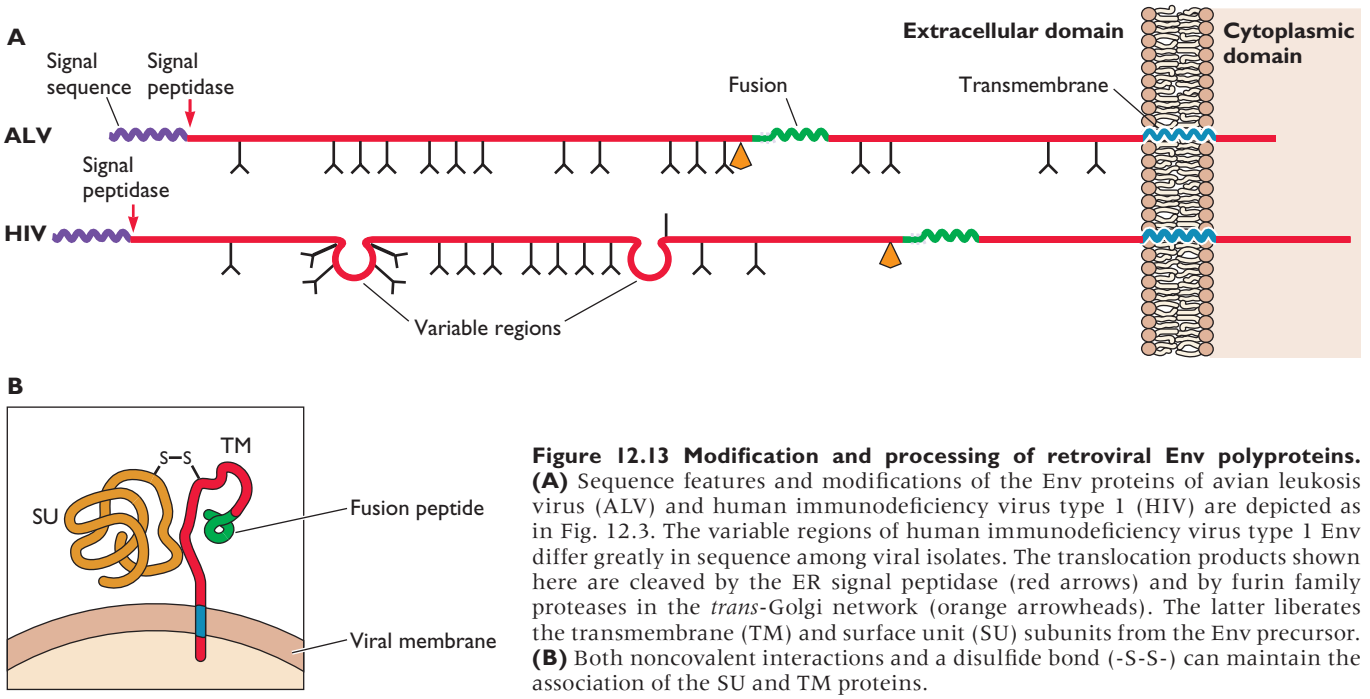


Figure 12.13 Modification and processing of retroviral Env polyproteins. **(A)** Sequence features and modifications of the Env proteins of avian leukosis virus (ALV) and human immunodeficiency virus type 1 (HIV) are depicted as in Fig. 12.3. The variable regions of human immunodeficiency virus type 1 Env differ greatly in sequence among viral isolates. The translocation products shown here are cleaved by the ER signal peptidase (red arrows) and by furin family proteases in the *trans*-Golgi network (orange arrowheads). The latter liberates the transmembrane (TM) and surface unit (SU) subunits from the Env precursor. **(B)** Both noncovalent interactions and a disulfide bond (-S-S-) can maintain the association of the SU and TM proteins.

Table 12.4 Examples of acylated or isoprenylated viral proteins

Virus	Protein	Lipid	Probable function
Envelope proteins			
Alphavirus			
Sindbis virus	E2	Palmitate	Required for efficient budding of virus particles
Coronavirus			
Severe acute respiratory syndrome virus	S	Palmitate	Fusion
Hepadnavirus			
Hepatitis B virus	L (pre-S1)	Myristate	Initiation of infection
Orthomyxovirus			
Influenza A virus	HA	Palmitate	Essential for fusion and infectivity
Retrovirus			
Human immunodeficiency virus type 1, Moloney murine leukemia virus	Env (TM)	Palmitate	Virion budding
Other viral proteins			
Hepatitis delta satellite virus	Large delta antigen	Geranylgeranol	Interaction with HBV L protein; assembly; inhibition of HDV RNA replication
Papovavirus			
Simian virus 40	VP2	Myristate	Virion assembly
Picornavirus			
Poliovirus	VP_0, VP_4	Myristate	Virion assembly; uncoating
Retrovirus			
Human immunodeficiency virus type 1, murine leukemia virus	Gag, MA	Myristate	Membrane association, assembly and budding
Rous sarcoma virus	$pp60^{src}$	Myristate	Membrane association, transformation

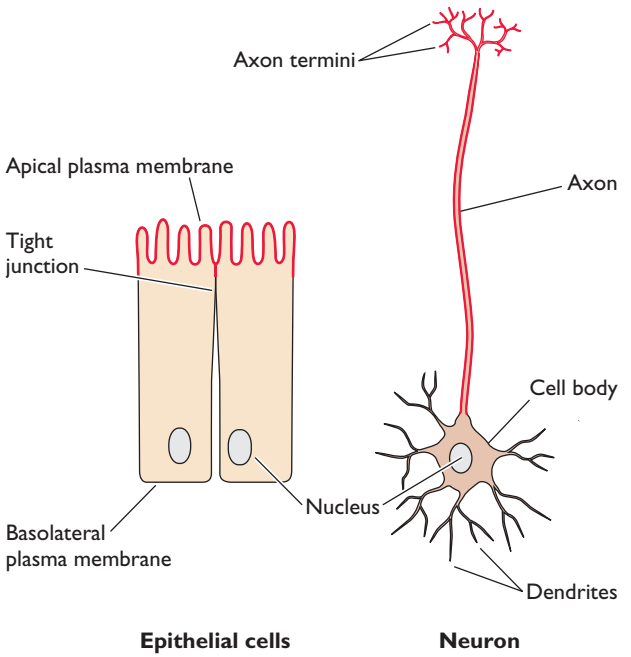


Figure 12.14 Polarized epithelial cells and neurons.

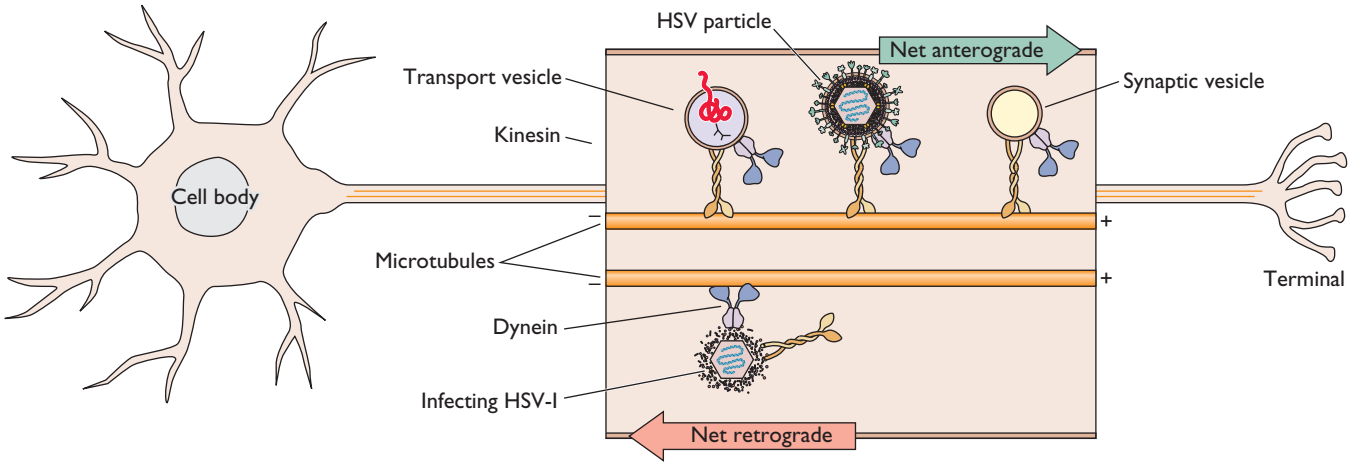
that includes herpes simplex virus type 1 and varicella-zoster virus. Upon infection of sensory neurons, herpesvirus capsids are transported to the nucleus by microtubule-based transport, probably mediated by (–) end-directed motors such as dynein (Fig. 12.15; Box 12.8). Later in the infectious cycle, virion components must be moved in the opposite direction upon association with protons of the kinesin family. How nucleocapsids and other virion proteins are targeted to exons for anterograde transport remains controversial (Box 12.9). The spread of herpesviruses from neuron to neuron occurs at or near sites of synaptic contact, indicating that virus particles must be targeted to specific areas within neurons for egress. As discussed in Volume II, Chapter 1, this attribute can be exploited to define neuronal connections in a living animal by using the virus as a self-amplifying tracer.

Disruption of the Secretory Pathway in Virus-Infected Cells

Inhibition of Transport of Particular Cellular Proteins

The genomes of several viruses encode proteins that interfere with the transport to the plasma membrane of specific cellular proteins, notably MHC class I molecules (Table 12.5; Volume II, Chapter 4). For example, the adenovirus E3 glycoprotein gp19 (Appendix, Fig. 1B) binds to these important components of immune defense in the ER and prevents their exit from this compartment, probably by inhibiting folding and assembly. Several herpesviral proteins also block transport of MHC class I molecules to the cell surface. The human cytomegalovirus US11 and US2 gene products induce transport of the cellular proteins from the ER to the cytosol for rapid degradation by the cellular proteasome. When this activity was first discovered, such retrotranslocation from the ER to the

Figure 12.15 Axonal transport in neurons. Vesicles containing cargo destined for synaptic vesicles (e.g., neurotransmitters) become associated with molecular motors of the kinesin family, and are transported down the axon on the tracks formed by axonal microtubules. The force generated by kinesin family motors allows transport at rates of 1 to 2 μm/s. Transport toward the cell body is thought to be mediated by the cytoplasmic motor dynein. In infected neurons, herpes simplex virus type 1 (HSV-1) components are transported in opposite directions at the beginning and end of the infectious cycle.



BOX 12.8 DISCUSSION

The dynamics of herpesvirus capsid transport in axons

The core structures of many viruses move within cells by association with host cytoskeletal motor proteins; however, the mechanisms by which intracellular viral particles are transported toward sites of replication or the cell periphery at distinct stages of infections remain to be understood.

In this study, green fluorescence protein-labeled capsids of pseudorabies virus (an alphaherpesvirus) were visualized in living, unfixed axons following infections of chicken sensory dorsal root ganglia neurons. Images of individual green fluorescent protein puncta (capsids) were captured by laser-scanning confocal microscopy at multiple frames per second.

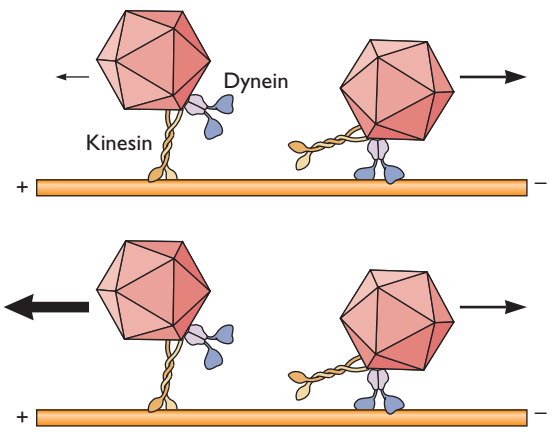
After entry of the capsids into axons, the motion of capsids was bidirectional and saltatory, i.e., proceeding by starts and stops. Nevertheless, the net direction of movement was toward the cell body (retrograde). The dynamics of capsids immediately after entry was unperturbed by the presence of egressing virus particles produced during a prior infection. This observation indicates that transport direction is modulated not by viral gene expression, but rather by a component of the subviral particle. The motion of newly formed capsids later in infection was also bidirectional and saltatory, but the net flow of capsids was reversed, and was predominantly toward the cell body (anterograde). It was suggested that the control of net directional transport of capsids occurs by modulation of plus-end, but not minus-end, motors.

Smith, G. A., L. Pomeranz, S. P. Gross, and L. W. Enquist. 2004. Local modulation of plus-end transport targets herpesvirus entry and egress in sensory axons. *Proc. Natl. Acad. Sci. USA* **101:**16034–16039.

A Nucleocapsid motion: egress versus entry in neurons

Direction	Rate (μm/s)	Run length (μm)
Egress		
Anterograde	1.97	13.1
Retrograde	1.28	6.8
Entry		
Anterograde	0.55	0.5
Retrograde	1.17	8.1

B



Coordination of a bidirectional motor complex directs capsid transport. The model depicts contrasting transport dynamics of herpesvirus capsids during entry and egress. Dynein and a kinesin family motor are illustrated associated with individual capsids simultaneously as part of a coordinated bidirectional motor complex. A direct interaction of capsids with motor complexes is shown for illustrative purposes only: the interaction of motors with cargo or each other may be direct, or indirect by means of additional proteins and capsids may be inside vesicles during egress. Net retrograde motion required for entry results when kinesin-mediated movement is reduced **(top).** Alternatively, increasing the kinesin contribution results in the net enterograde motion required for egress **(bottom).** In this model, the contribution of the dynein motor does not change during entry and egress.

cytoplasm appeared very unusual. However, as discussed previously, it is now clear that this is a normal activity of uninfected cells. The human immunodeficiency virus type 1 Vpu protein (Appendix, Fig. 19), a transmembrane phosphoprotein, also induces selective degradation of newly synthesized MHC class I proteins, and of CD4, by a similar mechanism (Table 12.5). Such degradation of CD4, the major receptor for this virus, is important for assembly and release: tight binding of this cellular protein to the viral Env glycoprotein in the ER prevents transit of both proteins to the cell surface.

Drastic Effects on Compartments of the Secretory Pathway

Proteins encoded by certain other viruses exert more drastic effects on the cellular secretory pathway. For example, rotaviruses, which lack a permanent envelope but are transiently membrane enclosed during assembly, encode

BOX 12.9 DISCUSSION

How are herpesviral particles targeted to axons for anterograde transport?

Alphaherpesviruses (e.g., herpes simplex virus [HSV] and pseudorabies virus [PRV]) replicate and traffic within polarized neurons, a strategy conducive to their lifestyles in the host peripheral nervous system. Infection begins with virion entry at mucosal surfaces and spread of infection between cells of the mucosal epithelium. The peripheral nervous system is infected via axon termini innervating this region, and subsequent trafficking of nucleocapsids to the cell body. It is here that a reactivatable, latent infection that persists for the life of the host is established. A well-known but poorly understood phenomenon is that, upon reactivation from latent infection, alphaherpesviruses rarely enter the central nervous system, despite having what seems to be two rather similar choices: cross one synapse and infect the central nervous system (rare) or traffic back down the axon and cross to the initial site of (peripheral) infection, mucosal epithelial cells (very common). Inherent in this choice is the fact that viral proteins must be targeted to axons, a highly specialized neuronal compartment restricted to only a subset of neuronal proteins. The primary problem is to identify the mechanisms that gather and sort the many viral structural proteins to this compartment.

The mechanisms by which newly synthesized structural proteins are sorted to axons for anterograde transport have been the subjects of considerable controversy. In fact, different processes have been proposed for herpes simplex virus and pseudorabies virus, separate transport of the nucleocapsid, plus tegument and viral glycoprotein and transport of enveloped virions, respectively. Similar methods have been used to examine anterograde transport of the two viruses in several laboratories. The methods include confocal microscopy, imaging of nucleocapsids and glycoproteins that carry distinguishable fluorescent labels in live cells, and immunoelectron microscopy. Nevertheless, the controversy has not been resolved. Although counterintuitive, it is possible that different processes operate in cells infected by these two herpesviruses, which are the most distantly related among the alphaherpesviruses. This hypothesis implies that envelopment of naked herpes simplex virus nucleocapsids takes place at the membrane of axonal growth cones, whereas unenveloped pseudorabies virus nucleocapsids can travel only in the retrograde direction.

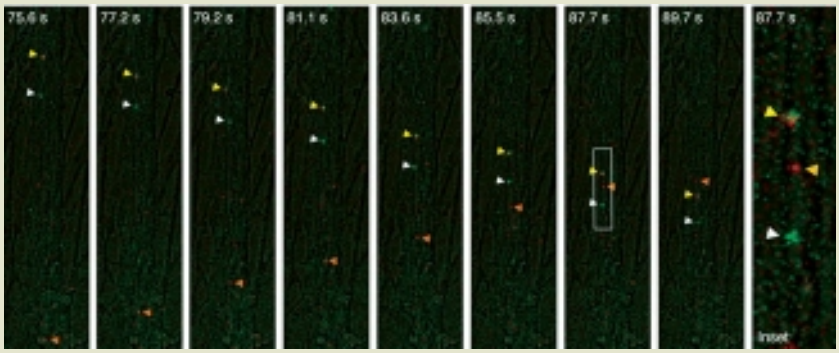
Antinone, S. E., and G. A. Smith. 2006. Two modes of herpesvirus trafficking in neurons: membrane acquisition directs motion. *J. Virol.* **80:**11235–11240.

Diefenback, R. J., M. M. Saksena, M. Douglas, and A. Cunningham. 2007. Transport and egress of herpes simplex virus neurons. *Rev. Med. Virol.* **18:**35–51.

Feierbach, B., M. Bisher, J. Goodhouse, and L. W. Enquist. 2007. In vitro analysis of trans-neuronal spread of an alpha-herpesvirus infection in peripheral nervous system neurons. *J. Virol.*, **81:**6846–6857.

Snyder, A., B. Bruun, H. Browne, and D. Johnson. 2007. A herpes simplex virus gD-YFP fusion glycoprotein is transported seperately from viral capsids in neuronal axons. *J. Virol.* **81:**8337–8340.

Sympathetic neurons from superior cervical ganglia of Sprague-Dawley rats were cultured in a chamber system that separates cell bodies (outside) from axons (in the chamber). Cell bodies outside the chamber were infected with a dually fluorescent derivative of pseudorabies virus: the envelope is labeled green by a gD-GFP fusion protein, and the nucleocapsid is labeled red by fusion of a structural protein with mRFP. Live neurons within the chamber were examined by confocal video microscopy from 15.5 h after infection. Shown are individual images, taken at the intervals indicated. The inset shows an enlargement fo the area outlined in white. The axon runs along the longitudinal axis of the frame. The yellow puncture (yellow arrowhead) represents an enveloped (green) nucleocapsid (red, green plus red = yellow). This particle moved in the opposite direction to a red punctum (orange arrowhead), that is, a naked nucleocapsid. Eventually, the paths of these two particles cross within the same axon. Adapted from B. Feierbach et al., *J. Virol.* **81:**6846–6857, 2007, with permission.



a protein that disrupts the ER membrane. This protein is thought to allow removal of the temporary envelope formed during virion assembly. The replication of most (+) strand RNA viruses takes place in membranous structures derived from various cytoplasmic membranes of the host cell. Such remodeling of cytoplasmic membranes is best understood for poliovirus and other enteroviruses, which induce a dramatic reorganization of compartments of the secretory pathway.

Three functionally distinct alterations of internal cell membranes occur in cells infected with poliovirus (Fig. 12.16). The Golgi complex is disrupted, an effect that is also observed when the viral protein 2B is synthesized in cells. Golgi disassembly is not required for viral replication.

Table 12.5 Viral proteins that inhibit transport of cellular proteins through the secretory pathway

Viral protein	Properties and activities
Adenovirus	
Ad2 E3gp19	ER transmembrane glycoprotein; blocks exit of MHC class I proteins from the ER; probably inhibits folding and assembly
Herpesvirus	
Human cytomegalovirus US2 and US11	ER transmembrane proteins; induce retrotranslocation of MHC class I proteins from ER to cytoplasm for proteasomal degradation
Picornavirus	
Poliovirus 2BC, 3A	Inhibit vesicular transport from ER; induce accumulation of infected-cell-specific vesicles derived from ER and autophagosomes; associated with membranes of the viral replication complex
Retrovirus	
Human immunodeficiency virus type 1	
Vpu	Transmembrane protein with phosphorylated cytoplasmic domain; induces degradation of MHC class I and CD4 proteins
Nef	Induces decreased cell surface accumulation of MHC class I proteins and retention in intracellular compartments; increases rate of endocytosis of CD4

Poliovirus infection also leads to inhibition of protein traffic to the cell surface, a consequence of blocked transport from the ER intermediate compartment of the Golgi complex. This effect may be phenocopied by synthesis of viral protein 3A in cells. Because poliovirus particles lack an envelope, the cellular secretory pathway is not needed for virus reproduction. However, inhibition of protein secretion interferes with some antiviral responses, such as those mediated by MHC class I molecules and cytokines, and may allow evasion of immune surveillance.

Independent of the disruption of the Golgi complex and inhibition of protein secretion is the accumulation in the cytoplasm of double-membrane membranous vesicles 200 to 400 nm in diameter. These vesicles resemble autophagosomes, and serve as the site of viral RNA replication (Chapter 6). They may also play a role in nonlytic release of virus from cells (Chapter 13). The synthesis of double-membrane vesicles can be induced in uninfected cells by the cosynthesis of viral proteins 2BC and 3A. The vesicles that serve as scaffolds for formation of replication complexes in cells infected by coronaviruses also exhibit properties of autophagosomes. The mechanisms by which infection by these viruses override the cellular circuits that normally prevent autophagy are not yet known. Nevertheless, it is clear that formation of autophagosomes facilitates virus replication: virus yield is reduced when synthesis of cellular proteins required for autophagy is prevented.

Signal Sequence-Independent Transport of Viral Proteins to the Plasma Membrane

As discussed in Chapter 4, many enveloped viruses contain matrix or tegument proteins lying between, and making contact with, the inner surface of the membrane of the particle and the capsid or nucleocapsid. In contrast to the integral membrane proteins of enveloped viruses, such internal virion proteins do not enter the secretory pathway, but are synthesized in the cytoplasm of an infected cell and directed to membrane assembly sites by specific signals.

Lipid-plus-Protein Signals

It has been known for many years that cytoplasmic proteins can be modified by the covalent addition of lipid chains (Table 12.4). Best characterized are the addition of the 14-carbon saturated fatty acid myristate to N-terminal glycine residues, or of unsaturated polyisoprenes, such as farnesol (C_{15}) or geranylgeranol (C_{20}), to a specific C-terminal sequence (Fig. 12.17). Palmitate is also added to some viral proteins that do not enter the secretory pathway. The discovery that transforming proteins of oncogenic retroviruses, the Src and Ras proteins, are myristoylated and isoprenylated, respectively, led to a resurgence of interest in these modifications. In this section, we focus on myristoylation and isoprenylation of viral structural proteins.

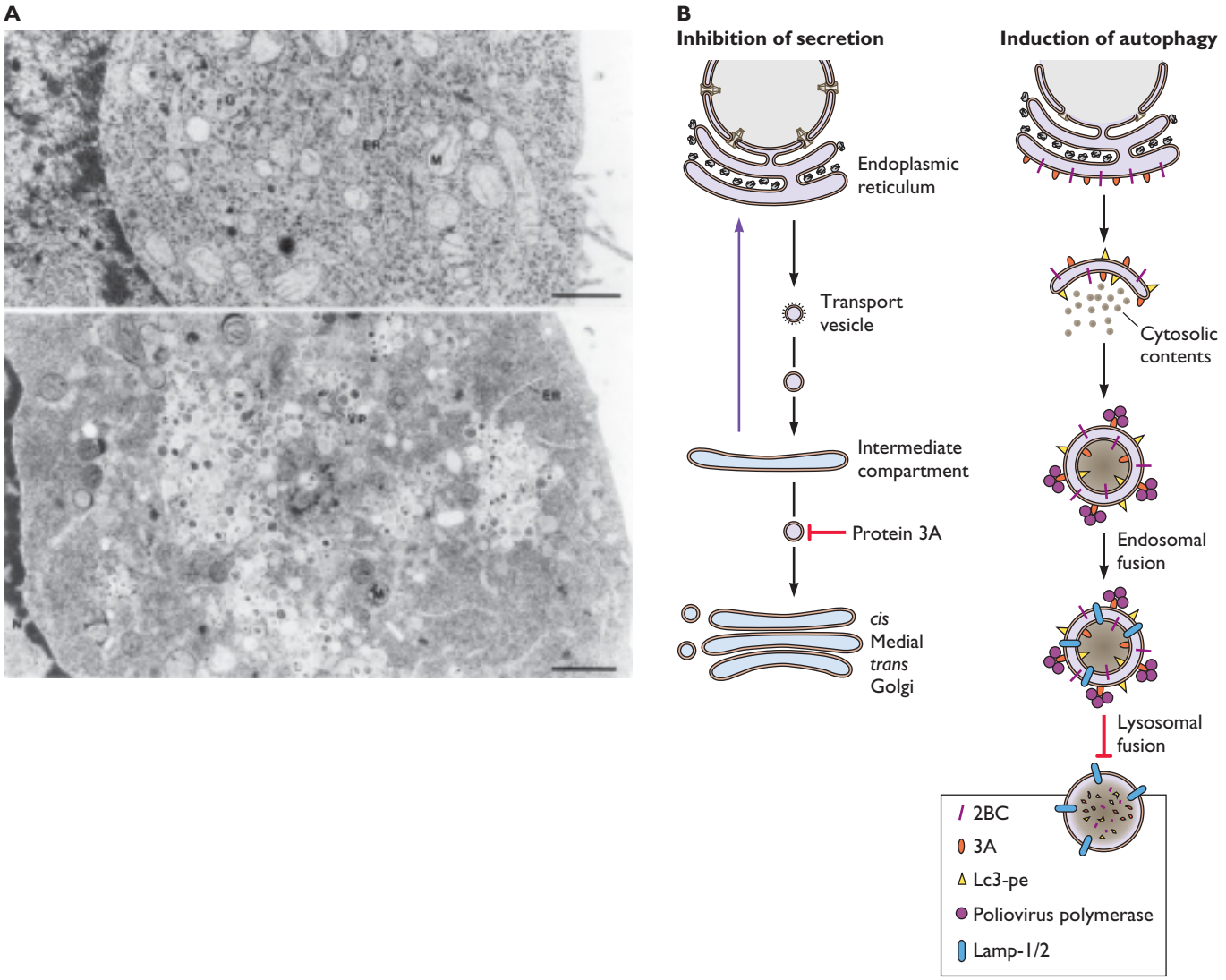


Figure 12.16 Inhibition of the cellular secretory pathway in poliovirus-infected cells. (A) Electron micrographs of uninfected HeLa cells (left) and HeLa cells 5 h after poliovirus infection (right) preserved by high-pressure freezing. Many infected cell-specific vesicles can been seen in the infected cells. G, Golgi apparatus; M, mitochondrion; VP, virus particles. The bars indicate 1 μm. Adapted from A. Schlegel et al., *J. Virol.* **70:**6576–6588, 1996, with permission. Courtesy of Karla Kirkegaard, University of Colorado, Boulder. **(B)** Models for inhibition of vesicular transport from the ER to the cell surface (left) and formation of poliovirus-induced vesicles (right). The viral 3A protein blocks transport between the ER-Golgi intermediate compartment and *cis*-Golgi compartments, and trafficking of proteins to the cell surface via the secretory pathway. The Golgi is also fragmented during polio infection, but this is not needed for viral replication or inhibition of secretion. When Golgi dispersion is prevented by nocodazole treatment in poliovirus-infected cells, the secretory pathway is still blocked. As shown at the right, the viral 3A and 2BC proteins induce the formation of double-membrane vesicles that resemble autophagosomes. These infected cell specific vesicles carry cellular proteins that are required for autophagy, such as Lc3-pe and Lamp1/2.

Myristoylation of the cytoplasmic Gag proteins of retroviruses and its consequences have been examined in detail. The internal structural proteins of these viruses, MA (matrix), CA (capsid), and NC (nucleocapsid), are produced by proteolytic cleavage of the Gag polyprotein following virus assembly. The Gag proteins of the majority of retroviruses are myristoylated at their N-terminal glycines. Mutations that alter the sequence at which murine leukemia virus or human immunodeficiency virus type 1 Gag proteins are myristoylated prevent interaction

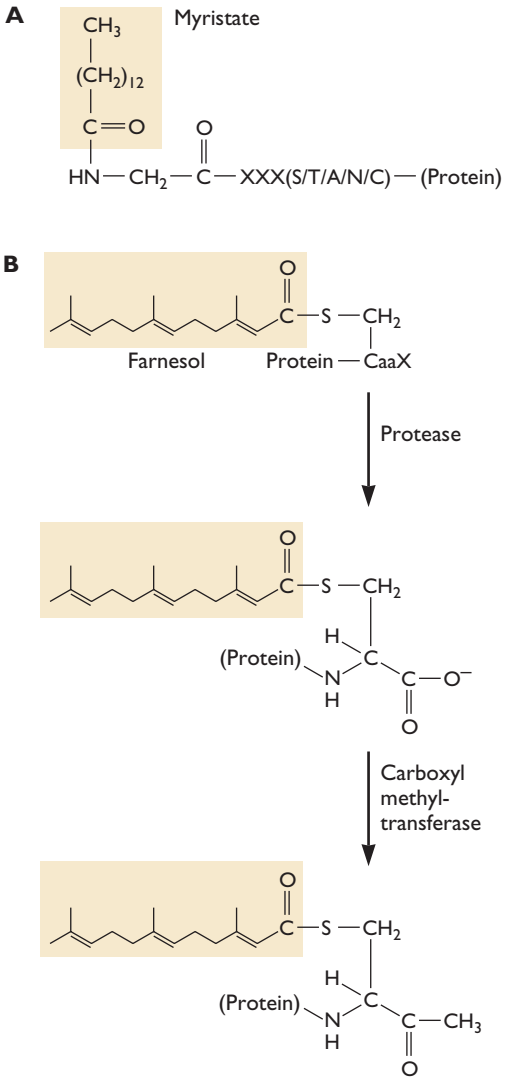


Figure 12.17 Addition of lipids to cytoplasmic proteins. (A) N-terminal myristoylation. An amide bond links the saturated fatty acid myristate to an N-terminal glycine present in the myristoylation site consensus sequence (X is any amino acid except proline). The initiating methionine must be removed, a reaction that is facilitated by uncharged amino acids in the positions denoted X. **(B)** C-terminal isoprenylation. A thioether bond links the unsaturated lipid farnesol to a cysteine in the isoprenylation consensus sequence (a is an aliphatic amino acid). In many proteins, isoprenylation is followed by proteolytic cleavage to expose the C-terminal cysteine, which is then methylated.

of the protein with the cytoplasmic face of the plasma membrane, induce cytoplasmic accumulation of Gag, and inhibit virus assembly and budding. In the case of the human immunodeficiency virus type 1 Gag protein, the myristoylated N-terminal segment and a highly basic sequence located a short distance downstream (Fig. 12.18) form a bipartite signal, which allows membrane binding *in vitro* and virus assembly and budding *in vivo*. The MA domain of the Gag protein of this protein binds to phosphatidylinositol-(4,5)-bisphophate, a lipid found only in the plasma membrane. This interaction accounts for the preferential association of Gag with the plasma membrane. It also induces a conformational change that leads to exposure of the N-terminal myristate, and presumably tighter association of Gag with the membrane.

The hepatitis B virus large surface (L) protein is also myristoylated at its N terminus. However, in contrast to retroviral Gag, the L protein is present in the envelope of the virion. Modification of its N terminus must therefore occur while it traverses the secretory pathway. In this case, myristoylation is not necessary for assembly or release of virions, but is required for infection of primary hepatocytes, presumably because it contributes to the initial interaction of the virus with, or its entry into, the host cell. More surprising is the myristoylation of structural proteins of poliovirus (VP4) and polyomavirus (VP2): although these virions do not contain an envelope, such modification is necessary for efficient assembly of both viruses. In mature poliovirus particles, the myristate chain at the N terminus of VP4, which is processed from VP0, interacts with the VP3 protein on the inside of the capsid (Fig. 4.13B). The hydrophobic lipid chain must therefore facilitate protein-protein interactions necessary for the assembly of virions. The fatty acid is also important during entry into cells of poliovirus particles and their uncoating at the beginning of an infectious cycle (see Chapter 5).

Among viral structural proteins, only the large delta protein of the hepatitis delta satellite virus has been found to be isoprenylated. Formation of the particles of this satellite virus depends on structural proteins provided by the helper virus, hepatitis B virus. The isoprenylation of large delta protein is necessary, but not sufficient, for its binding of the hepatitis B virus S protein during assembly of the satellite. This hydrophobic tail of large delta protein seems likely to facilitate interaction with the plasma membrane adjacent to the helper envelope of S protein in cells infected by the two viruses.

The addition of lipid chains allows the association of virion proteins with the cytoplasmic face of the plasma membrane, providing opportunities for interactions with viral membrane glycoproteins. Therefore, we can now explain (at least in principle) how cytoplasmic structural proteins, such as the Gag and Gag-Pol polyproteins of most retroviruses, associate with the plasma membrane.

Protein Sequence Signals

The matrix proteins of members of several families of (−) strand RNA viruses lie between the nucleocapsid and

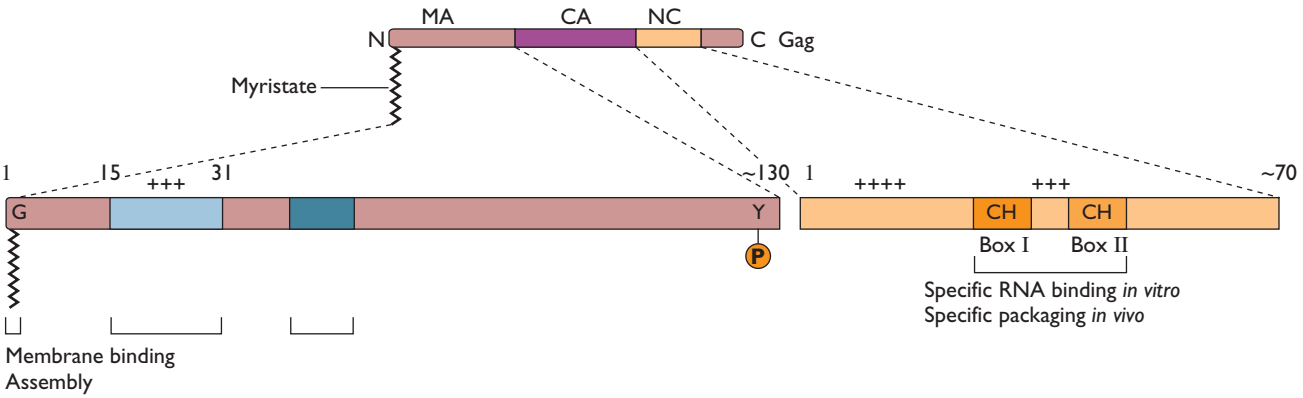
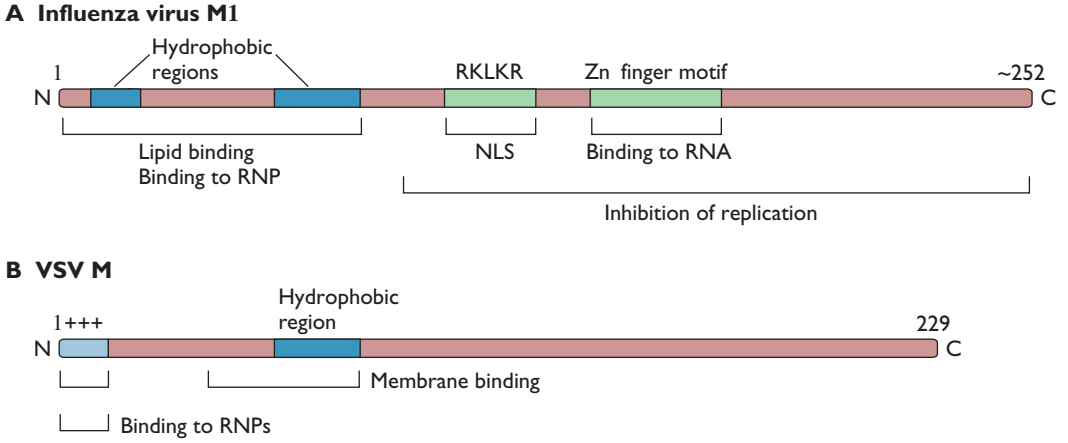


Figure 12.18 Human immunodeficiency virus type 1 Gag proteins and their targeting signals. The locations of the internal structural proteins MA (matrix), CA (capsid), and NC (nucleocapsid) in the Gag polyprotein are shown at the top. Sequence features, localization signals (MA), and the RNA-binding domain (NC) are shown below. The lengths of the MA and NC proteins are listed as approximate because of the variation among virus isolates. Specific amino acids are given in the single-letter code in the boxes, and a plus sign indicates a basic amino acid. The basic region of MA of simple retroviruses, such as avian sarcoma virus, is not required for membrane binding. The CH boxes of NC contain three cysteines and one histidine, and each coordinates one Zn^{2+} ion. CH box I is conserved among retroviruses, but CH box II is not.

the envelope, and are essential for correct localization and packaging of RNA genomes. During assembly, matrix proteins, such as M of vesicular stomatitis virus and M1 of influenza A virus, must bind to the plasma membrane of infected cells. These proteins are produced in the cytoplasm but, as far as is known, receive no lipid after translation. Direct membrane binding *in vivo* appears to be an intrinsic property of matrix proteins. For example, when the influenza virus M1 protein is synthesized in host cells in the absence of any other viral proteins, a significant fraction is tightly associated with cellular membranes. Both this protein and the vesicular stomatitis virus M protein contain specific sequences that are necessary for their interaction with the plasma membrane *in vivo* or with lipid vesicles *in vitro*. This region of the influenza A virus M1 protein contains two hydrophobic sequences (Fig. 12.19A), which might form a hydrophobic surface structure in the folded protein. In addition to hydrophobic segments, membrane association of the vesicular stomatitis virus M protein requires a basic N-terminal sequence (Fig. 12.19B). This latter segment might participate directly in membrane binding, like the basic

Figure 12.19 Targeting signals of matrix proteins of influenza virus (A) and vesicular stomatitis virus (B). Sequence features of specific segments of the proteins and the boundaries of targeting and RNP-binding domains are shown. Amino acids are written in the one letter code, and a plus sign indicates a basic amino acid. VSV, vesicular stomatitis virus; NLS, nuclear localization signal.



sequence of the human immunodeficiency virus type 1 Gag membrane-targeting signal, or it might stabilize a conformation of the internal sequence favorable for interaction with the membrane.

Assembly of (−) strand RNA viruses requires association of their matrix proteins with the plasma membrane. In some cases, such as the vesicular stomatitis virus M protein, specificity for the plasma membrane is an intrinsic property. However, binding of matrix proteins to the cytoplasmic tails of viral envelope glycoproteins can also be an important determinant of membrane association. The cytoplasmic domains of both the NA and HA proteins of influenza A virus stimulate membrane binding by M1 protein. Similarly, membrane binding by the matrix protein of Sendai virus (a paramyxovirus) is independently stimulated by the presence of either of the two viral glycoproteins (F or HN) in the membrane.

Interactions with Internal Cellular Membranes

The envelopes of a variety of viruses are acquired from internal membranes of the infected cell, rather than from the plasma membrane. The majority assemble at the cytoplasmic faces of compartments of the secretory pathway (Table 12.6). Although a single budding reaction is typical, the more complex herpesviruses and poxviruses interact with multiple internal membranes of the cell during assembly and exocytosis. And components of the enveloped hepatitis C virus associate with both the ER and the membrane-bound lipid droplets, in which cells store triacylglycerols and cholesterol (Box 12.10). Even some viruses with mature forms that lack an envelope, such as rotaviruses, can enter the secretory pathway transiently.

The diversity of the internal membranes with which these viruses associate during envelope acquisition and exocytosis is the result of variations on a single mechanistic theme: the site of assembly is determined by the intracellular location of viral envelope proteins (Fig. 12.20), just as assembly at the plasma membrane is the result of transport of such proteins to that site. Assembly of viruses at internal membranes therefore requires transport of envelope proteins to, and their retention within, appropriate intracellular compartments.

Localization of Viral Proteins to Compartments of the Secretory Pathway

The bunyaviruses, a family that includes Uukuniemi and Hantaan viruses, are among the best-studied viruses that assemble by budding into compartments of the secretory pathway. Bunyavirus particles contain two integral membrane glycoproteins, called G1 and G2, which are encoded within a single open reading frame of the M genomic RNA segment. Like alphaviral envelope proteins, the bunyaviral polyprotein containing G1 and G2 is processed cotranslationally by signal peptidase as the precursor enters the lumen of the ER. However, association of G1 with G2 is required for transport to, and retention in, Golgi compartments. For example, when synthesized alone, G1 accumulates in the Golgi complex in normal fashion, but G2 fails to leave the ER. When both glycoproteins are made, the G2 protein is now transported to the Golgi complex. The G1 protein may therefore contain signals necessary for transport along the secretory pathway, or it may be required for correct

Table 12.6 Interactions of viruses with internal cellular membranes

Virus family	Example	Integral membrane protein(s)	Intracellular membrane(s)	Mechanism of envelopment
Bunyaviruses	Uukuniemi virus Hantaan virus	G1, G2	*cis*-medial Golgi cisternae	Budding into Golgi cisternae
Coronavirus	Mouse hepatitis virus	M, S	ER, *cis*-Golgi network	Budding into ER and *cis*-Golgi network
Flavivirus	Dengue virus West Nile virus	E, prM	ER	Budding into ER
Hepadnavirus	Hepatitis B virus	L, M, S	ER and other compartments	Budding into ER?
Herpesvirus	Herpes simplex virus type 1	gB, gH, UL34	Nuclear membrane	Primary envelopment; budding of capsids from inner nuclear membrane
		gE-gI	*trans*-Golgi cisternae	Budding at *trans*-Golgi membrane
Poxvirus	Vaccinia virus, immature	A14L, A13L, A17L	ER	Formation of mature virion
	Vaccinia virus, intracellular mature virus	A56R (HA), F13L, B5R	Late *trans*-Golgi cisternae and post-Golgi vesicles	Wrapping of mature virion
Rotavirus	Simian rotavirus	VP7, NS28	ER	Budding into ER

BOX 12.10

EXPERIMENTS

An unexpected site of viral genome replication and assembly: lipid droplets

Mammalian cells store uncharged lipids in lipid droplets. These structures contain a core of cholesterol esterified to triacylglycerols, bounded by a single phospholipid leaflet and an external protein layer. Recent studies have highlighted a crucial role for lipid droplets in replication of hepatitis C virus, a member of the family *Flaviviridae*.

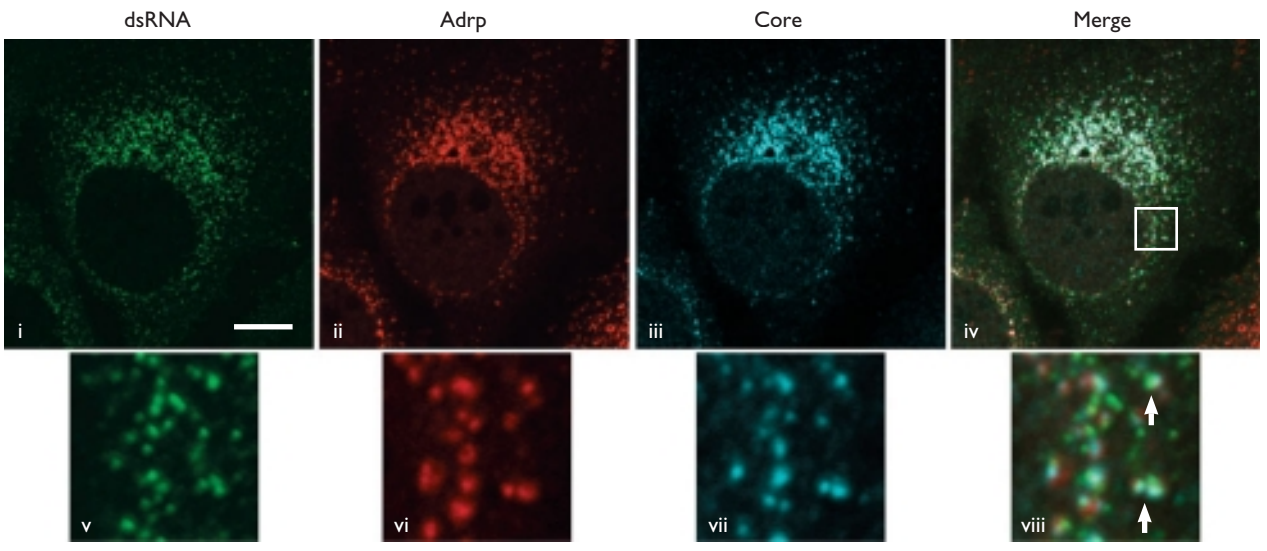
The hepatitis C virus (+) strand RNA genome contains a single open reading frame that encodes a large polyprotein. N-terminal sequences of the polyprotein become inserted into the ER membrane during translation, when cleavage by signal peptidase liberates the envelope proteins E1 and E2. The major structural protein, the core protein, is released on the cytoplasmic side of the ER membrane by cleavage by signal peptide peptidase. The several nonstructural proteins also remain in the cytoplasm, and interact directly or indirectly with the ER membrane. In contrast, the core protein becomes associated with lipid droplets.

Remarkably, lipid droplets appear to be the sites of viral genome replication (see, for example, the figure). Furthermore, substitutions in the core protein that prevent its association with lipid droplets block production of progeny virus particles. It is therefore thought that the core protein nucleates assembly of viral replication complexes on lipid droplets. The localization of this structural protein and newly synthesized RNA genomes to the same structure seems likely to facilitate the assembly of capsids. This arrangement would also be expected to promote acquisition of the envelope by budding into the ER: lipid droplets are attached to the ER membrane.

Boulant, S., P. Targett-Adams, and J. McLauchlan. 2007. Disrupting the association of hepatic C virus core protein with lipid droplets correlates with a loss of production of infectious virus. *J. Gen. Virol.* **88:**2204–2213.

Targett-Adams, P., S. Boulant, and J. McLauchlan. 2008. Visualization of double-stranded RNA in cells supporting hepatitis C virus replication. *J. Virol.* **82:**2182–2195.

Colocalization of double-stranded RNA, viral core protein and lipid droplets in hepatitis C virus-infected cells. Human cells infected with hepatitic C virus were examined by immunofluoresence with antibodies specific for double-stranded RNA (dsRNA), the lipid droplet restricted protein adipocyte differentiation-related protein (Adrp), or the viral core protein (core). The enlargements show the area boxed in white in panel iv. Scale bar, 10 μm. A variety of control experiments indicated that the anti-double-stranded RNA antibody detected sites of viral genome replication. All three molecules can be seen to be colocalized (merge), indicating that both viral RNA and the core protein are associated with lipid droplets. Adapted from P. Targett-Adams et al., *J. Virol.* **82:**2182–2195, 2008, with permission. Courtesy of P. Targett-Adams, Pfizer PGRD, Sandwich, Kent, United Kingdom.



folding of G2 and its exit from the ER. Both visualization of the intracellular distribution of the G1 and G2, and the structures of their N-linked oligosaccharides, indicate that they do not reach the *trans*-Golgi network, but accumulate in *cis* or medial compartments. The signals that specify such locations have not been identified precisely, but are included within the transmembrane domain and the adjacent segment of the cytoplasmic domain of the G1 protein.

Golgi cisternae are by no means the only compartments of the secretory pathway at which virus budding can occur. For example, rotaviruses transiently aquire an evelope by budding into and out of the ER, whereas coronaviruses

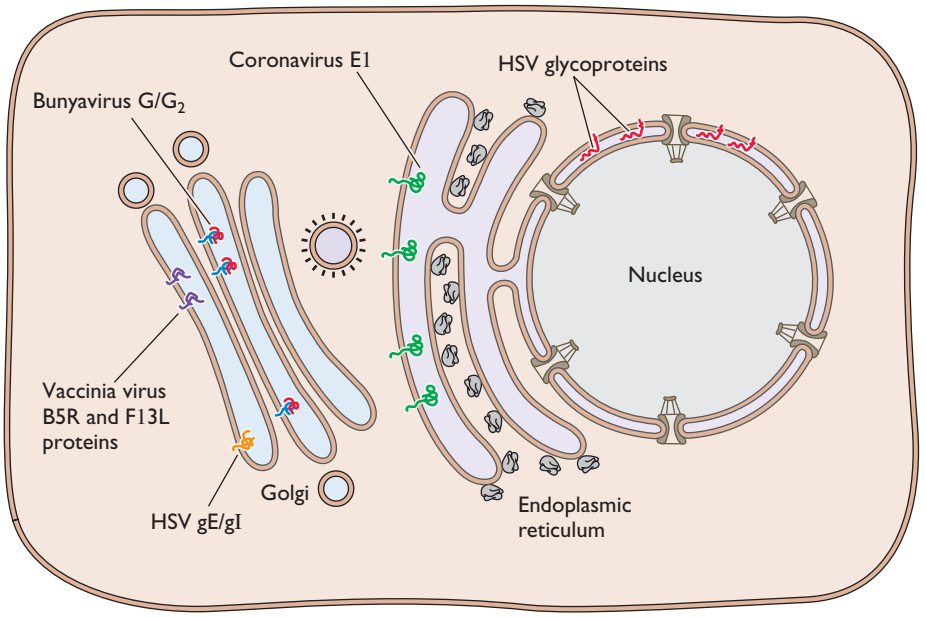


Figure 12.20 Sorting of viral glycoproteins to internal cell membranes. The destinations of membrane glycoproteins of viruses that bud into compartments of the secretory pathway (bunyaviruses and coronaviruses) or from the inner nuclear membrane and compartments of the *trans*-Golgi network (herpesviruses [HSV]) or are wrapped by cellular membranes during assembly (poxviruses) are indicated.

bud into both the ER and the *cis*-Golgi network. In these and other cases, it is the presence of viral membrane glycoproteins in specific cellular membranes (Table 12.6) that determines the site of assembly and budding.

Localization of Viral Proteins to the Nuclear Membrane

Herpesviruses such as herpes simplex virus type 1 are the only enveloped viruses that are known to assemble initially within, and bud from, the nucleus. The first association of an assembling herpesvirus with a cellular membrane is therefore budding of the nucleocapsid through the inner membrane of the nuclear envelope. This process, which is described in Chapter 13, depends on association of particular viral proteins with the inner nuclear membrane (Table 12.6).

Transport of Viral Genomes to Assembly Sites

Like the structural proteins and enzymes of the virion, progeny genomes must be transported to the intracellular site at which assembly takes place. Such transport is frequently coupled to mechanisms that prevent participation of the genome in biosynthetic reactions, i.e., replication, transcription, mRNA synthesis, or translation. The synthesis and packaging of most DNA genomes takes place in the infected cell nucleus, so no transport of progeny DNA molecules from one cellular compartment to another is required. For other DNA viruses, as well as for many with RNA genomes, both synthesis of the nucleic acid genome and assembly of virus particles take place in the cytoplasm of the host cell. However, many of these viruses assemble at membrane sites, necessitating transport of genomic nucleic acid from its site of synthesis to the cytoplasmic face of the appropriate membrane.

The membranes of both poxviruses and hepadnaviruses originate from internal compartments of the host cell (Table 12.6), and genome-bound proteins appear to mediate association of the genome with the appropriate membrane. For example, the hepadnaviral DNA genome is synthesized from the pregenomic RNA in the cytoplasm within cores containing the viral C protein. When infected cells contain a sufficient concentration of the large (L) viral glycoprotein in the ER membrane, core and L proteins can bind to one another, bringing the genome to the appropriate cell membrane and allowing budding of particles into the appropriate intracellular compartment. Similarly, many RNA genomes made in the cytoplasm must be transported to the plasma membrane for assembly of virions. Yet other RNA genomes must travel even further: both influenza virus and retroviral genomic RNAs are synthesized within the infected cell nucleus, but progeny virions bud from the plasma membrane.

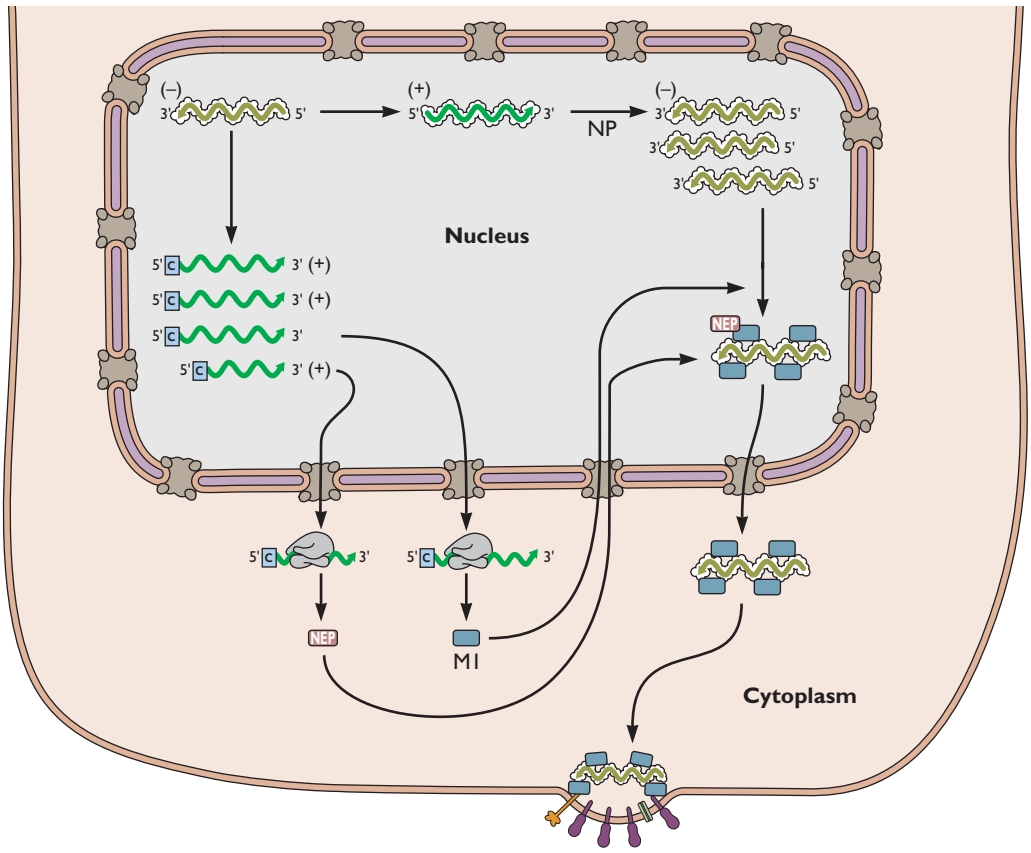
Transport of Genomic and Pregenomic RNA from the Nucleus to the Cytoplasm

Retroviral RNA genomes are unspliced RNA transcripts synthesized in infected cell nuclei by host cell RNA polymerase II, as are hepadnaviral pregenomic RNAs. These RNAs must be exported to the cytoplasm for assembly, a process which requires that the inefficient export of unspliced mRNAs

characteristic of host cells be circumvented. As discussed in Chapter 10, the genomes of complex retroviruses encode sequence-specific viral RNA-binding proteins that promote export of unspliced RNA, such as human immunodeficiency virus type 1 Rev protein. Specific sequences in the genomes of simple retroviruses and hepadnaviruses also direct export of genomic RNA, but these are recognized by cellular proteins.

Perhaps the most elaborate requirements for transport of viral RNA species between nucleus and cytoplasm are found in influenza virus-infected cells: both the direction of transport of genomic RNA and the nature of the viral RNA exported from the nucleus change as the infectious cycle progresses. When the cycle is initiated, viral genomic ribonucleoproteins (RNPs) released into the cytoplasm enter the nucleus under the direction of the nuclear localization signal of the NP protein. The mechanisms that ensure export of viral (+) strand mRNAs for translation are not fully understood. With the switch to replication, genomic (−) strand RNA segments are synthesized in infected cell nuclei, where they accumulate as viral RNPs containing the NP protein and the three P proteins. These RNPs must be exported to allow virus assembly and completion of the infectious cycle, a reaction that requires the viral M1 and NEP proteins. NEP provides the nuclear export signal, but its interaction with viral RNPs depends on the prior binding of M1 (Fig. 12.21). Because the M1 protein is not available in large quantities until the later stages of infection, such

Figure 12.21 Transport of influenza A virus genomic RNA segments from the nucleus to the plasma membrane. Genomic RNA segments are bound by the NP protein as they are synthesized (see Chapter 6) and subsequently by the M1 protein. M1 is the most abundant protein of the virus particle and enters the nucleus by means of a typical nuclear localization signal (Fig. 5.21). Binding of M1 to genomic RNPs (Fig. 12.19A) both inhibits RNA synthesis and promotes genomic RNP export. M1-continuing RNPs are directed to the cellular exportin-1 export pathway upon binding of NEP, which contains an N-terminal nuclear export signal. The NEP protein possesses no intrinsic RNA-binding activity, but includes a C-terminal M1-binding domain. This domain is thought to allow recognition of RNPs to which the M1 protein is bound. Once within the cytoplasm, viral RNPs are transported to the plasma membrane by a mechanism that is not well understood. The M1 protein interacts with both the membrane itself and the cytoplasmic domains of the HA and NA glycoproteins to initiate assembly and release of enveloped particles.



an indirect recognition mechanism probably ensures that genomic RNPs are not exported from the nucleus before sufficient quantities of viral mRNA and progeny genomic RNA have been synthesized. Furthermore, the RNPs enter the cytoplasm only when they have associated with the protein (M1) that is necessary for guiding them to the plasma membrane.

Transport of Genomes from the Cytoplasm to the Plasma Membrane

Like nuclear export, transport of the RNA genomes of enveloped viruses to the appropriate cellular membrane depends on signals present in viral proteins bound to the RNA. As we have seen, the influenza virus M1 protein contains an N-terminal sequence that allows it to bind to lipids (Fig. 12.19A). This membrane-binding domain of M1 allows association of the genomic RNPs with the plasma membrane. Although more limited in its RNA transport functions, the M protein of vesicular stomatitis virus shares several properties with the influenza virus M1 protein.

Replication of vesicular stomatitis virus RNA takes place in the cytoplasm, where newly synthesized genomic RNA molecules assemble with the N, L, and NS proteins to form helical RNPs. Genomic RNA molecules within RNPs can serve as templates for additional cycles of replication or for mRNA synthesis. However, these RNPs eventually must travel to the plasma membrane for association with the G protein and incorporation into virions. Entry into the latter pathway is determined by the viral M protein, which associates with RNPs containing genomic RNA (Fig. 12.19B). This interaction induces formation of a tightly coiled RNA-protein "skeleton," the final structure of the rhabdoviral nucleocapsid (Fig. 12.22). Formation of this structure precludes replication and mRNA synthesis and allows transport of nucleocapsids to the plasma membrane via microtubules (Box 12.11). The membrane-binding domains of the M protein described above, then mediate association with the plasma membrane.

The retroviral proteins that mediate membrane association of genomic RNA are similar to the matrix proteins of these (–) strand RNA viruses in several respects. Once within the cytoplasm, unspliced retroviral RNA is translated on polyribosomes into the Gag and, at low frequency, Gag-Pol polyproteins. The functions of Gag include transport of unspliced RNA molecules to membrane assembly sites and packaging of the RNA into assembling capsids. Consequently, whether unspliced genomic RNA molecules continue to be translated, or are redirected for assembly, is controlled by the cytoplasmic concentration of Gag. Accumulation of Gag may inhibit translation of the viral RNA genome directly: binding of the MA segment to the translational elongation protein EF1α inhibits translation *in vitro*.

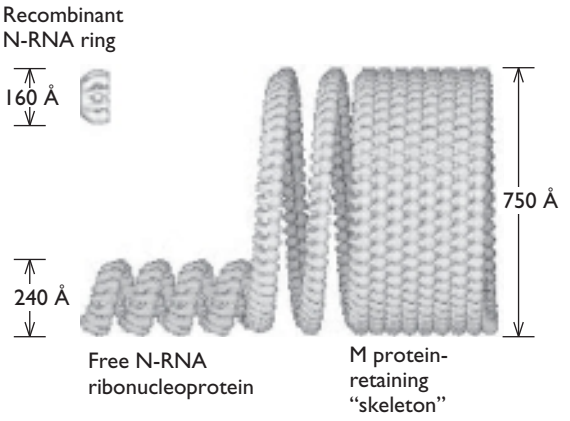


Figure 12.22 Models of the rabies virus nucleocapsid, showing the free nucleocapsid and the nucleocapsid present in virions. The models are based on cryo-electron microscopy and image reconstruction of the two forms of the nucleocapsid, as well as of rings of 9 or 10 molecules of the viral N protein and RNA assembled when the protein is produced in insect cells. The free nucleocapsid, which is the template for viral RNA synthesis, is a loosely coiled helix with a variable pitch and diameter of 240 Å. In contrast, the nucleocapsid helix incorporated into virus particles is tightly wound, with a small pitch and a much larger diameter. These structural transitions are induced by binding of the M protein to the free nucleocapsid. Adapted from G. Schoehn et al., *J. Virol.* **75:**490–498, 2001, with permission.

The NC (nucleocapsid) segment of Gag (Fig. 12.18) contains an RNA-binding domain required for specific recognition of the RNA-packaging sequences described in Chapter 13. The NC region, which functions as an independent protein in mature virions, is very basic and contains at least one copy of a zinc-binding motif (Fig. 12.18). This "zinc knuckle" domain makes a major contribution to the specificity with which Gag or NC proteins bind to unspliced retroviral RNA and, in conjunction with basic amino acids located nearby, is responsible for the RNA-packaging activity of Gag. The N-terminal MA portion of Gag contains the signals described above that target the polyprotein to the plasma membrane. Binding of Gag to unspliced retroviral RNA therefore allows delivery of the genome to assembly sites at the plasma membrane. There is accumulating evidence that more complicated mechanisms govern the fate of cytoplasmic retroviral genomes. For example, trafficking through the nucleus of Gag, which contains both nuclear localization and export signals, has been reported to be essential for efficient packaging of viral RNA during assembly.

Retroviral RNA and associated proteins travel to the appropriate cellular membrane (in most cases the plasma membrane) on recycling endosomal vesicles that move along microtubules. Cellular motor proteins that bind to Gag, such as kinesin, are thought to mediate such

BOX 12.11 EXPERIMENTS

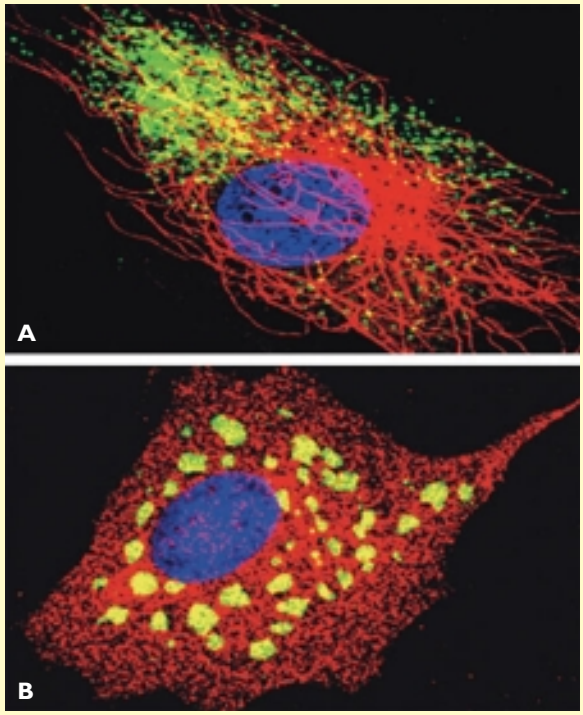
Movement of vesicular stomatitis virus nucleocapsids within the cytoplasm requires microtubules

Vesicular stomatitis virus nucleocapsids that must be transported to the plasma membrane for assembly and budding of virus particles contain the (–) strand RNA genome and several viral proteins, including the P protein (see the text). To examine intracellular trafficking of nucleocapsids, a sequence coding for a green fluorescent protein (eGFP) was inserted into that for the hinge region of the P protein. Control experiments established that the P-eGFP fusion protein catalyzed both viral mRNA synthesis and genome replication, although it exhibited somewhat reduced activity. Furthermore, mutant particles containing P-eGFP in place of the P protein were infectious.

In infected cells, P-eGFP colocalized with newly synthesized viral RNA, as well as with the N and L proteins, in cytoplasmic structures of the size predicted for nucleocapsids. Time-lapse imaging of these P-eGFP-containing structures indicated that nucleocapsids move toward the cell periphery and, unexpectedly, do so in close association with mitochondria. The significance of this association is not yet clear. However, as mitochondria are known to move on microtubules, it seemed likely that viral nucleocapsids also do so. Indeed, nucleocapsids were observed to be distributed throughout the cytoplasm in close association with microtubules (panel A below). Treatment of infected cells with drugs that disrupt microtubules, such as nocodazole, dramatically altered this pattern: nucleocapsids became clustered in the absence of microtubules in large aggregates and did not reach the plasma membrane (panel B). Such drugs also reduced virus yield significantly, confirming the importance of microtubules in the transport of vesicular somatitis virus nucleocapsids to sites of assembly.

Das, S. C., D. Nayak, Y. Zhou, and A. K. Pattnaik. 2006. Visualization of intracellular transport of vesicular stomatitis virus nucleocapsids in living cells. *J. Virol.* **80:**6368–6377.

Localization of P-eGFP-containing nucleocapsids (green) and microtubules (red) in cells infected by the mutant virus VSV-PeGFP and untreated (A) or treated with nocodazole prior to infection (B). Nuclei are in blue. Courtesy of Asit Pattnaik, University of Nebraska—Lincoln.



trafficking. Vesicle-associated transport of the viral RNA requires the packaging signal and the NC domain of Gag, which contains the RNA-binding motif described previously. Recruitment to vesicles appears to be via association of Gag with the viral Env protein, which is present in endosomal membranes. These observations suggest that the majority of the interactions among the retroviral components required for assembly are established during transport from the various sites of synthesis to the plasma membrane.

The influenza virus M1, vesicular stomatitis virus M, and retroviral Gag proteins each possess the ability to bind both to RNPs containing genomic RNA and to membranes. Such interactions commit genomic RNA to the assembly pathway, direct genomic RNA to the plasma membrane, and promote interactions among internal and envelope components of virions. These properties are essential at the end of an infectious cycle, when the primary task is assembly of progeny virions. On the other hand, they would be disastrous if the interactions could not be reversed before or at the beginning of a new cycle, when the infecting genome must reach nuclear (influenza virus) or cytoplasmic (vesicular stomatitis virus and retroviruses) sites distant from the plasma membrane. In the case of (–) strand viruses, matrix proteins are removed during virus entry. Matrix-free RNPs can then enter the nucleus for mRNA synthesis (influenza virus) or begin this process

in the cytoplasm (vesicular stomatitis virus). Retroviruses exhibit a more elegant mechanism: following virus assembly and budding, Gag (and Gag-Pol) polyproteins are processed by the viral protease to the individual structural proteins shown in Fig. 12.18. Such cleavages place the RNA-binding domain of NC in protein molecules separate from membrane-binding signals of MA, so that matrix-free core RNPs can be released into the cell to initiate a new infectious cycle.

Perspectives

The cellular trafficking systems described in this chapter are just as crucial for virus reproduction as are the host cell's biosynthetic capabilities. The trafficking requirements during the infectious cycle can be quite complex, with transport of viral macromolecules (or structures built from them) over large distances, or in opposite directions during different periods of the infectious cycle. Assembly of progeny particles of all viruses depends on the prior sorting of virion components by at least one cellular trafficking system.

This property has provided important tools (viral proteins or nucleic acids synthesized in large quantities in infected cells) with which to study these essential cellular processes. Indeed, the fundamental principle of protein sorting, that a protein's final destination is dictated by specific signals within its amino acid sequence and/or covalently attached sugars or lipids, was established by analyses of viral proteins. Furthermore, the study of viral proteins that enter the secretory pathway has provided much of what we know of the reactions by which proteins are folded and processed within the ER, as well as those that clear misfolded proteins from the pathway. It therefore seems certain that viral systems will provide equally important insights into the signals and sorting mechanisms that are presently less well characterized, such as those responsible for the direction of proteins to specialized membrane regions of polarized cells.

One of the greatest current challenges in this field remains the elucidation of the mechanics of the movement of proteins, nucleic acids, nucleoproteins, or transport vesicles from one cellular compartment or site to another. The development and application of techniques that exploit fluorescent proteins to visualize transport in living cells is providing important new insights into these processes. Motor protein-mediated movement of various viral proteins, nucleic acids, and assemblies has been observed, and seems likely to emerge as a major theme in the trafficking of viral components for assembly.

Infection by some viruses interferes with the normal transport of specific cellular proteins to the cell surface. Such inhibition can facilitate modulation of the host's immune defense systems, for example, because the display of viral antigens bound to MHC class I proteins on the surface of the infected cell is impaired or prevented. In the decade since the first edition of this book was published, we have come to appreciate that unanticipated and radical alterations in the structure of cellular organelles and transport processes also take place in cells infected by some viruses, to facilitate assembly or egress. Regardless of the degree to which replication and assembly of different viruses impinges on normal cellular processes, transport of components of virus particles to sites of assembly results in formation of microenvironments containing high concentrations of viral structural proteins and the nucleic acid genome. Such microenvironments are ideal niches for the assembly of progeny virions from their multiple parts.

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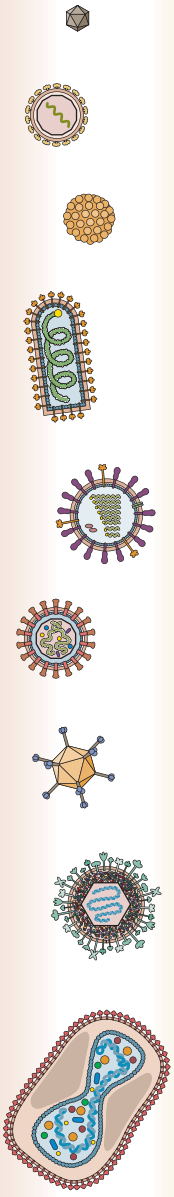


13

Assembly, Exit, and Maturation

The probability of formation of a highly complex structure from its elements is increased, or the number of possible ways of doing it diminished, if the structure in question can be broken down in a finite series of successively smaller substrates!

J. D. Bernal
A. I. Oparin (ed.), *The Origins of Life on Earth*
(Pergamon, Oxford, United Kingdom, 1959)

Introduction

Virus particles exhibit considerable diversity in size, composition, and structural sophistication, ranging from those comprising a single nucleic acid molecule and one structural protein to complex structures built from many different proteins and other components. Nevertheless, successful replication of all viruses requires execution of a common set of *de novo* assembly reactions. These processes include formation of the structural units of the protective protein coat from individual protein molecules, assembly of the coat by interaction among the structural units, incorporation of the nucleic acid genome, and release of newly assembled progeny virions (Fig. 13.1). In many cases, formation of internal virion structures must be coordinated with acquisition of a cellular membrane into which viral proteins have been inserted, or additional maturation steps must be completed to produce infectious particles. Assembly of even the simplest viruses is therefore a remarkable process requiring considerable specificity in, and coordination among, each of multiple reactions. Furthermore, virus reproduction is successful only if each of the assembly reactions proceeds with reasonable efficiency, and if the overall pathway is irreversible. The diverse mechanisms by which viruses assemble represent powerful solutions to the problems imposed by *de novo* assembly. Indeed, infectious virus particles are produced in prodigious numbers with great specificity and efficiency.

The structure of a virus particle determines the nature of the reactions by which it is formed (Fig. 13.1), as well as the mechanisms by which it enters a new host cell, reproduces, and promotes pathogenesis within a host animal. For example, the exceptionally stable poliovirus survives passage through the stomach to replicate in the gut. Despite such virus-specific variations in structure and biological properties, all viruses must be metastable structures well suited for protection of the nucleic acid genome in extracellular environments. They must also be built in a way that allows their ready disassembly during entry into a new host cell. A number of elegant mechanisms resolve the apparently paradoxical requirements for very stable associations among virion

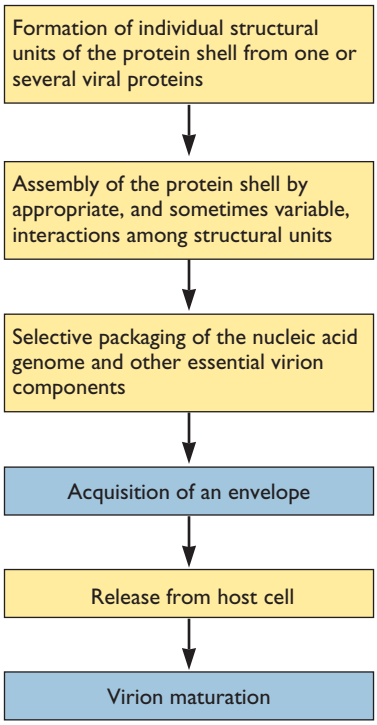


Figure 13.1 Hypothetical pathway of virion assembly and release. Reactions common to all viruses are shown in yellow, and those common to many viruses are shown in blue. The structural units that are often the first assembly intermediates are the homo- or hetero-oligomers of viral structural proteins from which virus particles are built (see Table 4.1). The arrows indicate a general sequence that strictly applies to only some viruses. Packaging of the genome can be coordinated with assembly of the capsid or nucleocapsid, and, for enveloped viruses, assembly of internal components can be coordinated with acquisition of the envelope.

components during assembly and transmission but facile reversal of these interactions when appropriate signals are encountered upon infection of a host cell.

Like synthesis of viral nucleic acids and proteins, production of virus particles depends on host cell components, such as the cellular proteins that catalyze or assist the folding of individual protein molecules. Furthermore, the components from which virions are built are transported to the appropriate assembly site by cellular pathways (see Chapter 12). Such localization of virion components to a specific intracellular compartment or region undoubtedly facilitates virus production: the concentration of virion components in specialized microenvironments must increase the rates of assembly reactions. It is also likely to restrict the number of interactions in which a particular virion component can engage, thereby increasing the specificity of assembly reactions.

A successful virus-host interaction, the survival and propagation of a virus in a host population, generally requires dissemination of the virus beyond the cells infected upon initial contact. Progeny virus particles assembled during the later stages of the infectious cycle must therefore escape from the infected cell for transmission to new host cells within the same animal or to new host animals. The majority of viruses leave an infected cell by one of two general mechanisms: they are released into the external environment (upon budding from, or lysis of, the cell), or they are transferred directly into a new host cell.

Methods of Studying Virus Assembly and Egress

Mechanisms of virus assembly and release can be understood only with the integration of information obtained by structural, biochemical, genetic, and imaging approaches. These methods are introduced briefly in this section.

Structural Studies of Virus Particles

The mechanisms by which viruses form within, and leave, their host cells are intimately related to their structural properties. Our understanding of these processes therefore improves dramatically whenever the structure of a virus particle is determined. An atomic-level description of the contacts among the structural units that maintain the integrity of the virus particle identifies the interactions that mediate assembly, and the ways in which these interactions must be regulated. For example, the X-ray crystal structure of the polyomavirus simian virus 40 particle described in Chapter 4 solved the enigma of how VP1 pentamers could be packed in hexameric arrays. Three distinct modes of interpentamer contact, mediated by conformationally flexible N- and C-terminal arms of VP1 subunits, were also identified. Assembly of the simian virus 40 capsid therefore must require specific variations in the way in which pentamers associate, depending on their position in the capsid shell. Such subtle, yet sophisticated, regulation of association of structural units during assembly was certainly not anticipated and could be revealed only by high-resolution structural information. Such information can also provide important insights into specific features of individual assembly pathways or the mechanisms which ensure that assembly is irreversible, as discussed for picornaviruses in subsequent sections.

The atomic structures of larger naked particles and enveloped viruses cannot yet be determined by X-ray crystallography. However, the newer methods for structural analysis, notably, cryo-electron microscopy and difference imaging, are rapidly improving our understanding of the mechanisms by which these more complex virions assemble.

Visualization of Assembly and Exit by Microscopy

While high-resolution structural studies of purified virions or virion proteins provide a molecular foundation for describing virus assembly, they offer no clues about how assembly (or exit) actually proceeds in an infected cell. Electron microscopy can be applied to investigation of these processes. Examination of thin sections of cells infected by a wide variety of viruses has provided important information about intracellular sites of assembly, the nature of assembly intermediates, and mechanisms of envelope acquisition and release of particles. This approach can be particularly useful when combined with immunocytochemical methods for identification of individual viral proteins, or the structures they form, via binding of specific antibodies attached to electron-dense particles of gold.

The labeling of viral proteins by fusion with the green fluorescent protein (Chapter 2) (or of membranes with fluorescent lipophilic dyes) allows direct visualization of assembly and egress, an approach inconceivable even a few years ago. Such chimeric proteins and virus particles containing them can be observed in living cells, and their associations and movements can be recorded by video microscopy. Consequently, these techniques overcome the limitations associated with traditional microscopic methods, which provide only static views of populations of proteins or virus particles.

Biochemical and Genetic Analysis of Assembly Intermediates

Although of great value, the information provided by X-ray crystallography or microscopy is not sufficient to describe the dynamic processes of virus assembly and release. An understanding of virus assembly requires identification of the intermediates in the pathway by which individual viral proteins and other virion components are converted to mature infectious virions.

When extracts are prepared from the appropriate compartment of infected cells under conditions that preserve protein-protein interactions, a variety of viral assemblies often can be detected by techniques that separate on the basis of mass and conformation (velocity sedimentation in sucrose gradients or gel filtration) or of density (equilibrium centrifugation). These assemblies range from structural units of the capsid or nucleocapsid (see Table 4.1 for the definition of structural units) to empty capsids and mature virions. Similar methods have identified various subcomplexes formed by viral structural proteins in *in vitro* reactions. Furthermore, such structures can be organized into a sequence logical for assembly, from the least to the most complex. On the other hand, it is often quite difficult to **prove** that structures identified by these approaches, such as empty capsids, are true intermediates in the pathway.

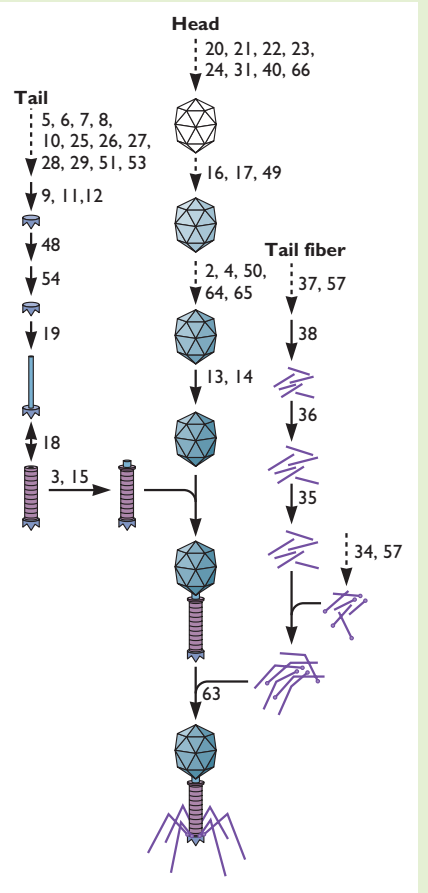
By definition, the intermediates in any pathway do not accumulate, unless the next reaction is rate limiting. For this reason, assembly intermediates are generally present within infected cells at low concentrations against a high background of the starting material (mono- or oligomeric structural proteins) and the final product (virus particles). This property makes it difficult to establish precursor-product relationships by pulse-chase experiments: the large pools of structural proteins initially labeled are converted only slowly and inefficiently into subsequent intermediates in the pathway. Genetic methods of analysis provide one powerful solution to this problem. Mutations that confer temperature-sensitive or other phenotypes and block a specific reaction have been invaluable in the elucidation of assembly pathways. A specific intermediate may accumulate in mutant virus-infected cells, and can often be purified and characterized more readily. The judicious use of temperature-sensitive mutants can allow the reactions in a pathway to be ordered, and second-site suppressors of such mutations can identify proteins that interact with one another. Of even greater value is the combination of genetics with biochemistry, an elegant approach pioneered more than 35 years ago during studies of assembly of the complex bacteriophage T4 with the development of *in vitro* complementation (Box 13.1).

The difficulties inherent in kinetic analyses are compounded by the potential for formation of dead-end products, and the unstable nature of some assembly intermediates. Dead-end assembly products are those that form by off-pathway (side) reactions. Because they are not true intermediates, they may accumulate in infected cells and be identified incorrectly as components in the pathway. By definition, such dead-end products differ from true intermediates in some structural property that prevents them from completing assembly. Furthermore, some authentic intermediates may be fragile structures, because they lack the complete set of intermolecular interactions that stabilize the virus particle. Less obvious is the conformational instability of some intermediates: such structures do not fall apart during isolation and purification but, rather, undergo irreversible conformational changes so that the structures studied experimentally do not correspond to **any** present in the infected cell. This kind of instability is not easy to detect, because monoclonal antibodies that distinguish specific structural features, rather than a simple linear sequence of amino acids, are needed. Consequently, such conformational change may well escape notice, as was initially the case for poliovirus empty capsids.

BOX 13.1 BACKGROUND

Late steps in T4 assembly

As illustrated, the head, tail, and tail fibers of this morphologically complex bacteriophage first form separately and then assemble with one another. The many genes encoding products that participate in building the T4 particle are listed by the reaction for which they are required. These gene products and the order in which they act were identified by genetic methods, including identification of second-site suppressors of specific mutations (see Chapter 3). The development of *in vitro* systems in which specific reactions were reconstituted was also of the greatest importance, allowing the development of biochemical complementation. For example, noninfectious T4 particles lacking tail fibers accumulate in infected cells when the tail fiber pathway (right part of figure) is blocked by mutation. These incomplete particles can be converted to infectious phage when mixed *in vitro* with extracts prepared from cells infected with T4 mutated in the gene encoding the major "head" protein. The fact that the phages formed in this way were infectious established that assembly was accurate. This type of system was used to identify the genes encoding proteins required for assembly of heads or tails, as well as scaffolding proteins essential for assembly of the head, but not present in the virus particle. Adapted from W. B. Wood, *Harvey Lect.* **73:**203–223, 1978, and W. B. Wood et al., *Fed. Proc.* **27:**1160–1166, 1968, with permission.



Methods Based on Recombinant DNA Technology

Modern methods of molecular biology and the application of recombinant DNA technology have greatly facilitated the study of virus assembly. Especially valuable is the simplification of this complex process that can be achieved by the synthesis of an individual viral protein or small sets of proteins in the absence of other viral components (Box 13.2).

Assembly of Protein Shells

Although virus particles are far simpler in structure than any cell, they are built from multiple components, such as a capsid (or nucleocapsid), a nucleoprotein core containing the genome, and a lipid envelope carrying viral glycoproteins. The first steps in assembly are therefore the formation of the various components of the virion from their parts. Such intermediates must then associate in ordered fashion, in some cases following transport to the appropriate intracellular site, to complete construction of the virus particle. Application of the techniques described in the previous section has allowed us to sketch the pathways by which many viruses are assembled, and describe some specific reactions in exquisite detail. In this section, we draw on this large body of information to illustrate mechanisms for the efficient assembly of protective protein coats for genomes, the first reaction listed in Fig. 13.1.

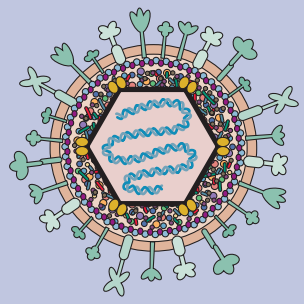
Formation of Structural Units

For some viruses, fabrication of a protein shell is coordinated with binding of structural proteins to the viral genome, as

BOX 13.2 METHODS

Assembly of herpes simplex virus type 1 nucleocapsids in a simplified system

The assembly and egress of herpesviruses from infected cells are complex processes that comprise multiple steps (Fig. 13.5 and 13.21). To facilitate analysis of the initial reactions that lead to assembly of the protein shell, the constituent proteins were produced by using baculovirus vectors. Formation of the nucleocapsid was examined by electron microscopy of insect cells infected with various combinations of the recombinant baculoviruses. Empty capsids indistinguishable from those formed in herpes simplex virus type 1-infected cells were observed when six viral genes were expressed together. Four of these encode the structural proteins VP5 (hexons and pentons), VP19C and VP23 (triplexes that link VP5 structural units), and VP26. By



omission of individual recombinant baculoviruses, it was shown that VP26 is not necessary for capsid assembly. Furthermore, only partial or deformed structures assemble in the absence of VP24, VP21, and VP22a, the protease and scaffolding proteins (see "Viral Scaffolding Proteins: Chaperones for Assembly").

Synthesis of subsets of viral proteins is also used widely to identify and characterize protein-protein and protein-membrane interactions critical for assembly.

Tatman, J. D., V. G. Preston, P. Nicholson, R. M. Elliot, and F. J. Rixon. 1994. Assembly of herpes simplex virus type 1 capsids using a panel of recombinant baculoviruses. *J. Gen. Virol.* **75:**1101–1113.

during assembly of the ribonucleoproteins of (–) strand RNA viruses. Consequently, structures built entirely from proteins do not accumulate. In other cases, the first assembly reaction is formation of the structural units from which the protein shell is constructed (Fig. 13.1). Compared to many other steps, this process is relatively simple: individual structural units contain a small number of protein molecules, typically two to six, that must associate appropriately following (or during) their synthesis. Nevertheless, several mechanisms have evolved for formation of structural units, and in some cases additional proteins are required to assist the reactions (Fig. 13.2).

Assembly from Individual Proteins

The structural units of some protein shells, including the VP1 pentamers of simian virus 40, assemble from their individual protein components (Table 13.1; Fig. 13.2A). This straightforward mechanism is analogous to formation of cellular structures containing multiple proteins, such as nucleosomes. In some cases, exemplified by adenoviral pentons, assembly is a two-step process (Fig. 13.2A). In this kind of reaction, the surfaces of individual protein molecules that contact either other molecules of the same protein or a different protein are formed prior to assembly of the structural unit. This arrangement facilitates specific binding when appropriate protein molecules encounter one another: no energetically costly conformational change is required, and subunits that come into contact can simply interlock. Production of these structural units generally can be reconstituted *in vitro*, or in cells that synthesize the component proteins. Such experiments confirm that all information necessary for accurate assembly is contained within the primary sequence, and hence the folded structure, of the protein subunits. On the other hand, the individual protein subunits must find one another in an intracellular environment, in which the concentration of irrelevant (cellular) proteins is as high as that attained in protein crystals (20 to 40 mg/ml). Such a milieu offers uncountable opportunities for nonspecific binding of viral proteins to unrelated cellular proteins. This problem may account for the synthesis of viral structural proteins in quantities far in excess of those incorporated in virus particles, a common feature of virus-infected cells. Such high concentrations must facilitate the encounter of viral proteins with one another by random diffusion, and would provide a sufficient reservoir to compensate for any loss by nonspecific binding to cellular components. Another benefit of high protein concentration is that formation of structural units proceeds efficiently (Fig. 13.2A), driving the assembly pathway in the productive direction.

Assembly from Polyproteins

An alternative mechanism for production of structural units is assembly while covalently linked in a polyprotein precursor (Fig. 13.2B). This mechanism circumvents the need for protein subunits to meet by random diffusion, and avoids competition from nonspecific binding reactions.

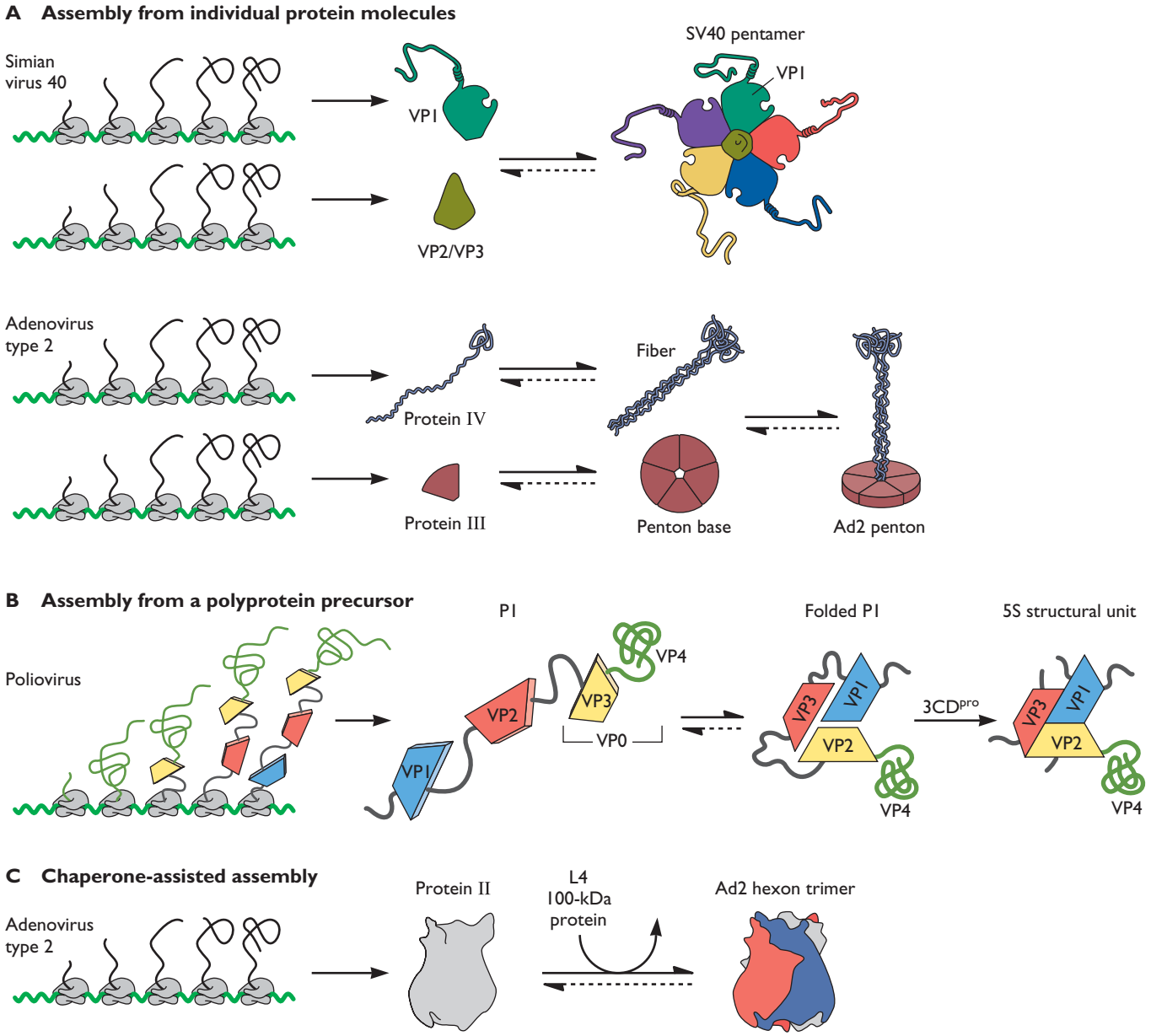


Figure 13.2 Mechanisms of assembly of the structural units of virion protein shells. (A) Assembly from folded protein monomers, illustrated with simian virus 40 (SV40) VP1 pentamers and adenovirus pentons. The assembly reactions shown are the result of specific interactions among the proteins that form structural units. In many cases, for example those among VP1 molecules in the simian virus 40 pentamer, the interactions have been described at atomic resolution (Chapter 4). These assembly reactions are driven in a forward direction by the high concentrations of protein subunits synthesized in infected cells, as indicated by the solid arrows. **(B)** Assembly from a polyprotein precursor, illustrated with the poliovirus polyprotein that contains the four proteins that form the heteromeric structural unit. The latter proteins are synthesized as part of the single polyprotein precursor from which all viral proteins are produced by proteolytic processing. For simplicity, only the P1 capsid protein precursor and its cleavage by the viral 3CD protease following folding and assembly of the proteins of the immature structural unit (VP0, VP3, and VP1) are shown. The flexible covalent connections between VP1, VP3, and VP0 in the P1 precursor, which are exaggerated for clarity, are severed by the protease to form the 5S structural unit. However, VP4 remains covalently linked to VP2 in VP0 until assembly is completed (see the text). **(C)** Assisted assembly. Some structural units are assembled only with the assistance of viral chaperones, such as the adenoviral L4 100-kDa protein, which is required for formation of the hexon trimer from the protein II monomer.

Table 13.1 Mechanisms of assembly of structural units

Mechanism	Virus	Structural unit
Association of individual protein molecules	Adenovirus (adenovirus type 2)	Protein IV trimer (fiber) and protein III pentamer (penton base) that forms pentons
	Hepadnavirus (hepatitis B virus)	C (capsid) protein dimers
	Papovavirus (simian virus 40)	VP1 pentamer, with one molecule of VP2 or VP1 in its central cavity
	Reovirus (reovirus type 1)	λ, σ2 (inner capsid protein) homo-oligomers; σ3-μ, (outer capsid protein) hetero-oligomers
Assisted assembly of protein subunits	Adenovirus (adenovirus type 2)	Hexon trimers of protein II, formed with assistance of the L4 100-kDa protein
	Herpesviruses (herpes simplex virus type 1)	VP5 pentamers and hexamers, formed with assistance of VP22a
Assembly from polyprotein precursors	Alphavirus (Sindbis virus)	Capsid (C) protein folds in, and cleaves itself from, a nascent polyprotein also containing glycoprotein sequences
	Picornavirus (poliovirus)	Immature 5S structural units, VP0-VP3-VP1
	Retrovirus (avian sarcoma virus)	NC, CA, and MA protein shells assembled via Gag polyprotein

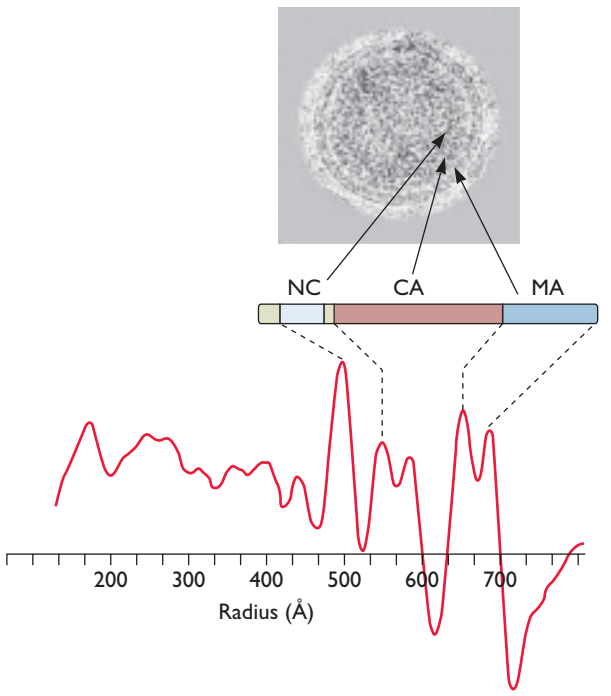
The structural units of several viruses are assembled by this mechanism (Table 13.1), which is exemplified by formation of picornaviral capsids. The first poliovirus intermediate, which sediments as a 5S particle, is the immature structural unit that contains one copy each of VP0, VP3, and VP1 (Fig. 13.2B). These three proteins are liberated from the capsid protein precursor P1 upon cleavage by the viral $3CD^{pro}$ protease. However, it is thought that folding of their central β-barrel domains (Fig. 4.12) takes place during synthesis of P1. The poliovirus structural unit can then form by intramolecular interactions among the surfaces of these β-barrel domains, before the covalent connections that link the proteins are severed.

Retrovirus assembly illustrates an elegant and effective variation on the polyprotein theme. Mature retrovirus particles contain three protein layers. An inner coat of NC protein, which packages the dimeric RNA genome, is enclosed within the capsid built from the CA protein. The capsid is in turn surrounded by the MA protein, which lies beneath the inner surface of the viral envelope (Appendix, Fig. 19). These three structural proteins are synthesized as the Gag polyprotein precursor, which contains their sequences in order of the protein layers they form in virus particles, with MA at the N terminus (Fig. 13.3). Retrovirus particles assemble from such Gag polyprotein molecules by a unique mechanism that allows orderly construction of the three protein layers and, as we shall see, coordination of this reaction with encapsidation of the genome and acquisition of the envelope.

Participation of Cellular and Viral Chaperones

The assembly of viral proteins into structural units is often assisted by cellular **chaperones**. These specialized proteins facilitate protein folding by preventing nonspecific, improper associations among exposed, sticky patches on nascent and newly synthesized proteins. The first chaperone to be identified, the product of the *Escherichia coli*

Figure 13.3 Radial organization of the Gag polyprotein in immature human immunodeficiency virus type 1 particles. The model for the arrangement of the Gag polyprotein shown below a cryo-electron micrograph of a virus-like particle assembled from Gag was deduced from radial density measurements of digitized images of the particles. The plot shows density as a function of distance from the particle center, in angstroms. Courtesy of T. Wilk, European Molecular Biology Laboratory.



groEL gene, was discovered because it is essential for reproduction of bacteriophages T4 and lambda. As discussed in Chapter 12, the participation of chaperones resident in the lumen of the endoplasmic reticulum (ER) in folding and assembly of oligomeric viral glycoproteins is well established. Cytoplasmic and nuclear chaperones seem likely to play equally important roles in the formation of structural units, or later reactions in assembly. A number of viral structural proteins have been shown to interact with one or more cellular chaperones (Table 13.2). In most cases, a role for these proteins in viral assembly is based on "guilt by association." However, some cellular chaperones have been directly implicated in assembly reactions (Table 13.2). For example, alterations in the Gag protein of the betaretrovirus Mason-Pfizer monkey virus that prevent binding to a cytoplasmic chaperone (Table 13.2) reduce the accumulation of stable Gag and capsids in cells transiently synthesizing the viral protein. These observations, and the dependence of association of Gag molecules with one another on the chaperone, indicate that the latter protein facilitates proper folding of the Gag polyprotein.

Chaperones are abundant in all cells, and some accumulate to concentrations even greater than that of ribosomes. Nevertheless, the genomes of several viruses encode proteins with chaperone activity, some with sequences and functions homologous to those of cellular proteins (Table 13.2). Some viral chaperones are essential participants in the reactions by which structural units are formed. For

Table 13.2 Cellular and viral proteins implicated in viral assembly reactions

Chaperone	Properties and function(s)	Viral protein(s) bound
Cellular chaperones		
Bacterial		
Chaperonins GroEL and GroES	GroEL comprises two rings of 8 identical subunits to which the single heptameric GroES ring binds and dissociates, regulated by the ATPase of GroEL; nonnative proteins enter the GroEL cavity, where they fold; required for assembly of phage/T4 and λ heads	Phage B protein, phage T4 gene 31 protein
Mammalian		
Chaperonin Tri C	Large, double-ring complexes of ~800 kDa surrounding a central cavity; encapsulates substrates upon ATP binding	Mason-Pfizer monkey virus Gag, implicated in productive folding of Gag
Hsp70 proteins	Cytoplasmic proteins synthesized constitutively and in response to stress; in conjunction with Hsp40 cochaperones, participate in ATP-dependent cycles of binding to, and release from, nascent proteins to prevent misfolding or nonspecific aggregation	Adenovirus protein IV Hepatitis B virus L protein Human immunodeficiency virus type 1 Gag Poliovirus P1 capsid protein precursor Simian virus 40 VP1
Hsp68	Contains ATP-binding motifs and an epitope present in a subunit of TriC; RNase L inhibitor	Human immunodeficiency virus type 1 Gag; facilitates a late reaction in assembly of Gag capsids *in vitro*
Viral chaperones		
Adenovirus type 2 L4 100-kDa protein	Formation of hexon trimers	Hexon monomer (protein II)
African swine fever virus CAP80	Productive folding of the major capsid protein, p73	Capsid protein p73
Herpes simplex virus type 1 VP22a	Formation of VP5 pentamers	VP5
Simian virus 40 LT antigen	N-terminal J-domain necessary for assembly of virions; binds to and stimulates activity of cellular Hsc70 proteins	None known
Viral scaffolding proteins		
Adenovirus type 2 L1 52/55-kDa proteins	Necessary for formation of capsids; present in immature particles, but not virions; may be required for encapsidation	IVa_2
Herpes simplex virus type 1 VP22a	Self-associates to form scaffold-like structure that organize assembly of the empty nucleocapsid	VP5 VP19-VP23 triplexes

example, production of adenoviral hexon trimers, which form the faces of the icosahedral capsid, depends on such an accessory protein, the viral L4 100-kDa protein (Fig. 13.2C). The latter protein facilitates folding of monomeric hexon subunits or their assembly into trimers, although its biochemical activity has not been identified.

Capsid and Nucleocapsid Assembly

The accumulation of virion structural units within the appropriate compartment of an infected cell sets the stage for the assembly of more complex capsids or nucleocapsids (see Table 4.1 for nomenclature). For reasons discussed previously, the reactions by which these structures are formed are often not understood in detail. Nevertheless, several different mechanisms for their assembly can be distinguished.

Intermediates in Assembly

A striking feature of well-characterized pathways of bacteriophage assembly (Box 13.1) is the sequential formation of progressively more complex structures: heads, tails, and tail fibers are each assembled in stepwise fashion via defined intermediates. Such an assembly line mechanism appears ideally suited for orderly formation of virus particles, which can be large and morphologically complex. Discrete intermediates also form during assembly of some icosahedral animal viruses. A stepwise assembly mechanism has been well characterized for poliovirus: the 5S structural unit described in the previous section is the immediate precursor of a 14S pentamer, which in turn is efficiently incorporated into virus particles (Fig. 13.4). The pentamer is stabilized by extensive protein-protein contacts and by interactions mediated by the myristate chains present on the five VP0 N termini (see Fig. 4.13C). The contribution of the lipids to pentamer stability is so great that this structure does not form at all when myristoylation of VP0 is prevented. The extensive interactions among its components result in molecular interlocking of the five structural units of the pentamer and impart great stability. Consequently, formation of the 14S assembly intermediate is irreversible under normal conditions in an infected cell, a property that imposes the appropriate directionality on the entire assembly pathway (Fig. 13.4).

For many viruses, discrete assembly intermediates like the poliovirus pentamer have been difficult to identify. In some cases, the absence of discrete intermediates can be attributed to coordination of assembly of protein shells with binding of the structural proteins to the nucleic acid genome. This mode of assembly is exemplified by the ribonucleoproteins of (−) strand RNA viruses, which assemble as genomic RNA is synthesized. Nucleocapsid formation depends on interactions of the protein components with both the nascent RNA and other protein molecules

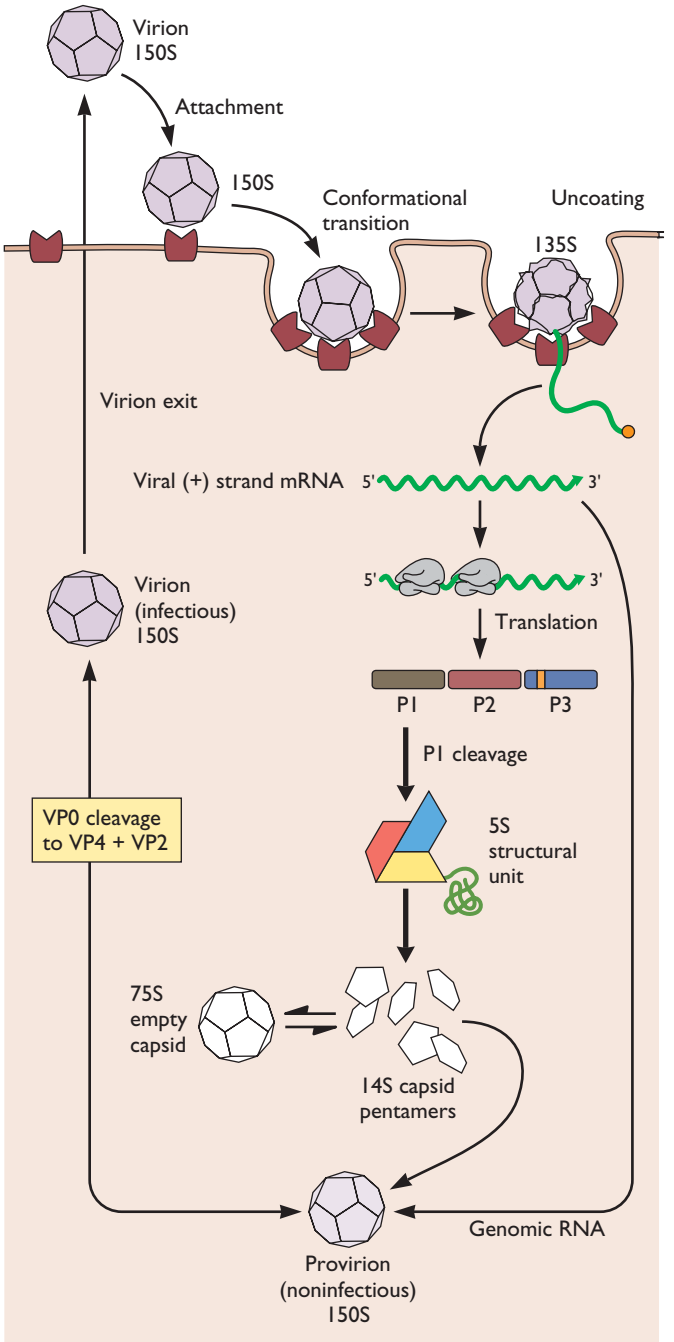


Figure 13.4 Assembly of poliovirus in the cytoplasm of an infected cell. Most of the assembly reactions are essentially irreversible, because of proteolytic cleavage (formation of 5S structural units and mature virions) or extensive stabilizing interactions in the assembled structure (formation of 14S pentamers and of provirions). Stable, empty capsids, originally considered the precursors of provirions, do not possess the same conformation as the mature virion, as symbolized by the white color, and are dead-end products. Formation of the capsid shell from 14S pentamers is coordinated with genome encapsidation, and requires replication of genomic RNA. The conformational transition upon attachment to the poliovirus receptor, for which the virion is primed by cleavage of VP0 to VP2 and VP4, is also illustrated.

previously bound to the RNA. Because the synthesis of genomic RNA molecules is an all-or-none process, so too is the assembly of ribonucleoproteins in infected cells.

Methods that permit synthesis of subsets of virion proteins have begun to provide insights into how such ribonucleoproteins assemble. The vesicular stomatitis virus N protein, which is a dimer in the helical nucleocapsid, aggregates when synthesized alone in *E. coli.* However, when the viral P protein is also made, aggregation does not occur, and discrete, disk-like oligomers assemble. The assembly contains 10 molecules of the N protein, 5 molecules of the P protein, and an RNA molecule of some 90 nucleotides (Fig. 4.7). The disk-like oligomer is equivalent to one turn of the ribonucleoprotein helix formed in vesicular stomatitis virus-infected cells, and the RNA (presumably of bacterial origin) is of the length required to bind to 10 molecules of the N protein. As no further assembly takes place in bacterial cells, even though they contain numerous, long RNA molecules, it has been proposed that assembly of the ribonucleoprotein containing genomic RNA requires a viral or mammalian cell assembly chaperone.

For many enveloped viruses, including retroviruses, assembly of a protein shell is coordinated with binding of structural proteins to a cellular membrane. This property makes isolation of intermediates a technically demanding task. Nevertheless, new methods for separation of intermediates make it possible to examine assembly reactions of these viruses. Specific assembly reactions can also be studied by using simplified experimental systems. For example, when synthesized in a cell-free transcription-translation system, the human immunodeficiency virus type 1 Gag protein multimerizes through a series of discrete intermediates to form 750S particles. These structures resemble virus-like particles released when Gag is the only viral gene expressed in mammalian cells. Conversion of an early intermediate to the 750S particle requires adenosine triphosphate (ATP) and the ATPase cellular Abcel (Hsp68) (Table 13.2). These observations illustrate the power of simplified approaches to the study of virus assembly. An important caveat is that such experimental systems must faithfully reproduce reactions that take place within infected cells. There is good reason to conclude that the *in vitro* assembly of Gag particles meets this crucial criterion, because assembly phenotypes exhibited by altered Gag proteins *in vitro* correspond closely to those observed *in vivo*. Furthermore, binding of Gag to Abcel is required for assembly of later intermediates in cells in culture.

The general dearth of structures simpler than empty capsids in cells infected by nonenveloped viruses might simply be a consequence of the properties that make such intermediates difficult to detect, notably low concentration and instability. In addition, the formation of assembly intermediates may be rate limiting, allowing stable structural units to be stockpiled before the final assembly reactions begin. While these possibilities cannot be excluded, it is more likely that assembly of many capsids is a highly cooperative, all-or-none process. Both simple and more complex icosahedral capsids are built by the repetition of interactions among multiple copies of one or a small number of structural units. Consequently, once the first few structural units were associated in the correct manner, assembly of the capsid would proceed rapidly to completion.

Self-Assembly and Assisted Assembly Reactions

The primary sequences of virion proteins contain sufficient information to specify assembly, including complex reactions like the alternative five- and sixfold packing of VP1 pentamers in the simian virus 40 capsid: when synthesized in *E. coli*, VP1 is isolated as pentamers that assemble into capsid-like structures *in vitro*. Such self-assembly is mediated by interactions between complementary surfaces in individual structural units, or in intermediates assembled from them. Self-assembly of structural proteins is the primary mechanism for formation of protein shells, but other viral components or cellular proteins can assist assembly.

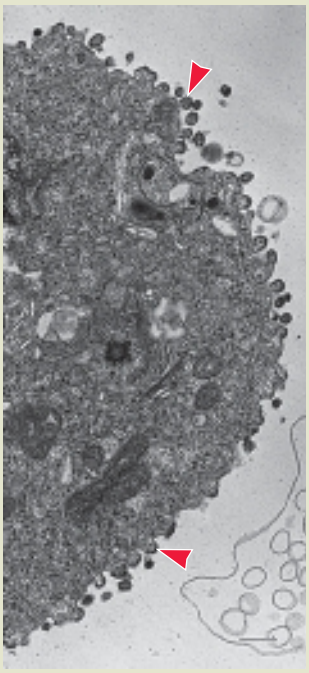
Viral and Cellular Components That Regulate Self-Assembly

Self-assembly of viral structural proteins may be the mortar for construction of virus particles, but other components of the virion often provide an essential foundation, or the blueprint for correct assembly. As we have seen, assembly of the nucleocapsids of (−) strand RNA viruses is both coordinated with, and dependent on, synthesis of genomic RNA. The RNA must serve as a template for productive and repetitive binding of nucleocapsid proteins to one another. Interactions of retroviral Gag proteins with RNA mediated by the NC RNA-binding domain also appear to be essential to initiate assembly of the Gag protein shell (Box 13.3). In other cases, the viral genome plays a more subtle yet equally important role, ensuring that the interactions among structural units are those necessary for infectivity. For example, poliovirus empty capsids lack internal structural features characteristic of the mature virion, because VP0 is not cleaved to VP4 and VP2. The RNA genome is thought to participate in the autocatalytic cleavage of this precursor, which is essential for the production of infectious particles.

Association of structural proteins with a cellular membrane is essential for the assembly of some protein shells, a situation exemplified by many retroviruses: the sequences of MA that specify Gag myristoylation and

BOX 13.3 DISCUSSION

A scaffolding function for RNA



Electron micrograph showing a thin section (fixed and stained) of a human T cell synthesizing the viral Gag-Pol protein. Prior to electron microscopy, viral particles (red arrowheads) were labeled with polyclonal antibodies (attached to gold beads) recognizing the CA protein. Bar, 1.0 μm. N, nucleus; M, mitochondrion. Courtesy of J. J. Wang, Institute of Biomedical Sciences, Academica Sinica, Taipei, Taiwan, and B. Horton and L. Ratner, Washington University School of Medicine, St. Louis, MO.

When synthesized in the absence of any other viral component, retroviral Gag polyproteins direct assembly and release of the virus-like particles shown in the figure. It was therefore assumed for many years that this protein contains all information necessary and sufficient for assembly of particles. However, the results of more recent experiments indicate that RNA acts as a scaffold during Gag assembly.

In vitro studies of the ability of altered Gag proteins to multimerize with the full-length protein initially underscored the importance of the nucleocapsid (NC) RNA-binding domain for efficient assembly. Association of Gag with RNA is also required for multimerization in this system. The apparent contradiction between these findings and efficient assembly of Gag in mammalian cells in the absence of genomic RNA was subsequently resolved: virus-like particles contain cellular RNAs when they form in cells infected by a Moloney murine leukemia virus mutant with a deletion in the signal that directs packaging of the RNA genome. Furthermore, when the Gag coding sequence was expressed from an alphavirus vector, which directs very efficient synthesis of genomic and subgenomic viral RNAs, these alphaviral mRNAs were readily detected in the Gag virus-like particles. Finally, RNase digestion of cores assembled from Gag in wild-type Moloney murine leukemia virus-infected cells was shown to dissociate these structures. This observation indicates that interactions of Gag molecules with RNA, as well as with one another, are required for assembly, and maintain particle stability.

In infected cells, interaction of the MA domain of Gag with the plasma membrane is necessary for efficient assembly (see the text). The high concentrations of Gag typically used in *in vitro* assembly reactions appears to overcome this requirement.

Campbell, S., and V. M. Vogt. 1995. Self assembly in vitro of purified CA-NC proteins from Rous sarcoma virus and human immunodeficiency virus type 1. *J. Virol.* **69:**6487–6497.

Muriaux, D., J. Mirro, D. Harvin, and A. Rein. 2001. RNA is a structural element in retrovirus particles. *Proc. Natl. Acad. Sci. USA* **98:**5246–5251.

binding to the cytoplasmic surface of the plasma membrane (described in Chapter 12) are also required for assembly of the core.

Binding of structural proteins to the genome or to a cellular membrane might simply raise their local concentration sufficiently to drive self-assembly, might organize the proteins in such a way that their interactions become cooperative, or might induce conformational changes necessary for productive association of structural units. These mechanisms, which are not mutually exclusive, have not been distinguished experimentally, but there is evidence for induction of conformational transitions in specific cases. We do not understand adequately the molecular mechanisms by which binding of structural proteins to other virion components directs or regulates particle assembly. However, such a requirement offers the important advantage of integration of assembly of protein shells with acquisition of other essential parts of the virion.

Cellular components can also modulate the fidelity with which viral structural proteins bind to one another. The capsid-like structures assembled when simian virus 40 VP1 is made in insect or mammalian cells are much more regular in appearance than those formed *in vitro* by bacterially synthesized VP1. Modification of VP1 (by acetylation and phosphorylation) or the participation of chaperones, such as Hsp70, must therefore improve the accuracy with which VP1 pentamers associate to form capsids. Similarly, *in*

vitro self-assembly of poliovirus structural proteins is very slow, proceeding at a rate at least 2 orders of magnitude lower than that observed in infected cells. Furthermore, the empty capsids that form have the altered conformation described previously, unless the reaction is seeded by 14S pentamers isolated from infected cells. This property indicates that the appropriate folding, modification, and/or interaction of the viral proteins that form the pentamer are critical for subsequent assembly reactions to proceed productively. Within infected cells, these crucial reactions are likely to be modulated by cellular chaperones, such as Hsp70, which is associated with the polyprotein during its folding to form 5S structural units. It is clear from such examples that host cells provide a hospitable environment for productive virus assembly, one that is not necessarily reproduced when viral structural proteins are made and assemble *in vitro*.

Viral Scaffolding Proteins: Chaperones for Assembly

Accurate assembly of some large icosahedral protein shells, such as those of adenoviruses and herpesviruses, is mediated by proteins that are not components of mature virions. Because these proteins participate in reactions by which the capsid or nucleocapsid is constructed, but are then removed, they are termed **scaffolding proteins**. Among the best characterized of such proteins is the precursor of the herpes simplex virus type 1 VP22a protein.

This protein is the major component of an interior core present in assembling nucleocapsids (Fig. 13.5A). In the absence of other viral proteins, it forms specific scaffold-like structures. In immature nucleocapsids isolated from infected cells, it appears as an ordered sphere that lacks the icosahedral symmetry of the mature nucleocapsid. Self-association of pre-VP22a stimulates binding of the scaffolding protein to VP5, the protein that forms the hexameric and pentameric structural units of the nucleocapsid. The VP5 and pre-VP22a proteins form a core via hydrophobic interactions, to which are added additional VP5 hexamers and the triplex structures formed by VP19 and VP23 (Table 4.7). The latter are also required for capsid assembly, which occurs by sequential formation of partial dome-like structures and the spherical immature nucleocapsid. The interactions of VP5 with the scaffolding protein guide and regulate the intrinsic capacity of VP5 hexamers (and other nucleocapsid proteins) for self-assembly: omission of the scaffolding protein from a simplified assembly system (Box 13.2) leads to the production of unclosed and deformed nucleocapsid shells.

One of the 12 vertices of the herpesviral nucleocapsid is formed by the portal through which the DNA enters, rather than a VP5 pentamer (Fig. 13.5A, see also Fig. 4.26). Consequently, this unique structural unit, a dodecamer of the UL6 protein, must be incorporated at just one vertex during assembly. This reaction requires interaction of the portal with the scaffolding protein: a small molecule that blocks this interaction prevents assembly of portal-containing nucleocapsids in infected cells. Although the portal is dispensable for formation of procapsids or nucleocapsids, the results of *in vitro* studies indicate that it can be incorporated only during the initial stages of assembly. The mechanism which ensures that each nucleocapsid contains only one portal remains an enigma.

Once nucleocapsids have assembled, scaffolding proteins must be disposed of, so that viral genomes can be accommodated (Fig. 13.5A). The virion protease (VP24), which is also present in the core of assembling nucleocapsids, is essential for such DNA encapsidation. This protein is incorporated into the assembling nucleocapsid as a precursor (Fig. 13.5B). The protease precursor possesses some activity and initiates cleavage to produce VP24. The protease cleaves a short C-terminal sequence from the scaffolding protein that is required for binding of the scaffolding protein to VP5. Such processing presumably disengages scaffolding from structural proteins, once assembly of the nucleocapsid is complete. The protease also degrades the scaffolding protein so that encapsidation of the genome can begin.

The proteolytic cleavages that liberate the VP5 structural units from their association with the scaffold also induce major changes in the organization of the nucleocapsid shell. Assembly of the nucleocapsid from its constituent proteins *in vitro* proceeds via a short-lived, spherical precursor to the mature structure. This intermediate possesses the $T = 16$ icosahedral symmetry characteristic of the mature nucleocapsid. However, it is unstable and dissociates at low temperature, because the strong interactions among VP5 structural units that form the floor of the nucleocapsid shell are absent. Similarly cold-sensitive particles accumulate at nonpermissive temperatures in cells infected by a mutant virus encoding a temperature-dependent viral protease, and can form infectious virions following shift to a permissive temperature. These properties suggest that the open structures initially assembled *in vitro* may correspond to *bona fide*, but short-lived, intermediates in herpesvirus assembly *in vivo*, analogous to the well-characterized **procapsids** formed during assembly of certain DNA-containing bacteriophages (Box 13.4).

Assembly of simpler protein shells can also depend critically on a viral protein. In addition to its many other functions, the simian virus 40 large T antigen (LT) participates in virion assembly. This protein does not form a scaffold, but an N-terminal domain of LT appears to be essential to

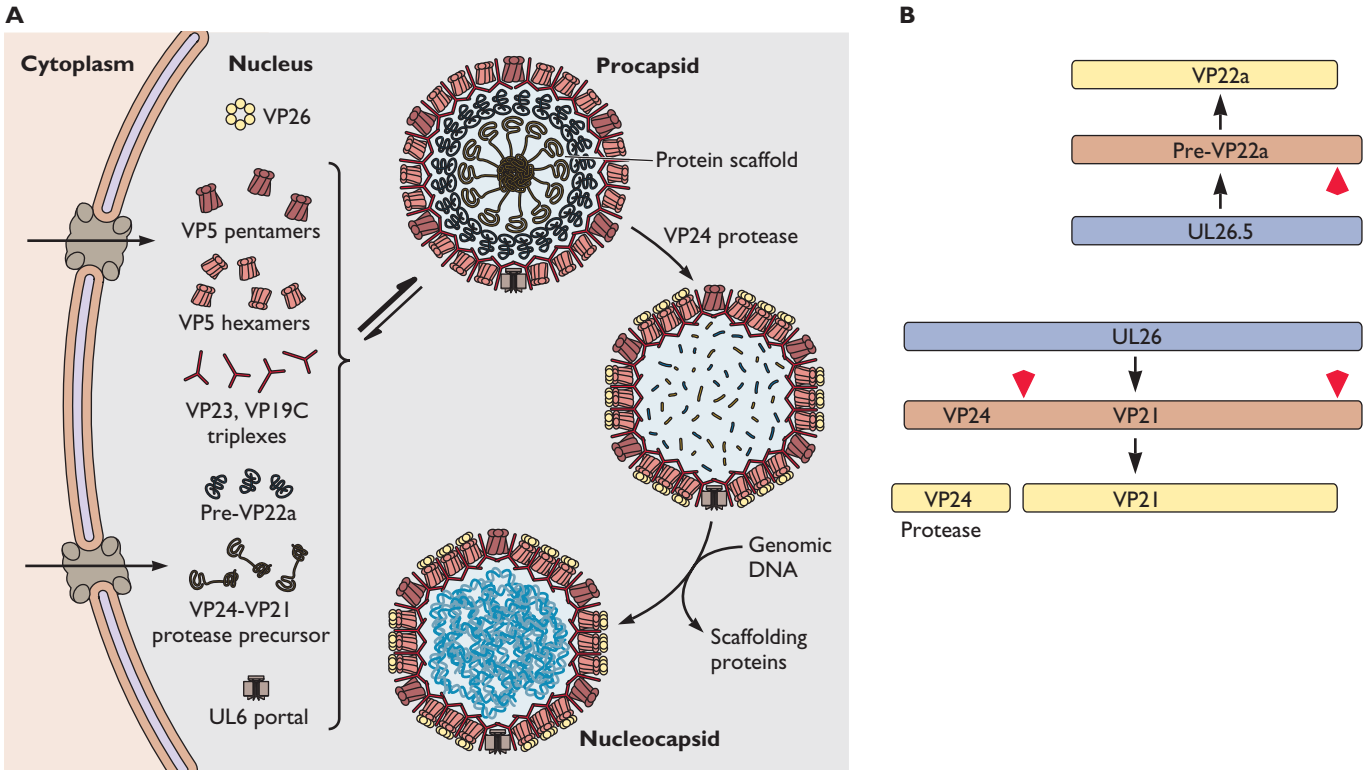


Figure 13.5 Assembly of herpes simplex virus type 1 nucleocapsids. (A) Assembly begins as soon as nucleocapsid proteins accumulate to sufficient concentrations in the infected cell nucleus. Intermediates include pentamers and hexamers of the major capsid protein VP5, which form pentons and hexons in the capsid, and triplexes of the minor proteins VP23 and VP19C. Whether structural units assemble prior to transport into the nucleus is not clear. Viral proteins essential for assembly of the nucleocapsid but not present in mature virions, the scaffolding protein (pre-VP22a) and the viral protease precursor (VP24-VP21), must also enter the nucleus. Assembly of nucleocapsids depends on the formation of an internal scaffold around which the protein shell assembles. Subsequent reactions require the viral protease to remove the scaffolding protein, allowing entry of the DNA genome and morphological transitions. As discussed in the text, encapsidation is concurrent with cleavage of the concatemeric products of herpesviral DNA replication. **(B)** Overlapping sequences of scaffolding proteins. The UL26 and UL26.5 reading frames are shown in purple, and their primary translation products are shown in light brown. The initiating methionine of VP22a protein is within the larger reading frame that encodes the VP24-VP21 polyprotein. Consequently, VP21 and VP22a are identical in sequence, except that the former contains a unique N-terminal segment. All proteolytic cleavages, at the sites indicated by the red arrowheads, including those that liberate the protease itself from the VP24-VP21 precursor, are carried out by the VP24 protease. The cleavage at the C-terminal site in VP22a disengages the scaffolding from the capsid proteins.

organize the capsid shell: alterations within this domain block production of virions and induce accumulation of an incomplete structure, not normally observed during virus assembly, that contains the viral chromatin and VP1. The N-terminal segment of LT possesses chaperone activity (Table 13.2). It is similar in sequence to a specific domain of cellular chaperones of the DnaJ family and, like these cellular proteins, stimulates the activity of Hsp70 chaperones. The chaperone activity of LT may ensure the productive binding of VP1 pentamers to one another, and to other components of the virion during assembly.

Selective Packaging of the Viral Genome and Other Virion Components

Concerted or Sequential Assembly

Incorporation of the viral genome into assembling particles is often called **packaging**. This process requires specific recognition of genomic RNA or DNA molecules (see below). It is clear that all viral genomes are packaged by one of two general mechanisms, concerted or sequential assembly.

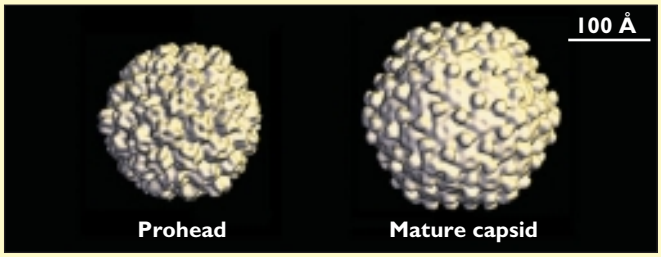
In concerted assembly, the structural units of the protective protein shell assemble productively only in association

BOX 13.4 EXPERIMENTS

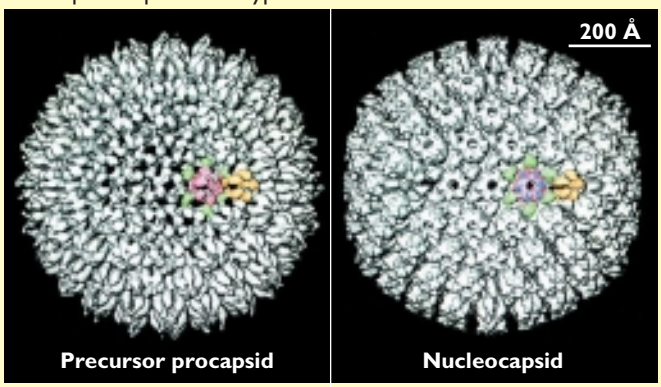
Visualization of structural transitions during assembly of DNA viruses

The assembly of viruses that package double-stranded DNA genomes into a preformed protein shell exhibits several common features, regardless of the host organism. These include the presence of a portal for DNA entry in the capsid or nucleocapsid precursor and probably the mechanism of DNA packaging (see the text). In addition, as illustrated for bacteriophage λ and herpes simplex virus type 1, assembly of DNA-containing structures is accompanied by major reorganizations of the protein shell.

A Phage λ



B Herpes simplex virus type I



(A) Cryo-electron micrographs of the phage lambda prohead and the DNA-containing mature capsid. The former comprises hexamers and pentamers of the capsid protein gpE organized with $T = 7$ icosahedral symmetry, and is assembled prior to encapsidation of the DNA genome. It is smaller than the mature capsid (270 and 315 Å in diameter, respectively), but its protein shell is considerably thicker. Packaging of the DNA genome leads to an expansion of the capsid shell, as a result of reorganization of gpE hexamers. This change is accompanied by binding of the gpD protein, which contributes to capsid stabilization. Adapted from T. Dokland and H. Murialdo, *J. Mol. Biol.* **233:**682–694, 1993, with permission. **(B)** Cryo-electron micrographs of herpes simplex virus type 1 nucleocapsid precursor and mature nucleocapsid, viewed along a twofold axis of icosahedral symmetry. Some copies of the proteins that form the particles' surfaces are colored as follows: VP5 hexons, red; VP5 pentons, yellow; triplexes containing one molecule of VP19C and two of VP23, green. The precursor nucleocapsid is spherical and less angular than the mature, DNA-containing structure, and its protein shell is thicker. Furthermore, the VP5 hexamers are not organized in a highly regular, symmetric manner in the precursor, resulting in a more open protein shell. The precursor nucleocapsid also lacks the VP26 protein, which binds to the external surface of VP5 hexamers, but not pentamers, in the mature nucleocapsid. Adapted from A. C. Steven et al., *FASEB J.* **10:**733–742, 1997, with permission.

with the genomic nucleic acid. The nucleocapsids of (−) strand RNA viruses form by a concerted mechanism (Fig. 13.6), as do retrovirus particles (Fig. 13.7). In the alternative mechanism, sequential assembly, the genome is inserted into a preformed protein shell. The formation of herpesviral nucleocapsids provides a clear example of this type of assembly (Fig. 13.5). Neither mutations that inhibit viral DNA synthesis nor those that prevent DNA packaging block assembly of capsid-like structures that lack DNA. These phenotypes establish unequivocally that the DNA genome must enter preformed protein shells. In contrast to concerted assembly, encapsidation of the genome in a preformed structure requires specialized mechanisms to pull or push the genome into the capsid (see the next section), as well as to maintain or open a portal for entry of the nucleic acid. The herpesviral portal UL6, which is present at only 1 of the 12 vertices of the nucleocapsid (Fig. 13.5; see also Fig. 4.26), fulfills the latter function.

Despite the clear differences between concerted and sequential assembly pathways, it can be quite difficult to decide which mechanism applies to some viruses with icosahedral symmetry. A classic case in point is poliovirus assembly, which has been studied for more than 30 years. In the first scheme to be proposed, an empty capsid containing 60 copies of the VP0-VP3-VP1 structural unit but lacking the RNA genome was viewed as the precursor of the **provirion** (Fig. 13.4), the RNA-containing but immature virion. Such 75S empty capsids, initially called procapsids, are detected readily in infected cell extracts and also form by self-assembly of 14S pentamers *in vitro*. In the alternative pathway, pentamers are proposed to condense around the RNA genome (Fig. 13.4). In this

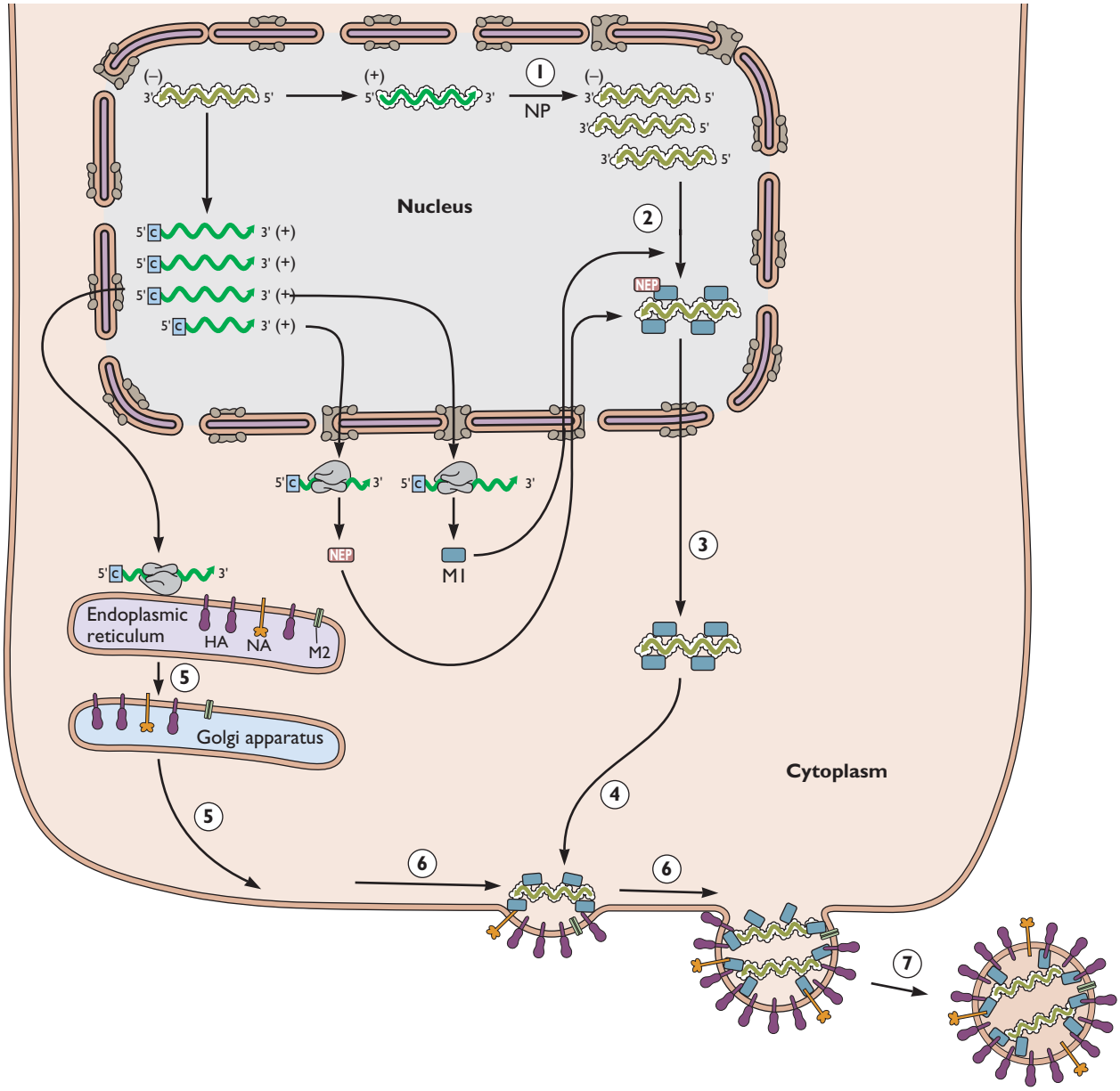


Figure 13.6 Assembly of influenza A virus. Assembly proceeds in stepwise fashion within different compartments of an infected cell. As (−) strand genomic RNA is synthesized in the nucleus, it is packaged by the NP RNA-binding protein (step 1). These ribonucleoproteins may serve as templates for mRNA synthesis, participate in further cycles of replication, or bind the M1 protein (step 2). The latter interaction prevents further RNA synthesis, and allows binding of NEP and export of the nucleocapsid to the cytoplasm (step 3). The M1 protein also binds to the cytoplasmic face of the plasma membrane via specific sequences and directs the nucleocapsid to the plasma membrane (step 4). The plasma membrane carries the viral HA, NA, and M2 proteins, which reach this site via the cellular secretory pathway (step 5). The M1 protein probably controls budding (step 6) via recruitment of cellular components (see the text). Fusion of the membrane bud releases the enveloped particle (step 7). Only two of the eight genome segments are illustrated for clarity.

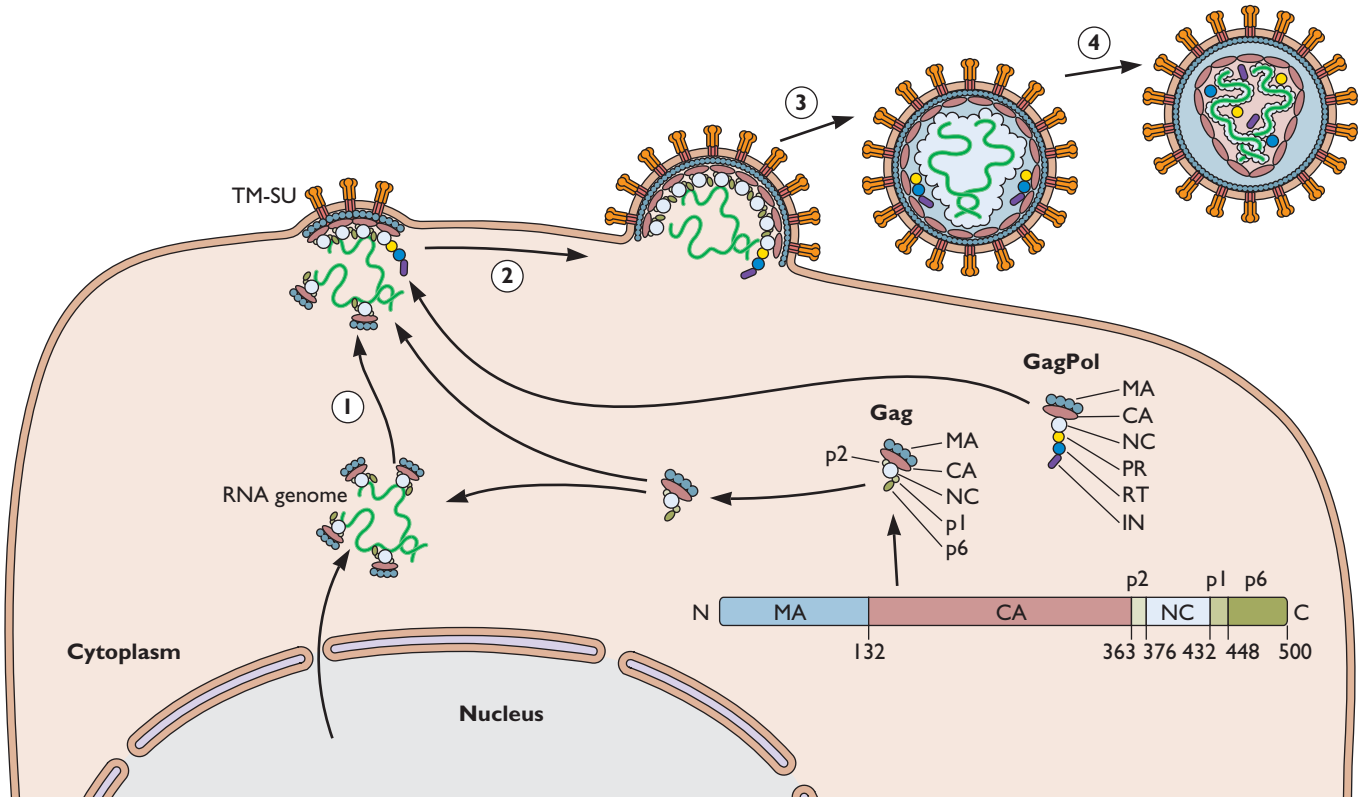


Figure 13.7 Assembly of a retrovirus from polyprotein precursors. The Gag polyprotein of all retroviruses contains the MA, CA, and NC proteins linked by spacer peptides that are variable in length and position. The proteins are in the order (from N to C terminus) of the protein shells of the virus particle, from the outer to the inner. The organization of human immunodeficiency virus type 1 Gag is summarized on the right. A minor fraction, about 1 in 10, of Gag translation products carry the retroviral enzymes, denoted by PR, RT, and IN, at their C termini. The association of Gag molecules with the plasma membrane, with one another, and with the RNA genome via binding of NC segments initiates assembly at the inner surface of the plasma membrane (step 1). In some cases, such as human immunodeficiency virus type 1, the MA segment also binds specifically to the internal cytoplasmic domain of the TM-SU glycoprotein. Assembly of the particle continues by incorporation of additional molecules of Gag (step 2). This pathway is typical of many retroviruses, but some (e.g., betaretroviruses) complete assembly of the core in the interior of the cell prior to its association with the plasma membrane. The dimensions of the assembling particle are determined by interactions among Gag polyproteins. Eventually, fusion of the membrane around the budding particle (step 3) releases the immature noninfectious particle. Cleavage of Gag and Gag-Pol polyproteins by the viral protease (PR) produces infectious particles (step 4) with a morphologically distinct core (see Fig. 13.23).

mechanism, capsid assembly and packaging of the RNA genome are coupled, and empty capsids are considered dead-end. The concerted pathway is supported by several experimental observations. These include the ability of radioactively labeled pentamers to form virions without the appearance of a procapsid intermediate, and the demonstration that stable empty capsids are produced by irreversible, conformational changes that take place during their extraction from infected cells. Furthermore, in a cell-free system for synthesis of infectious poliovirus particles, exogenously added 14S pentamers assemble with newly synthesized viral (+) strand RNA to form virions with antigenic sites characteristic of virions produced in infected cells. In contrast, exogenously added empty capsids undergo no further assembly, even when genomic RNA is synthesized, confirming that they are dead-end products. In this case, there is now strong evidence for the concerted pathway, but in others, such as adenovirus assembly (Fig. 13.8), there is still debate about which mechanism is used.

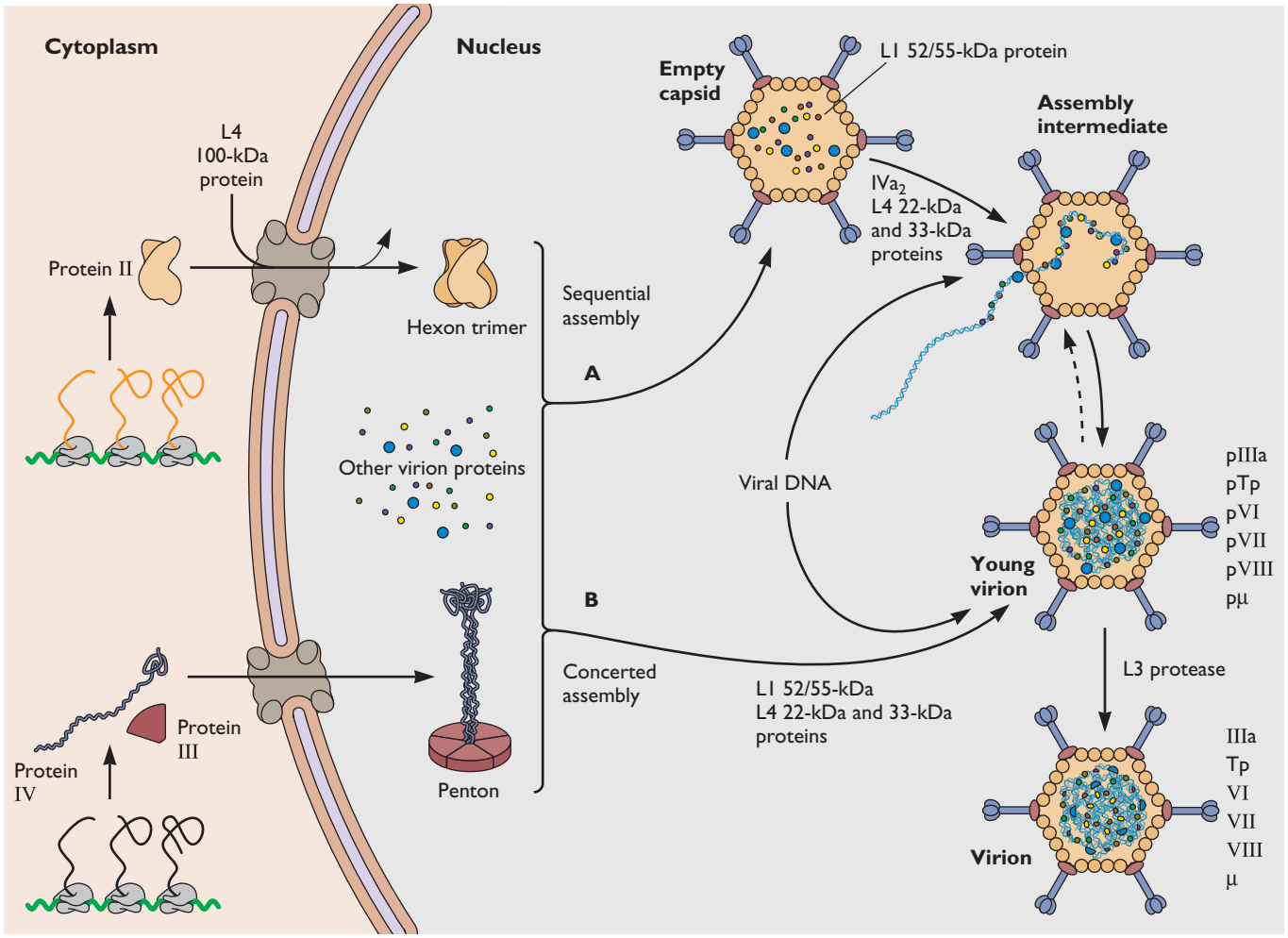


Figure 13.8 Adenovirus assembly pathways. Synthesis and assembly of hexons and pentons and their transport into the nucleus set the stage for assembly. The L4 100-kDa protein is required for formation of hexons, but its molecular function is not known. **(A)** In the pathway originally proposed, these structural units and the proteins that stabilize the capsid assemble into empty capsids. The L1 52/55-kDa proteins are necessary for the formation of structures that can complete assembly, and decrease in concentration as assembly proceeds. The DNA is then inserted into this structure via the packaging signal located near the left end of the genome. The viral IVa_2 and L4 22-kDa proteins bind specifically to this sequence *in vitro* and are required for assembly in infected cells. Premature breakage of DNA in the process of insertion would yield the structure designated "Assembly intermediate," in which an immature capsid is associated with a DNA fragment derived from the left end of the viral genome. Core proteins are encapsidated with the viral genome to yield noninfectious young virions. Mature virions are produced upon cleavage of the precursor proteins listed to the right of the young virion. **(B)** The alternative pathway is based on the failure of any capsid-like structures to assemble in cells infected by viruses with mutations that eliminate the packaging signal, prevent synthesis of the IVa_2 protein, or remove the C-terminal segment of the L4 33-kDa protein. In this model, capsid assembly and encapsidation of the genome are concerted reactions. Empty capsids and the assembly intermediate would then be viewed as dead-end products, or artifacts of the methods by which particles are extracted from infected cells. For example, the structure designated "Assembly intermediate" in pathway A could represent unstable structures with DNA genomes that were partially extruded and broken during extraction of intermediates (dashed arrow).

Recognition and Packaging of the Nucleic Acid Genome

The assembly mechanisms described in previous sections are fruitless unless the viral genome is packaged within progeny virions. Despite diversity in size, composition, and morphology, specific incorporation of the genome during assembly of virus particles is achieved by a limited repertoire of mechanisms, which are discussed below. The special problems imposed by segmented genomes are considered subsequently.

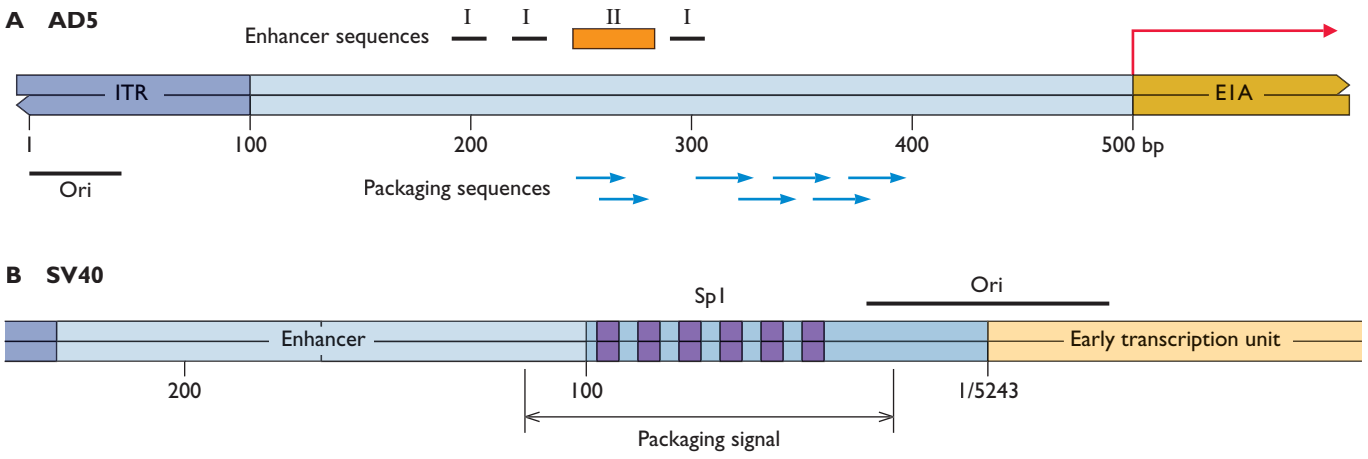
During encapsidation, viral nucleic acid genomes must be distinguished from the cellular DNA or RNA molecules present in the compartment in which assembly takes place. This process requires a high degree of discrimination among similar nucleic acid molecules. For example, retroviral genomic RNA constitutes much less than 1% of an infected cell's cytoplasmic RNA population and bears all the hallmarks of cellular messenger RNAs (mRNAs). Yet it is **the** RNA packaged in the great majority of retrovirus particles. Such discrimination is the result of specific recognition of sequences or structures unique to the viral genome, termed **packaging signals**. These can be defined by genetic analysis as the sequences necessary for incorporation of the nucleic acid into the assembling virion, or sufficient to direct incorporation of foreign nucleic acid. The organization of the packaging signals of several viruses is therefore quite well understood.

Nucleic Acid Packaging Signals

DNA signals. The products of polyomaviral or adenoviral DNA synthesis are genomic DNA molecules that can be incorporated into assembling virus particles without further modification. These DNA genomes contain discrete packaging signals with several common properties. The signals comprise repeats of short sequences, some of which are also part of viral promoters or enhancers; they are positioned close to an origin of replication, and their ability to direct DNA encapsidation depends on this location. They differ in whether they are recognized directly or indirectly by viral proteins.

The encapsidation signal of the adenoviral genome, which is located close to the left inverted repeat sequence and origin, comprises a set of repeated sequences. Several of these sequences overlap enhancers that stimulate transcription of viral genes (Fig. 13.9A). The packaging signal is recognized by viral proteins, the late IVa_2 and L4 22-kDa proteins. Cooperative binding of the proteins to the repeated sequence is thought to form a higher-order nucleoprotein structure that promotes packaging of the genome. The results of genetic experiments have established the importance of these proteins in assembly. For example, genomes and structural proteins are synthesized efficiently in cells infected by IVa_2 null mutants, but neither mature virions nor any other particles are produced. This phenotype also suggests that packaging of the genome is

Figure 13.9 Viral DNA-packaging signals. (A) Human adenovirus type 5 (AD5). The location of the repeated sequences (blue arrows) of the packaging signal relative to the left inverted terminal repeat (ITR), the origin of replication (Ori), and the E1A transcription unit is indicated. The repeated sequences are AT rich and functionally redundant. The viral IVa_2 protein binds directly to the 3′ portion of the sequence that is conserved to each of the repeats. Once the IVa_2 protein is associated, the L22-kDa protein interacts with the 5′ segment of the conserved sequence. The positions of transcriptional enhancers within this region are also shown. Enhancer I stimulates transcription of the immediate-early E1A gene, whereas enhancer II increases the efficiency of transcription of all viral genes. **(B)** Simian virus 40 (SV40). The region of the genome containing the enhancer, origin of replication (Ori) and packaging signal is shown, with positions (bp) in the circular genome indicated below. The Sp1-binding sites within the packaging sequence are required for genome packaging.



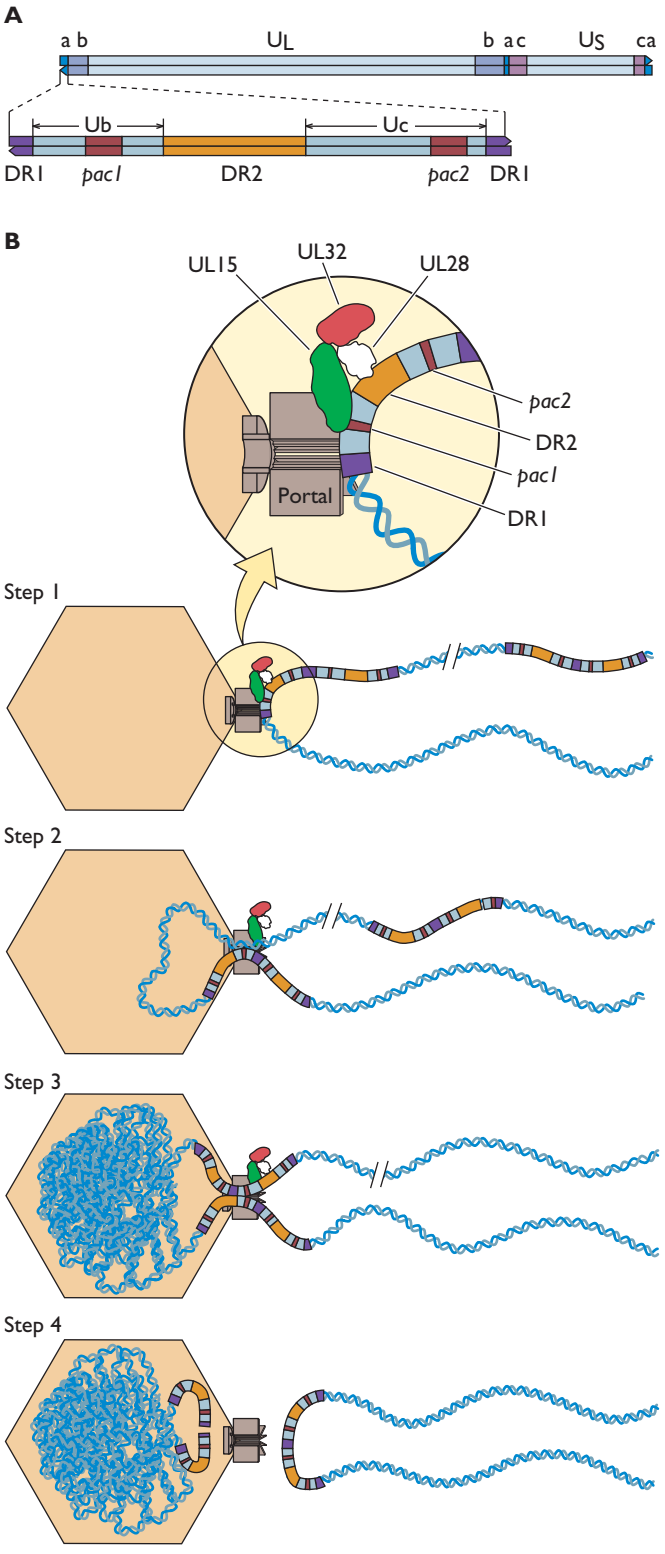


Figure 13.10 Packaging of herpes simplex virus type 1 DNA. (A) Organization of the *a* repeats of the viral genome, showing the location of the *pac1* and *pac2* sequences within the nonrepeated sequences Ub and Uc, and relative to the flanking direct repeats DR1 and DR2. One to several copies of the *a* sequence are present at the end of the unique long (U_L) segment and at the internal L-S junction, but only one copy lies at the end of the U_S region. **(B)** Model of herpes simplex virus type 1 DNA packaging, in which encapsidation is initiated by formation of a terminase complex, which includes the proteins indicated, on the packaging sequence. This protein-DNA complex is oriented to interact with the portal of the nucleocapsid (step 1). The DNA is then reeled into the capsid (steps 2 and 3) until a headful threshold is reached and an *a* sequence in the same orientation (i.e., one genome equivalent) is encountered (step 3), when cleavage in DR1 sequences takes place (step 4). When *a* sequences are tandemly repeated, adjacent copies share a single intervening DR1 sequence. The unit-length DNA genomes packaged in virions are therefore assumed to be released from the concatemeric products of viral DNA synthesis by cleavage at a specific site within shared DR1 sequences.

the result of a concerted assembly mechanism, but exactly how such assembly proceeds is not known. At least one additional viral protein, the L1 52/55-kDa protein mentioned previously, is associated with the packaging signal in infected cells, although it does not directly to the repeat sequence *in vitro*.

The simian virus 40 DNA-packaging signal is located in the regulatory region of the genome containing the origin of replication, the enhancer, and early and late promoters. Multiple sequences within this region contribute to the encapsidation signal. It includes multiple binding sites for the cellular transcriptional regulator Sp1. Although the cellular genome contains numerous binding sites for Sp1, the particular arrangement of sequences recognized by Sp1 in the packaging signal is unique to the viral genome. Neither the internal proteins of the simian virus 40 capsid (VP2/3) nor the major capsid protein (VP1) binds specifically to DNA. However, VP2/3 and Sp1 bind cooperatively to the packaging signal with high affinity and specificity. This cellular protein stimulates the *in vitro* assembly of infectious virus particles by an order of magnitude, consistent with a role in mediating indirect recognition of the packaging signal by capsid proteins. Subsequently, highly cooperative interactions among the structural units appear to drive converted assembly of the capsid, concomitant with displacement of Sp1 and nonspecific binding of capsid proteins to viral minichromosomes.

The replication of herpesviruses produces not genomic DNA molecules but, rather, concatemers containing many head-to-tail copies of the viral genome. Individual genomes must therefore be liberated from such concatemers. The herpes simplex virus type 1 packaging signals *pac1* and *pac2,* which lie within the terminal *a* repeats of the genome, are necessary for both recognition of the viral DNA and its cleavage within the adjacent DR repeats (Fig. 13.10A). It is generally thought that cleavage is

concomitant with genome encapsidation. In one model (Fig. 13.10B), it is proposed that a protein complex formed on the unique short *pac* sequence interacts with the portal in the nucleocapsid. Following the first DNA cleavage, a unit-length genome is reeled into the nucleocapsid prior to the second DNA cleavage. This mechanism is analogous to that by which concatemeric DNA products of bacteriophage T4 replication are cleaved and packaged by a terminase complex, which hydrolyzes ATP and associates transiently with the portal protein of the preformed capsid. The products of at least seven herpes simplex virus type 1 genes are dedicated to stable encapsidation of the viral genome.

The UL15, UL28, and UL33 proteins, which interact with one another and with the portal protein, exhibit the properties predicted for the terminase. For example, the UL15 protein contains a sequence motif characteristic of ATPases that is essential for encapsidation of viral genomes, while the UL28 gene product binds to *pac* sequences required for DNA cleavage. The UL17 protein is also essential for DNA cleavage, but its function in this process is not known. In addition, it is necessary for recruitment of the UL15 protein to the nucleocapsid, resulting in formation of a complex present only on mature DNA-containing nucleocapsids (Box 13.5). In the absence of the UL25 protein, DNA cleavage does occur, but fewer nucleocapsids are formed. It has therefore been suggested that one function of this protein complex is to stabilize the protein shell so that it can withstand the pressure exerted by the encapsidated genome.

RNA signals. Because it is also an mRNA, the retroviral genome must be distinguished during encapsidation from both cellular mRNA and subgenomic viral mRNA. In addition, two genomic RNA molecules must interact with one another, for the retroviral genome is packaged as a dimer. This unusual property is thought to help retroviruses survive extensive damage to their genomes (see Chapter 7). In virions, the dimeric genome is in the form of a 70S complex held together by many noncovalent interactions between the RNA molecules. However, most attention has focused on sequences that allow formation of stable dimers, termed the **dimer linkage sequence**. *In vitro* experiments with human immunodeficiency virus type 1 RNA have provided evidence for base pairing between loop sequences of a specific hairpin (SL1) within the dimer linkage sequence (Fig. 13.11A) and the formation of an intermolecular four-stranded helical structure (known as a G tetrad or G quartet). The effects of mutations in, or duplication of, this sequence indicate that it nucleates formation of genome RNA dimers *in vivo*, and that dimerization is required for efficient genome packaging. Indeed, the dimer linkage sequence lies with the RNA-packaging signal (Fig. 13.11A), and it has been proposed that dimerization results in conformational change to expose sequences recognized by the RNA-binding portion of Gag (see below).

Sequences necessary for packaging of retroviral genomes, termed Psi (ψ), vary considerably in their complexity and location. In some cases, exemplified by Moloney murine leukemia virus, a contiguous ψ sequence of about 350 nucleotides (Fig. 13.11B) is both necessary and sufficient for RNA encapsidation. As this sequence lies downstream of the 5′ splice site, only unspliced genomic RNA molecules are recognized for packaging. The human immunodeficiency virus type 1 genome also contains a primary RNA-packaging sequence (Fig. 13.11A) that distinguishes the full-length genome from spliced viral RNA molecules. However, this sequence fails to direct packaging of heterologous RNA species into retrovirus particles, indicating that it is not sufficient. Additional sequences required for genomic RNA encapsidation lie within the TAR sequence and adjacent sequences (Fig. 13.11A), and at more distant locations. Presumably, such dispersed packaging sequences form a distinctive structural feature in folded RNA molecules.

The NC domain of Gag, a basic region that contains at least one copy of a zinc-binding motif (Fig. 13.12A), mediates selective and efficient encapsidation of genomic RNA during retroviral assembly. The central region of NC containing the zinc-binding motif(s) and adjacent basic sequences binds specifically to RNAs that contain ψ sequences *in vitro* and is necessary for selective packaging of the genome in infected cells. It is clear from structural studies of NC proteins bound to RNA-packaging signals that NC binds specifically to short RNA sequences, UCUG in the case of Moloney murine leukemia virus (Fig. 13.12B). The zinc-binding motifs form a compact structure, termed a zinc knuckle, which makes specific contacts with bases and is complementary in charge and shape to the bound RNA. Two features of the Moloney murine leukemia virus ψ signal appear to promote selection of dimeric RNA genomes. First, this region contains 13 copies of the UCUG sequence recognized specifically by the NC protein, a higher frequency than elsewhere in the genome. Second, the UCUG sequences within the regions most important for efficient packaging are not bound by NC when the RNA is monomeric: they are sequestered within base-paired regions of stem-loops, which have been shown to undergo substantial changes in base pairing upon dimerization of the genome. It is therefore thought that dimerization induces conformational transitions that expose high-affinity NC-binding sites (Fig. 13.12C). Whether analogous mechanisms promote selective packaging of dimeric RNAs of retroviruses with more complex

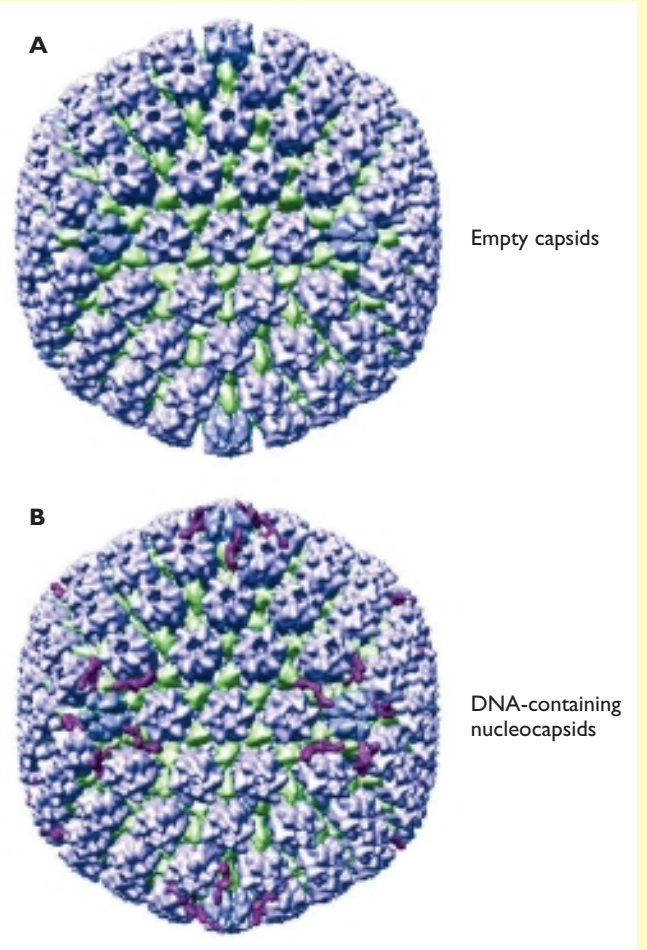
BOX 13.5 EXPERIMENTS

An allosteric transition that may stabilize the herpesviral nucleocapsid

The herpes simplex virus type 1 UL17 and UL25 proteins are not required for assembly of nucleocapsid protein shells, but are necessary for stable encapsidation of the DNA genome. Biochemical studies indicated that approximately equal quantities of these two proteins are exposed on the outer surface of mature, DNA-containing nucleocapsids. Cryo-electron microscopy of such nucleocapsids and shells that contain no DNA and retain or lack the assembly scaffold (so-called B and A capsids, respectively) revealed a structural component present only on nucleocapsids (see the figure). As mature nucleocapsids are also called C capsids, this structure was called the C capsid-specific component (CCSC). Formation of the CCSC correlated with the presence of the UL17 and UL25 proteins. For example, treatment of nucleocapsids with 0.5 M guanidinium hydrochloride for 30 min induced both dissociation of the UL17 and UL25 proteins, and disappearance of CCSCs. The CCSC is most likely to be a heterodimer of these two proteins.

The absence of the CCSC from A and B capsids suggested that its formation depends on encapsidation of the DNA genome. Consistent with this view, the genome is also released by the treatment described above that dissociates the UL17 and UL25 proteins. It has therefore been proposed that pressure exerted on the inner surface of the nucleocapsid as DNA enters induces a structural transition on the outer surface to create a site for stable binding of the CCSC.

Genetic analyses of the functions of the UL25 protein indicate that this protein has no role in cleavage of viral DNA concatemers, but is necessary for stable genome encapsidation, presumably because it stabilizes the protein shell. It may also permit binding of specific tegument proteins.



Cryo-electron microscopy reconstructions of the surfaces of mature nucleocapsids (A) and UL25-lacking A capsids (B) and viewed along a twofold axis of icosahedral symmetry. Hexons plus associated VP26 molecules, pentons, and triplexes are in light blue, blue, and green, respectively. The CCSCs present only on nucleocapsids, which are organized around the pentamers, are in purple. Bar, 20 nm. Adapted from B. L. Trus et al., *Mol. Cell* **26:**479–489, 2006, with permission. Courtesy of A. Steven, National Institutes of Health.

Stow, N. D. 2001 Packaging of genomic and amplicon DNA by the herpes simplex virus type 1 UL25-null mutant KUL25NS. *J. Virol.* **75:**10755–10765.

Trus, B. L., W. W. Newcomb, N. Cheng, G. Gardone, L. Marekov, F. L. Huma, J. C. Brown, and A. C. Steven. 2006. Allosteric signaling and nuclear exit strategy: binding of UL25/U17 heterodimers to DNA-filled HSV-1 capsids. *Mol. Cell* **25:**479–489.

packaging signals, such as human immunodeficiency virus type 1, is not yet clear.

As noted previously, binding of Gag to retroviral RNA is coordinated with assembly of the protein shell. One domain of the polyprotein of several retroviruses that is required for polyprotein assembly is located within the NC portion, and RNA binding by NC is necessary for efficient assembly of virus particles. Furthermore, particles that do form when RNA binding is prevented by alterations in NC are of low density, indicating that they contain fewer molecules

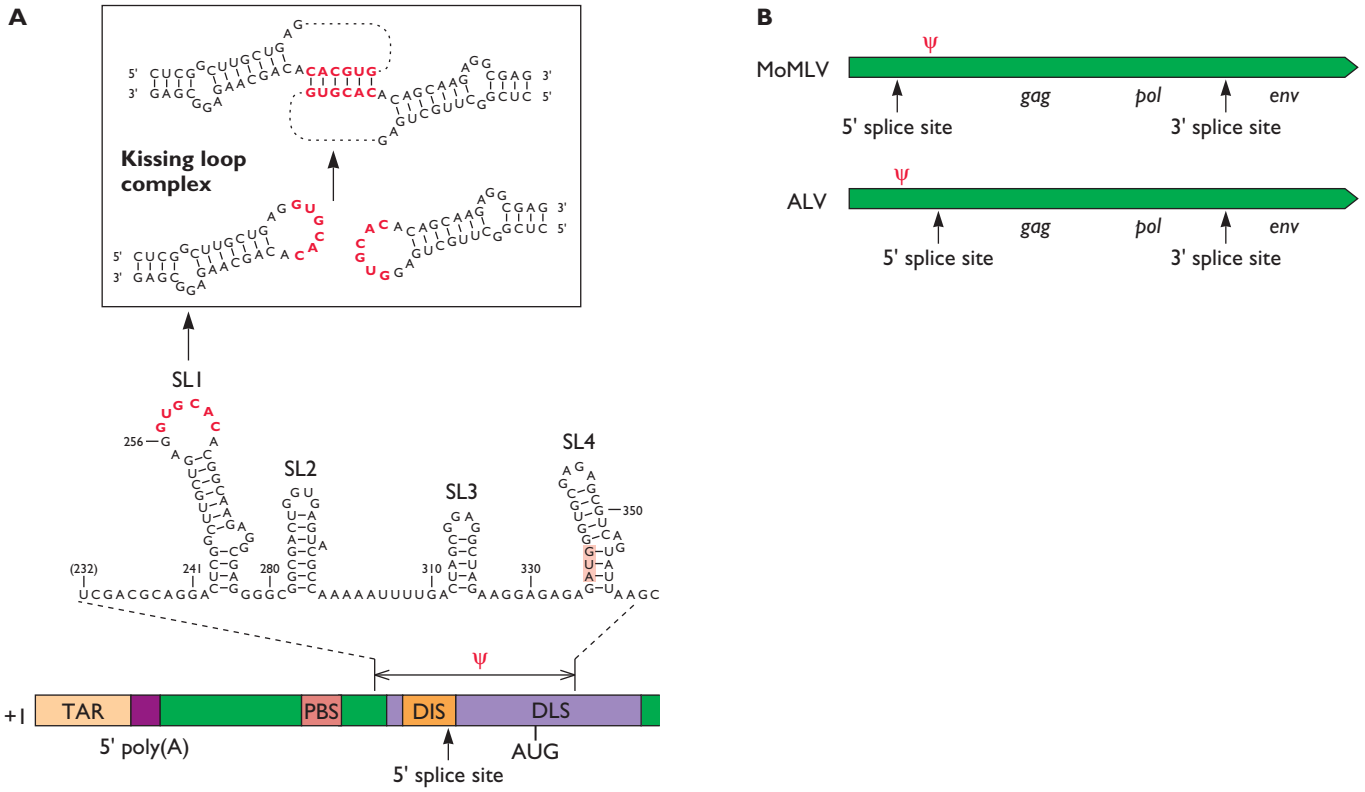


Figure 13.11 Sequences important in packaging of retroviral genomes. (A) The 5′ end of the human immunodeficiency virus type 1 genome is shown to scale at the bottom, indicating the positions of TAR, the 5′ polyadenylation signal [5′ poly (A)], the tRNA primer-binding site (PBS), the 5′ splice site, a packaging signal designated ψ, the sequence that forms the dimer linkage structure (DLS), and the dimerization initiation site (DIS), which can initiate dimerization *in vitro*. The four hairpins (SL1 to SL4) formed by the ψ sequence are shown above. The SL1 hairpin is the dimer initiation sequence. The loop-loop "kissing" complex proposed to form when two genomic RNA molecules dimerize via the self-complementary sequence shown in red is depicted at the top. The ψ sequence, which includes intronic sequences and therefore is present only in unspliced RNA, appears to be necessary but not sufficient for encapsidation of genomic RNA. A hairpin containing the 5′ poly(A)-addition signal and the bottom of the TAR hairpin, as well as more distantly located sequences, are also required. **(B)** Locations within the RNA genomes of sequences necessary for encapsidation of Moloney murine leukemia virus (MoMLV) and avian leukosis virus (ALV) RNAs, designated ψ. The latter ψ signal resides only upstream of the 5′ splice site. Even though both genomic and subgenomic RNAs contain this sequence, spliced mRNA molecules are not encapsidated efficiently.

of Gag. It has been demonstrated that nonspecific binding of Gag to nucleic acids induces Gag dimerization. The current model proposes that this reaction is followed by conformational change in RNA-bound Gag dimers to a form competent for multimerization and hence assembly (Box 13.6).

Other parameters that govern genome encapsidation. Specific signals may be required to mark a viral genome for encapsidation, but their presence does not guarantee packaging. The fixed dimensions of the closed icosahedral capsids or nucleocapsids of many viruses impose an upper limit on the size of viral nucleic acid that can be accommodated. Consequently, nucleic acids that are more than 5 to 10% larger than the wild-type genome cannot be encapsidated, even when they contain appropriate packaging signals. This property has important implications for the development of viral vectors. In some cases, the length of the DNA that can be accommodated in the particle (a "headful") is a critical parameter. This mechanism is exemplified by the coupled cleavage and encapsidation of genomic herpes simplex virus type 1 DNA molecules from the concatemeric products of replication, when both specific sequences and a headful of DNA are recognized. Indeed, the packaging of some viral DNA genomes, such as T4 DNA, depends solely on the latter parameter (Box 13.7).

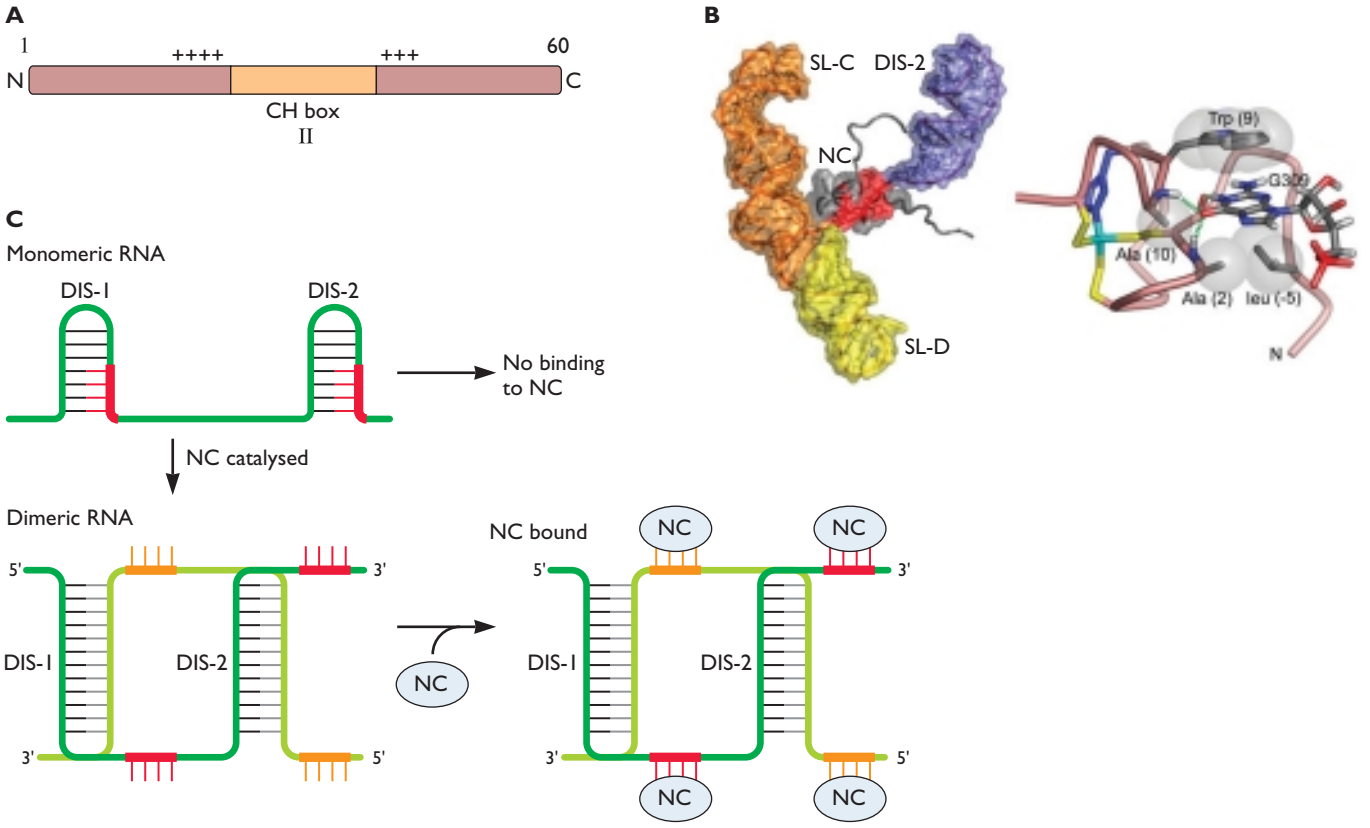


Figure 13.12 The retroviral NC RNA-packaging protein. (A) Schematic illustration of the Moloney murine leukemia virus NC protein, showing the locations of the Cys-His (CH) box and basic regions necessary for both nonspecific binding to RNA and specific recognition of the packaging signal. **(B)** Structural model of the Moloney murine leukemia virus NC protein bound to the 101-nucleotide core element of the packaging signal determined by nuclear magnetic resonance methods (left). Stem-loops are shown in purple, orange, and yellow, and the UCUG with which the NC zinc knuckle interacts is shown in red. The close-up view of the UCUG sequence bound to NC (right) shows hydrogen bonding of the exposed G residue with backbone atoms of the proteins. Adapted from V. D'Souza and M. F. Summers, *Nature* **431:**586–590, 2004, with permission. Courtesy of M. F. Summers, University of Maryland, Baltimore County. **(C)** Model for selective recognition of the dimeric RNA genome. The secondary structures of the monomeric and dimeric Moloney murine leukemia virus packaging signal are shown schematically. These structures are based on mapping the accessibility of RNA sequences to various chemicals, phylogenetic comparisons and free energy calculations, as well as structural studies. Adapted from V. D'Souza and M. F. Summers, *Nat. Rev. Microbiol.* **3:**643–655, 2005, with permission.

The coupling of encapsidation of a viral nucleic acid with its synthesis may also contribute to the specificity with which the viral genome is incorporated into assembling structures. As mentioned previously, such coordination is typical of the assembly of (–) strand RNA viruses (see, e.g., Fig. 13.6). However, the mechanisms by which nascent genomic RNA molecules are recognized by RNA-binding proteins are not well understood. Coordination of replication and encapsidation may also contribute to the great specificity with which picornaviral genomes are packaged: not only abundant cytoplasmic cellular RNA species (transfer RNAs [tRNAs], ribosomal RNAs [rRNAs], and mRNAs), but also (–) strand poliovirus RNA and poliovirus mRNA lacking VPg are excluded from virions. No packaging signal has yet been identified in the poliovirus genome. As discussed in Chapter 12, poliovirus infection induces the formation of cytoplasmic vesicles, with which both viral replication and virion assembly are associated. Such sequestration of genomic RNA molecules with the viral proteins that must bind to them clearly could make a major contribution to packaging specificity, by reducing competition from cytoplasmic cellular RNAs. There is accumulating evidence that encapsidation of the genome is coupled with RNA

BOX 13.6 DISCUSSION

Dimerization-induced conformational change forms assembly-competent Gag polyproteins

The model for higher-order assembly of the Gag polyprotein shown in the figure is based largely on *in vitro* studies of assembly of Rous sarcoma virus and human immunodeficiency virus type 1 Gag.

- Assembly of Gag synthesized in *E. coli* or made by *in vitro* translation into virus-like particles requires the NC domain and nucleic acid
- Heterologous RNA is present in virus-like particles released from Gag-producing cells (see the text), and DNA oligonucleotides can support Gag assembly in *in vitro* reactions
- Oligonucleotides that support such Gag assembly **must** contain at least two binding sites for the NC domain of Gag

These properties suggested that nonspecific binding of the NC domain of Gag to nucleic acid (the viral RNA genome during assembly in infected cells) promotes Gag dimerization. The hypothesis is consistent with the fact that basic sequences of NC, but not the zinc finger domain(s) that binds specifically to packaging sequences, are necessary for assembly and release of virus-like particles. The demonstrations that replacement of NC by either a heterologous, leucine zipper dimerization domain or cysteine residues that can be covalently linked (by chemical crosslinkers or oxidation) allows efficient assembly *in vitro* provides strong evidence for the crucial role of dimerization.

It has been proposed that efficient assembly also depends on conformational change following dimerization. Such assembly

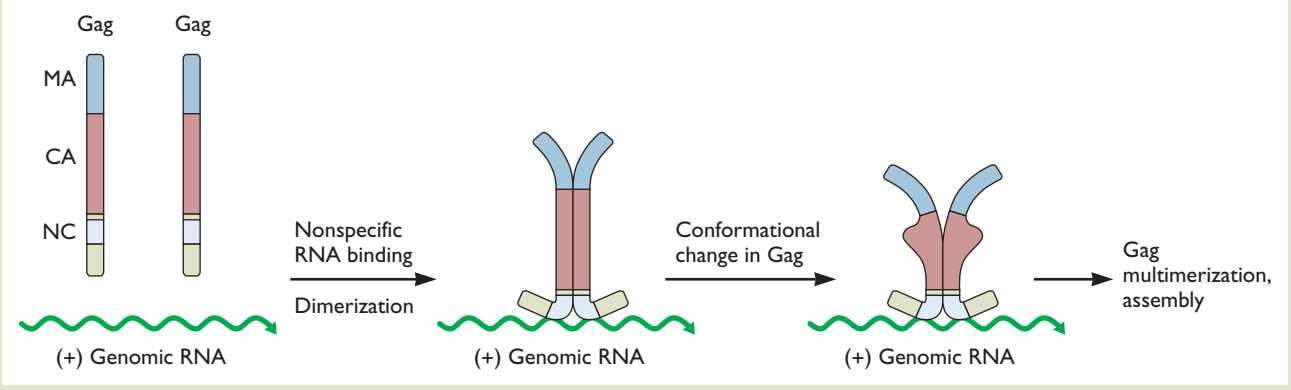
- requires not only NC and the CA dimerization interface (see the text), but also more N-terminal sequences, notably the N-terminal domain of CA
- has been reported to be temperature dependent: Gag assembly *in vitro* take place at 37°C, but not at 23°C. As protein rearrangements occur more readily at the higher temperature, this property implicated conformational change as a prerequisite for Gag multimerization. The temperature dependence can be eliminated by artificial tethering of N-terminal segments of CA to one another.

These observations argue that dimerization of RNA-bound Gag induces conformational change to form dimers competent for assembly into higher-order multimers, via alignment of N-terminal domains of CA.

Alfadhli, A., T. C. Dhenub, A. Still, and E. Barklis. 2005. Analysis of human immunodeficiency virus type 1 Gag dimerization-induced assembly. *J. Virol.* **79:**14498–14506.

Ma, Y. M., and V. M. Vogt. 2004. Nucleic acid binding-induced Gag dimerization in the assembly of Rous sarcoma virus particles *in vitro*. *J. Virol.* **78:**52–60.

The sequential formation of Gag molecules competent for assembly into virus-like particles (VLPs) is shown schematically.



replication and that this step in assembly is assisted by the poliovirus 2C protein. This protein is a nucleoside triphosphatase that might facilitate release of RNA from membrane-bound replication complexes. As packaging of flavivirus RNA also depends on replication of genomic RNA, coincident genome synthesis and assembly may be a general feature of (+) strand RNA viruses.

Packaging of Segmented Genomes

Segmented genomes pose an intriguing packaging problem. The best-studied example among animal viruses is the influenza A virus genome, which comprises eight molecules of RNA. It has been appreciated for many years that production of an infectious virus particle requires incorporation of at least one copy of each of the eight genomic segments. However, it has proved difficult to distinguish random packaging from a selective mechanism for inclusion of a full complement of genomic RNAs.

Packaging of the bacteriophage ϕ6 genome provides clear precedent for a selective mechanism. The genome of this bacteriophage comprises one copy of each of three double-stranded RNA segments designated S, M, and L.

BOX 13.7 BACKGROUND

Packaging a headful of viral DNA

During assembly of herpesvirus and several bacteriophages with large, double-stranded DNA genomes, including T4, the linear genome is cleaved from concatemeric products of viral genome replication during insertion into a preformed protein shell, the head in the case of T4. Furthermore, encapsidation of T4 DNA is coordinated with cleavage of concatemers. However, the T4 genome exhibits several unusual features.

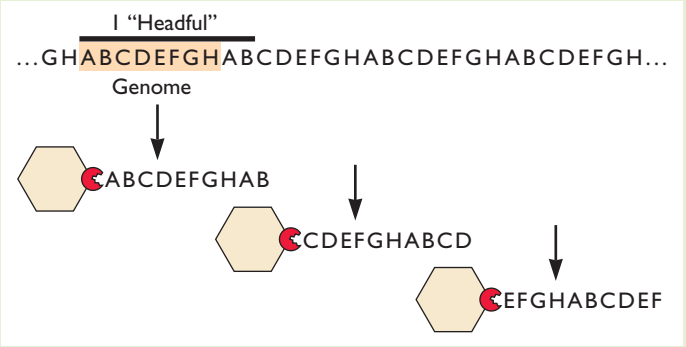
- The linear T4 genomes do not have unique terminal sequences
- The genetic map is circular, even though the genome is linear
- The terminal sequences, which are different in each DNA molecule, are repeated at each end of DNA

It was deduced from these properties that the T4 genome is circularly permuted and terminally redundant. These properties can be accounted for by essentially random cleavage of head-to-tail concatemers (the preferred substrate for DNA packaging) that results in encapsidation of DNA molecules that are **longer** than the unique sequence in the genome (see the figure). No specific DNA sequence dictates the cleavages that liberate linear DNA during encapsidation. Rather, the first cleavage occurs randomly, and the second takes place once the phage T4 head has been filled with DNA. As predicted by the "headful" packaging mechanism, when head size is increased or decreased by mutation in specific genes (or other manipulations), longer and shorter DNA molecules, respectively, are encapsidated. Furthermore, when sequences are deleted from, or inserted into, the genome, the length of the terminal repeats increases or decreases to the corresponding degree: when x bp are removed, the total length of the terminal repeats increases by x bp. These properties demonstrate directly that a fixed length of DNA, a headful, is incorporated during assembly.

A headful of DNA is packaged during assembly of other bacteriophage and animal viruses with double-stranded DNA genomes, including herpesviruses. Structural studies of bacteriophage P22 virions revealed that tight spooling of DNA in the nucleocapsid induces major conformational change in the portal, through which DNA enters. It has therefore been proposed that the change in portal structure provides the signal that the nucleocapsid is full, to activate termination of DNA encapsidation.

Lander, G. C., L. Tang, S. R. Casjens, E. B. Gilcrease, P. Prevelige, A. Poliakov, C. S. Potter, B. Carragher, and J. E. Johnson. 2006. The structure of infectious P22 virion shows the signal for headful DNA packaging. *Science* **312:**1791–1795.

A head-to-tail concatemer, in which the unique genome sequence is represented by ABCDEFGH. Initial cleavage between H and A is followed by packaging of a headful length that is longer than the length of the unique genome sequence, and the second cleavage. Repetition of this process yields a population of particles with encapsidated DNA molecules of the same length, but that are circularly permuted and terminally redundant.



The (+) strand of each segment is packaged prior to synthesis of complementary (−) strands within particles, a mechanism analogous to synthesis of the double-stranded RNA segments of the reovirus genome (Chapter 6). The particle-to-plaque-forming-unit ratio of ϕ6 is close to 1, indicating that essentially all particles contain a complete complement of genome segments. Such precise packaging appears to be the result of the serial dependence of packaging of the (+) strand RNA segments. In *in vitro* reactions, the S segment packages alone, but entry of M RNA requires the presence of S RNA within particles, and packaging of the L segment is dependent on prior entry of both S and M RNAs.

A random packaging mechanism, in which any eight RNA segments of the influenza virus genome were incorporated into virions, would yield a maximum of 1 infectious particle for every 400 or so assembled ($8!/8^8$). This ratio might seem impossibly low, but is within the range of ratios of noninfectious to infectious particles found in virus preparations. Furthermore, if packaging of more than eight RNA segments were possible, the proportion of infectious particles would increase significantly. For example, with 12 RNA molecules per virion, 10% of the particles would contain the complete viral genome. Particles containing more than eight RNA segments have been isolated, consistent with random packaging. In a plasmid-based system for production of infectious influenza A virus particles that included differentially marked versions of three of the genomic RNA segments, some of the virions produced contained two copies of the **same** RNA segment.

This result can be best explained by a random packaging mechanism. On the other hand, the results of more recent studies favor selective packaging.

This mechanism implies that each of the eight (−) strand genome RNAs (vRNAs) carries a unique signal that ensures its packaging. In the past few years, RNA sequences required for incorporation of the individual genome segments have been identified. Such packaging signals comprise the untranslated regions and adjacent coding sequences at both the 5′ and 3′ ends of the vRNAs. Furthermore, these sequences appear to interact to form structures unique to each segment: artificial vRNAs carrying a 5′ end from one genome segment and a 3′ end from a second are packaged with only low efficiency. The observation that defective derivatives of a particular vRNA compete for packaging of only the corresponding normal RNA segment is also consistent with selective packaging of genome RNA segments. The mechanisms by which vRNA packaging signals are recognized and that ensure incorporation of one copy of each segment are not clear. However, there are hints that a multisegment vRNA complex (analogous to the retroviral RNA genome dimer) may form prior to assembly.

Incorporation of Virion Enzymes and Other Nonstructural Proteins

In many cases, the production of infectious particles requires incorporation into the assembling virion of essential viral enzymes, or other proteins that are important in establishing an efficient infectious cycle. Some of these proteins are also structural proteins of the virion. For example, the herpes simplex virus type 1 VP16 protein is both a major component of the virion tegument and the activator of transcription of viral immediate-early genes.

A simple, yet elegant, mechanism ensures entry of retroviral enzymes (protease [PR], reverse transcriptase [RT], and integrase [IN]) into the assembling core. In most cases, these enzymes are synthesized as C-terminal extensions of the Gag polyprotein. The organization and complement of these translation products, here designated Gag-Pol, varies among retroviruses, but the important point is that they contain not only Pol but also the sequences specifying Gag-Gag interactions. These are presumed to direct incorporation of Gag-Pol molecules into assembling particles (Fig. 13.8). The low efficiency with which Gag-Pol polyproteins are translated determines their concentrations relative to Gag in the cell and in virions (1:9).

The enzymes present in other virus particles, such as the RNA-dependent RNA polymerases of (−) strand RNA viruses (see Table 4.8), are synthesized as individual molecules and therefore must enter assembling particles by noncovalent binding to the genome or to structural proteins.

All retroviral cores also contain the cellular tRNA primer for reverse transcription, brought into particles by its base pairing with a specific sequence in the RNA genome and by specific binding to RT. In some cases, including human immunodeficiency virus type 1, the host amino acyl tRNA synthetase that aminoacylates the particular tRNA used as primer is also encapsidated. The absence from virions of other amino acid tRNA synthetases, and the similar concentrations of the enzyme and its tRNA substrate in human immunodeficiency virus type 1 particles, suggest that the synthetase may be recognized by viral proteins during packaging.

Acquisition of an Envelope

Formation of many types of virus particle requires envelopment of capsids or nucleocapsids by a lipid membrane carrying viral proteins. Most such enveloped viruses assemble by virtue of specific interactions among virion components at a cellular membrane before budding and pinching off of a new virus particle. However, they vary in the intracellular site at which the envelope is acquired and therefore in the relationship of envelopment to release of the virus particle. As discussed in Chapter 12, whether particles assemble at the plasma membrane or internal membranes is determined by the destination of viral proteins that enter the cellular secretory pathway. Enveloped viruses assemble by one of two mechanisms, distinguished by whether acquisition of the envelope follows assembly of internal structures, or whether these processes take place simultaneously.

Sequential Assembly of Internal Components and Budding from a Cellular Membrane

For most enveloped viruses, the assembly of internal structures of the virion and their interaction with a cellular membrane modified by insertion of viral proteins are spatially and temporally separated. This class of assembly pathways is exemplified by (−) strand RNA viruses, such as influenza A virus (Fig. 13.6) and vesicular stomatitis virus. Influenza A virus ribonucleoproteins containing individual genomic RNA segments, NP protein, and the polymerase proteins are assembled in the infected cell nucleus as genomic RNA segments are synthesized. They are then transported to the cytoplasm in the M1- and NEP-dependent reactions described in Chapter 12. The viral glycoproteins HA and NA and the M2 membrane protein travel separately to specialized regions in the plasma membrane (lipid rafts) via the cellular secretory pathway (Fig. 13.6; see Chapter 12). The M1 protein interacts with both viral nucleocapsids and the inner surface of the plasma membrane to direct

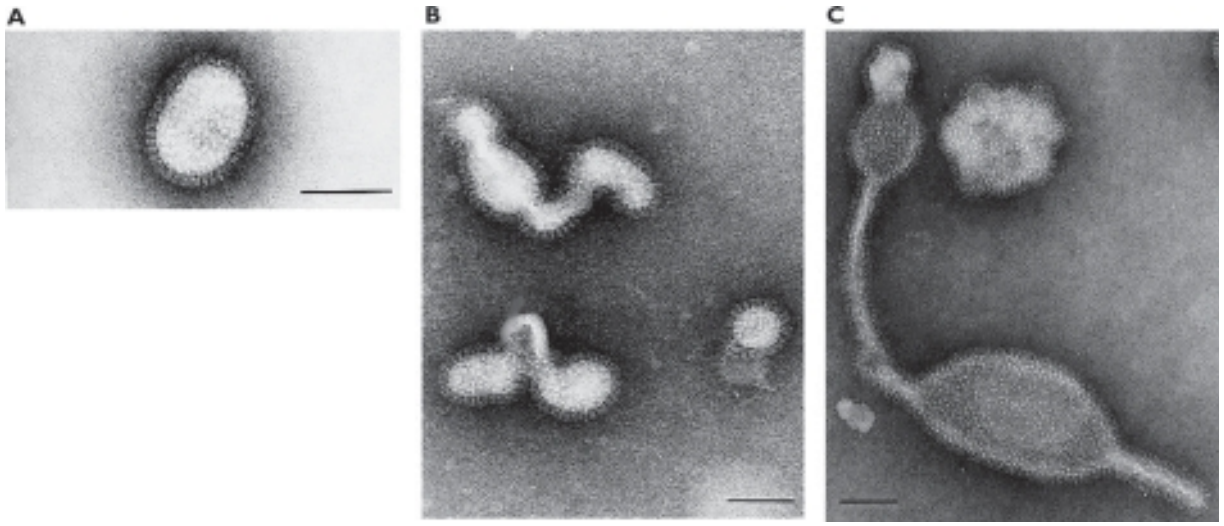
assembly of progeny particles at that membrane. Vesicular stomatitis virus assembles in a similar fashion, although no transport of the ribonucleoprotein from nucleus to cytoplasm is required. The matrix proteins of these (–) strand RNA viruses therefore provide the links among ribonucleoproteins and the modified cellular membrane necessary for assembly and budding.

The cellular membranes destined to form the envelopes of virus particles contain viral integral membrane proteins that play essential roles in the attachment of virus particles to, and their entry into, host cells. In simple enveloped alphaviruses, direct binding of the cytoplasmic portions of the viral glycoproteins to the single nucleocapsid protein (see Fig. 4.23) is necessary for acquisition of the envelope during budding from the plasma membrane. The crucial role and specificity of these interactions in the final steps in assembly are illustrated by the failure of a chimeric Sindbis virus containing the coding sequence for the E1 glycoprotein of Ross River virus, a second togavirus, to bud efficiently. The pE2 and E1 glycoproteins of the chimeric virus form heterodimers that are correctly processed (by cleavage of pE2 to E2) and transported to the plasma membrane. However, such chimeric glycoproteins exhibit an altered conformation and fail to bind to nucleocapsids at the plasma membrane. Binding of viral glycoproteins to internal components also appears to be important for production of more complex enveloped viruses. Interactions between the influenza virus M1 protein and the cytoplasmic tails of the HA and NA glycoproteins are necessary for formation of virus particles with normal size and morphology (Fig. 13.13), and with the appropriate concentration of genomic RNA. The cytoplasmic domain of the M2 ion channel protein is also necessary for efficient incorporation of viral ribonucleoproteins and production of infectious particles.

Coordination of the Assembly of Internal Structures with the Acquisition of the Envelope

The alternative pathway of acquiring an envelope, in which assembly of internal structures and budding from a cellular membrane are largely coincident in space and time, is exemplified by many retroviruses. Assembling cores of the majority first appear as crescent-shaped patches at the inner surface of the plasma membrane. These structures extend to form a closed sphere as the plasma membrane wraps around and eventually pinches off the assembling particle (Fig. 13.7). Formation of the assembling particles depends on the interaction of Gag polyprotein molecules with one another to form the protein core, with the RNA genome via the NC portion, and with the plasma membrane via the MA segment.

Figure 13.13 Deformed influenza A virus particles. These particles assemble when the cytoplasmic (internal) domains of the NA glycoprotein **(B)** or both NA and HA glycoproteins **(C)** are truncated. Purified wild-type **(A)** and mutant virions were negatively stained with phosphotungstic acid and examined by electron microscopy. Bars, 100 μm. The mutant particles are also considerably larger than wild-type virus particles. It is therefore assumed that binding of the internal portions of HA, and particularly of NA, to internal virion components (M1 protein and/or the ribonucleoproteins) determines the spherical shape and characteristic size of these virus particles, as well as the efficiency of budding. From H. Jin et al., *EMBO J.* **16:**1236–1247, 1997, with permission. Courtesy of R. A. Lamb, Northwestern University.



Specific segments of Gag mediate the orderly association of polyprotein molecules with one another, and are required for proper assembly. These sequences include an essential C-terminal multimerization domain of the CA segment: substitutions that disrupt the CA dimer interface block assembly of the CA protein *in vitro* and severely inhibit Gag assembly and formation of virus particles *in vivo*. The capsids of retroviruses can be spherical, conical or cylindrical (Fig. 4.19). Specific CA sequences that determine the morphology of mature particles or of structures formed by CA *in vitro* have also been identified. For example, insertions within an N-terminal CA sequence that forms a loop at the outer surface of CA hexamers in crystals block the formation of spherical Moloney murine leukemia virus cores. Certain sequences present only in the Gag polyprotein also govern morphology, for their removal results in assembly of misshapen particles.

As discussed previously, Gag multimerization during particle assembly is regulated by binding of the NC domain to the RNA genome. This process is also promoted by interaction of Gag with the plasma membrane via the MA membrane-binding signals (Fig. 12.18). Elimination of the signal for myristoylation prevents assembly, as does alteration of the sequence predicted to lie at the interfaces of the MA trimers formed in crystals (Fig. 4.24). It has been suggested that MA trimerization increases the accessibility of the myristate chain. Conversely, efficient membrane binding of Gag depends on sequences other than the membrane-binding region of MA, such as a sequence in the N-terminal portion of NC. Because this sequence is not required for production of stable Gag or its transport to the plasma membrane, it may promote Gag-Gag or Gag-RNA interactions that lead to cooperative and stable binding of Gag molecules to the membrane. In this context, it is noteworthy that in recent studies of assembly in live cells, Gag-Gag interactions were observed only at the plasma membrane.

In some cases, the MA segment of Gag also binds to the cytoplasmic tail of the viral envelope glycoprotein. For example, association of the assembling human immunodeficiency virus type 1 core with the TM-SU glycoprotein requires the N-terminal 100 amino acids of MA. Such Gag-Env interactions ensure specific incorporation of viral glycoproteins into virions. Nonetheless, they do not appear to be universal: glycoprotein-containing virions are produced even when the C-terminal tails of TM of other retroviruses (e.g., avian sarcoma virus) are deleted. Nor can a model based solely on Gag-Env interactions account for the ease with which "foreign" viral and cellular glycoproteins are included in the envelopes of all retroviruses. It is therefore thought that as a particle assembles at the plasma membrane, Gag-membrane interactions displace cellular membrane proteins connected to internal components of the cell. Such displacement would allow lateral diffusion of viral (and cellular) glycoproteins that are not connected in this way into these regions of the cellular membrane, and hence their passive incorporation into assembling virus particles. The final reaction, fusion of membrane regions juxtaposed as the particle assembles (Fig. 13.7), is shared with other viruses that assemble at the plasma membrane. This process is considered in the next section.

Release of Virus Particles

Many enveloped viruses assemble at, and bud from, the plasma membrane. Consequently, the final assembly reaction, fusion of the bud membrane, releases the newly formed virus particle into the extracellular milieu. When the envelope is derived from an intracellular membrane, the final step in assembly, budding, is also the first step in egress, which must be followed by transport of the particles to the cell surface. The assembly of enveloped viruses is therefore both mechanistically coupled and coincident with (or at least shortly followed by) their exit from the host cell. The egress of some viruses without envelopes from certain types of host cell also occurs by specific mechanisms. However, replication of such viruses more commonly results in destruction (lysis) of the host cell. Large quantities of assembled virions may accumulate within infected cells for hours, or even days, prior to release of progeny on cell lysis. The release of many enveloped viruses also destroys the cells that support their replication. However, in some cases nondestructive budding permits a long-lasting relationship with the host cell. The progeny of many simple retroviruses are released throughout the lifetime of an infected cell, which is not harmed (but may be permanently altered; see Volume II, Chapter 7).

Release of Nonenveloped Viruses

The most usual fate of host cells permissive for reproduction of nonenveloped viruses is death and destruction (but see Volume II, Chapter 5). In natural infections, the host defenses are an important cause of infected-cell destruction. However, infection by these viruses destroys host cells more directly: they are cytopathic to cells in culture. In general, we are remarkably ignorant about the mechanisms by which replication of nonenveloped viruses induces death and lysis of host cells.

Infection by many viruses, including poliovirus and adenovirus, leads to inhibition of expression of cellular genetic information by specific effects on cellular transcription, RNA export from the nucleus, or translation. In the case of adenovirus, with a one-step infectious cycle that is

a relatively long 1 to 2 days, the shutdown of production of cellular proteins during the late phase of infection makes an important contribution to the eventual destruction of the infected cell. However, more specific mechanisms have also been implicated, as several viral proteins induce degradation of structural components of the host cell. For example, late in the infectious cycle, cytoplasmic intermediate filament components (specific cytokeratins) are cleaved by the L3 protease into polypeptides that can no longer polymerize. Such disruption of intermediate filament networks, which requires inhibition of cellular protein synthesis so that the network cannot be rebuilt, seems likely to damage the structural integrity of the cell and facilitate virus release. In addition, a small E3-encoded glycoprotein is necessary for efficient nuclear disruption and lysis of cells in culture. This viral glycoprotein is made in large quantities late in infection and accumulates in the nuclear envelope, but its mechanism of action is not yet known. One or more poliovirus nonstructural proteins have been implicated in host cell lysis, but this process occurs more rapidly (within approximately 8 h) than can be explained by inhibition of synthesis of cellular macromolecules. One idea currently under investigation is that cells infected by poliovirus, and other viruses with short infectious cycles, succumb to apoptotic lysis.

While cell lysis is the most common means of escape of naked viruses, there is evidence that some are released in the absence of any cytopathic effect. Under certain conditions, polioviruses and other picornaviruses are released without lysis of the infected cell. When poliovirus replicates in polarized epithelial cells resembling those lining the gastrointestinal tract (a natural site of infection), progeny virions are released exclusively from the apical surface by a nondestructive mechanism. The viral 2BC and 3A proteins induce the formation of infected cell-specific vesicles that closely resemble autophagosomes (see Chapter 12). It has been proposed that these vesicles, which contain two membranes and virions (late in infection), provide a route for nonlytic release of particles assembled to the cytoplasm (Fig. 13.14). Simian virus 40 is also released from permissive cells before induction of cytopathic effects and leaves polarized epithelial cells at their apical surfaces, via the secretory transport pathway of a cell. It will be of considerable interest to learn how such nonenveloped virions enter the membrane-bound compartments of this pathway.

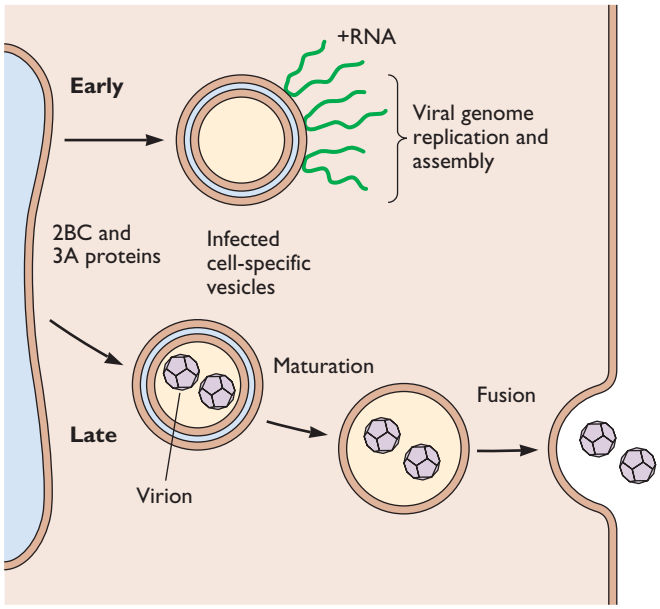


Figure 13.14 Model for nonlytic release of poliovirus particles. As discussed in Chapter 12, synthesis of the viral 2BC and 3A proteins leads to formation of infected cell-specific vesicles that resemble autophagosomes. The surfaces of these vesicles, which are bound by two lipid bilayers, are the sites of genome replication and assembly (top). It has been proposed that as autophagosome-like vesicles form later in infection, they enclose virions present in the cytoplasm. Maturation of such virion-containing vesicles analogous to maturation of autophagosomes would result in complete or partial degradation of the inner membrane. Subsequent fusion of the mature vesicle with the plasma membrane would release virions. This model is based largely on the observation that RNA interference-mediated knockdown of proteins required for formation of autophagosome-like vesicles reduced the yield of extracellular virus particles to a greater degree than the yield of intracellular particles.

Assembly at the Plasma Membrane: Budding of Virus Particles

The release of enveloped virus particles from the plasma membrane is a complex process that comprises induction of membrane curvature by viral components (bud formation), bud growth, and fusion of the bud membrane. Although budding has been visualized repeatedly, often in striking images, the mechanisms that underlie this crucial process are not fully understood.

For many years, it was generally accepted that bud formation was driven by interactions among viral envelope glycoproteins and internal components of viral particles, a mechanism exemplified by alphaviruses such as Sindbis virus. Such a mechanism (Fig. 13.15) can account nicely for how a bud site is determined, by initial binding of the internal nucleocapsid to a cluster of the heterodimeric E1-E2 glycoproteins within the membrane. The bud then expands by cooperative, lateral interactions among glycoprotein spikes and between their internal segments and the capsid protein. Nevertheless, as described in the previous section and summarized in Fig. 13.15, it is clear that there is greater variety in the viral proteins required

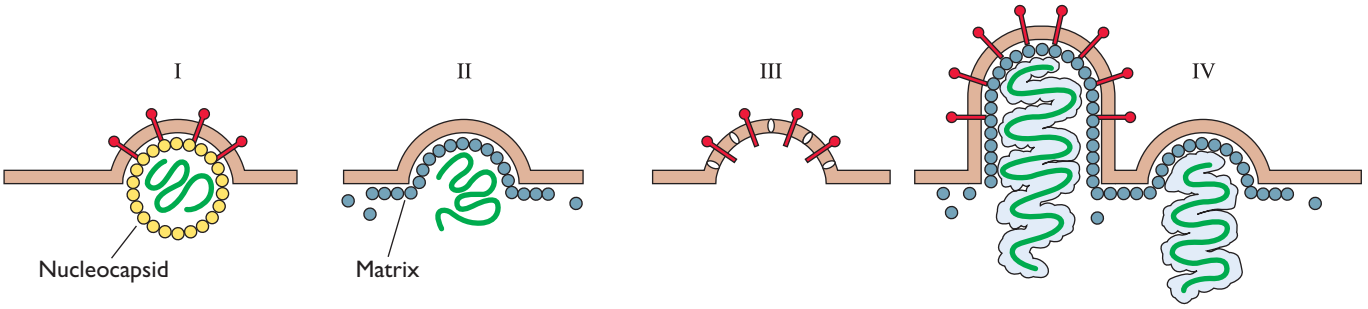


Figure 13.15 Interaction of viral proteins responsible for budding at the plasma membrane. Four distinct budding strategies have been identified. In type I budding, exemplified by alphaviruses such as Sindbis virus, both the envelope glycoproteins and the internal capsid are essential. Quite detailed structural pictures of alphaviruses are now available (Chapter 4). Certain altered or chimeric envelope proteins that reach the membrane normally do not support budding. These properties indicate that lateral interactions among the envelope heterodimers, as well as those of the heterodimers with the capsid, cooperate to drive budding. Type II budding, such as Gag-dependent budding of many retroviruses, requires only the internal matrix protein. For other viruses, type II budding requires only capsid proteins. Conversely, budding can be driven solely by envelope proteins (type III), a mechanism exemplified by the envelope proteins of the coronavirus mouse hepatitis virus. Type IV budding is driven by matrix proteins, but its proper functioning depends on additional components. For example, in the case of rhabdoviruses and orthomyxoviruses, internal matrix proteins alone can drive budding. However, this process is inefficient, or results in deformed or incomplete particles in the absence of envelope glycoproteins or the internal ribonucleoprotein. Adapted from H. Garoff et al., *Mol. Microbiol. Rev.* **62:**1171–1190, 1998, with permission.

for budding than was originally appreciated. Despite such diversity, recent studies of viral protein sequences required for budding have identified mechanistic themes common to different virus families.

Common Sequence Motifs Are Required for Budding at the Plasma Membrane

A major breakthrough in our understanding of how virus particles bud from the plasma membrane came with the identification of mutants of human immunodeficiency virus type 1 with an unusual assembly phenotype: mutations in the coding sequence for the p6 region unique to the Gag polyprotein did not impair assembly of immature particles, but the particles remained attached to the host cell by a thin membrane stalk (Fig. 13.16A). It was therefore concluded that these Gag sequences are required for the fusion reaction that separates the viral envelope from the plasma membrane. Subsequently, functionally analogous sequences, termed late-assembly (L) domains, were identified in Gag proteins of several other retroviruses. These L domains are not conserved in their location within Gag or in amino acid sequence, but nevertheless can substitute for one another to promote budding.

Retroviral L domains contain a small number of short, core sequence motifs, such as PTAP and PPXY (Table 13.3). The recognition of such motifs, and their ability to function independently of position or sequence context, led to definition of L domains containing such motifs in the proteins required for budding of viruses of several different families (Table 13.3). Furthermore, it is now clear that **L domain sequences** promote budding by recruitment of cellular proteins that participate in specific steps in vesicular trafficking.

The Activity of Viral L Domains Depends on Vascular Sorting Proteins

The autonomous activity (and in some cases the sequence) of L domains suggested that these sequences mediate protein-protein interactions. Cellular proteins that bind to each of the prototype sequences have now been identified. The PTAP motif was first shown to recruit the product of tumor susceptibility gene 101, Tsg101, an interaction that is essential for budding of human immunodeficiency virus type 1. This discovery provided the key to identification of the cellular machinery that promotes budding of this and several other enveloped viruses.

Mammalian Tsg101 participates in sorting and trafficking of cellular proteins from late endosomes to structures called multivesicular bodies, which fuse with lysosomes. As their name implies, multivesicular bodies contain vesicles within vesicles. Formation of these structures and budding of virus particles are topologically equivalent processes: in both cases, membranes invaginate away from the cytoplasm and fusion releases vesicles with cytoplasmic contents into a lumen or the extracellular space. Recruitment of Tsg101

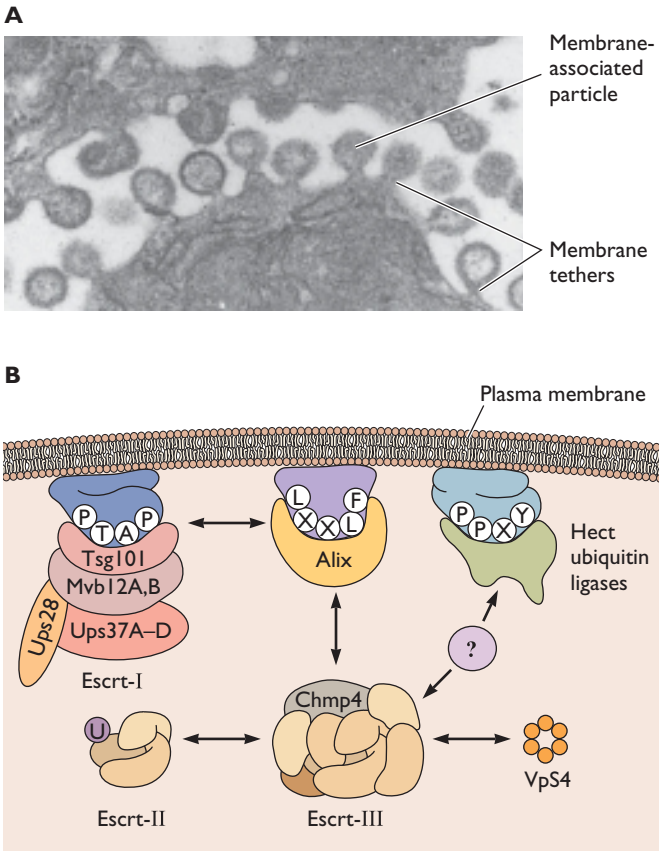


Figure 13.16 Release of retroviral particles. (A) Electron micrograph of monkey Cos-7 cells containing a human immunodeficiency virus type 1 mutant provirus from which the Gag p6 cannot be expressed. The plasma membrane-associated particles exhibit normal morphology, but remain tethered to the membrane. Adapted from H. G. Göttlinger et al., *Proc. Natl. Acad. Sci. USA*, **88:**3195–3199, 1991, with permission. Courtesy of H. Göttlinger, University of Massachusetts Medical Center. **(B)** Summary of association of cellular trafficking proteins with core sequence motifs of L domains in viral proteins required for release of viral particles. Interactions are shown by direct contact between motifs and proteins, and by double-headed arrows. It is thought that an unidentified adapter protein links Hect ubiquitin ligases with EscrtIII. This complex and Vps4 (vacuolar protein sorting 4) are thought to mediate membrane reorganization and fusion during release of enveloped virus particles. Alix binds to endophilins, proteins that induce membrane curvature. Its interaction with Chmp4 (charged multivesicular body protein 4) is required for budding of human immunodeficiency virus type 1.

by the PTAP L domain therefore suggested that the cellular machinery that mediates sorting and trafficking of endocytic vesicles is diverted to promote budding and release of virus particles. In fact, Tsg101 proved to be the human homolog of one subunit of a complex required for sorting of yeast proteins to the vacuole/lysosome, termed endosomal sorting complex required for transport-I (Escrt-I). The other subunits of human Escrt-I are also required

Table 13.3 Common sequence motifs required for budding of enveloped virus particles

Core motif	L domain sequence[a]	Viral protein
PT/SAP	…PE<u>PTAP</u>PEE…	Human immunodeficiency virus type 1 Gag
	…VE<u>PTAP</u>QV…	Human immunodeficiency virus type 1 Gag
	…ILP<u>TAP</u>EY…	Ebola virus M
	…YA<u>PSAP</u>…	Vesicular stomatitis virus M
PPXY	…A<u>PPPPY</u>VG…	Rous sarcoma virus Gag
	…IA<u>PPPY</u>EE…	Vesicular stomatitis virus M
LXXLF	…AS<u>LRSLF</u>G…	Human immunodeficiency virus type 1 Gag
	…VR<u>LDLLL</u>L…	Sendai virus M

[a]The amino acids of the L domain motifs are underlined.

for release of human immunodeficiency virus type 1. As summarized in Fig. 13.16B, Escrt-I is associated with two other Escrt assemblies, via adapter proteins such as Alix. Importantly, the other protypical L domain sequences (Table 13.3) recruit this **same** cellular machine, although they bind directly to different components (Fig. 13.16B). It therefore appears that formation and release of virus particles with very different structures, genomes, and composition are driven by the same cellular components and mechanism.

Many mechanistic questions about the precise molecular functions of the many proteins shown in Figure 13.16B remain to be addressed. However, Escrt-III is thought to provide the machinery for membrane budding and fusion. Consistent with this view, dominant negative derivatives of Escrt-III subunits inhibit budding of all retroviruses examined, irrespective of which L domain sequence is present in Gag, and of the rhabdovirus vesicular stomatitis virus.

A small fraction of retroviral Gag is ubiquitinylated, and the PPXY L domain sequence recruits specific ubiquitin ligases (Fig. 13.16B). A catalytically active ubiquitin ligase is necessary for release of retrovirus with this Gag L domain sequence. Furthermore, ubiquitinylation of human immunodeficiency virus type 1 Gag at sites C-terminal to the CA domain is necessary for efficient release: substitutions that prevent modification at these sites lower the rate of release, and induce the accumulation of virus particles tethered to the plasma membrane. As ubiquitin is recognized by several of the endocytic trafficking proteins, this modification might promote assembly of the machine that mediates budding and release of retroviruses. A significant fraction of human immunodeficiency virus type 1 Gag localizes to late endosomes and multivesicular bodies, particularly in macrophages. It has therefore been suggested that Gag is

sorted to such intracellular compartments prior to transport to the plasma membrane. However, analysis of Gag trafficking by pulse-chase labeling has shown recently that the polyprotein is sorted primarily to the plasma membrane. Much Gag is then released in assembled particles, and the remainder is subsequently internalized, accumulating in late endosomes and multivesicular bodies.

Release from the plasma membrane can also depend on viral proteins other than major structural proteins. For example, in some cell types, efficient release of human immunodeficiency virus type 1 requires the viral Vpu protein. This function of Vpu is distinct from the induction of proteolysis of CD4 in the ER described in Chapter 12. In the absence of Vpu, particles accumulate in intracellular vacuoles, or we tethered to the infected cell surface. The Vpu protein was shown to counteract the action of an antiviral protein that tethers virus particles to the cell surface, and is produced when cells are exposed to alpha interferon. This protein was subsequently identified, based on the pattern of expression of its gene, and termed tetherin. Its organization suggests that interactions between tetherin molecules inserted in the plasma membrane and the viral envelope are responsible for retaining virus particles at the cell surface. How this interaction is disrupted by Vpu is not yet known.

Assembly at Internal Membranes: the Problem of Exocytosis

Cytoplasmic Compartments of the Secretory Pathway

Several enveloped viruses are assembled at the cytoplasmic surfaces of compartments of the secretory pathway under the direction of specifically located viral glycoproteins (Table 12.4). The final step in virion production is budding of the particle into the lumen of one of these compartments, for example, of bunyaviruses into Golgi cisternae. These particles therefore lie within membrane-bound organelles. It is generally assumed that such virus particles must be packaged within cellular transport vesicles for travel along the secretory pathway to the cell surface. Despite almost universal acceptance of this mechanism, we have little direct evidence for this mode of transport. Indeed, in some cases there is evidence indicating that the secretory pathway is not required. Immature capsids of the betaretrovirus Mason-Pfizer monkey virus assemble at internal cytoplasmic sites, near the centrioles. Their transport to the plasma membrane depends on the viral Env protein, implicating some type of vesicular transport. However, this process is refractory to inhibition of ER-to-Golgi transport. Rather, it is blocked by disruption of endocytic trafficking.

The budding of virus particles into internal compartments of the secretory pathway is initiated by interactions among the cytoplasmic domains of viral membrane proteins and internal components of the particle. Consequently, this process generally begins as soon as the integral membrane and cytoplasmic viral proteins attain sufficient concentrations in the infected cell. For example, the concentration of viral membrane proteins (surface proteins) determines the fate of hepadnaviral cores, which contain the capsid (C) protein (Table 13.1), a DNA copy of the pregenomic RNA, and the viral polymerase (Appendix, Fig. 3). Early in infection, the concentration of the large surface protein (L) in membranes is too low for efficient envelopment of cores (Fig. 13.17); these structures therefore enter the nucleus, where they contribute to the pool of viral DNA templates for transcription. As the concentration of the L protein increases, it interacts with cores, and enveloped particles form. The ability of hepadnaviral cores to bind to this viral glycoprotein is also regulated by the nature of the nucleic acid they contain: synthesis of DNA from the pregenomic RNA induces significant conformational changes in the exterior surface of core particles. These induce changes in the shape of the hydrophobic pocket that is formed by residues required for normal assembly and release of virus particles.

An intriguing question is why some viruses formed by a budding mechanism assemble at intracellular membranes rather than at the plasma membrane, where budding ensures release of the virion from the host cell. One possible advantage of intracellular budding is that the concentration of viral glycoproteins exposed on the surface of the infected cell is reduced. This property would decrease the likelihood that the infected cell would be recognized by components of the immune system before the maximal number of progeny virions were assembled and released. Alternatively, the simpler cytoplasmic surfaces of internal membranes, which are not burdened with cytoskeletal structures and the proteins that attach them to the extracellular matrix, may make for more facile assembly or budding reactions. Or the distinctive lipid composition of internal membranes may confer some (as yet unknown) special property that is advantageous to these viruses.

Envelopment by a Virus-Specific Mechanism

The interaction of components of the poxvirus vaccinia virus with internal cellular membranes during assembly is most unusual. One remarkable feature is the assembly of two **different** infectious particles, the intracellular mature and the extracellular enveloped virions, that differ in the number and origin of lipid membranes. Furthermore, the initial acquisition of a membrane early in assembly occurs by an apparently unique, virus-specific mechanism.

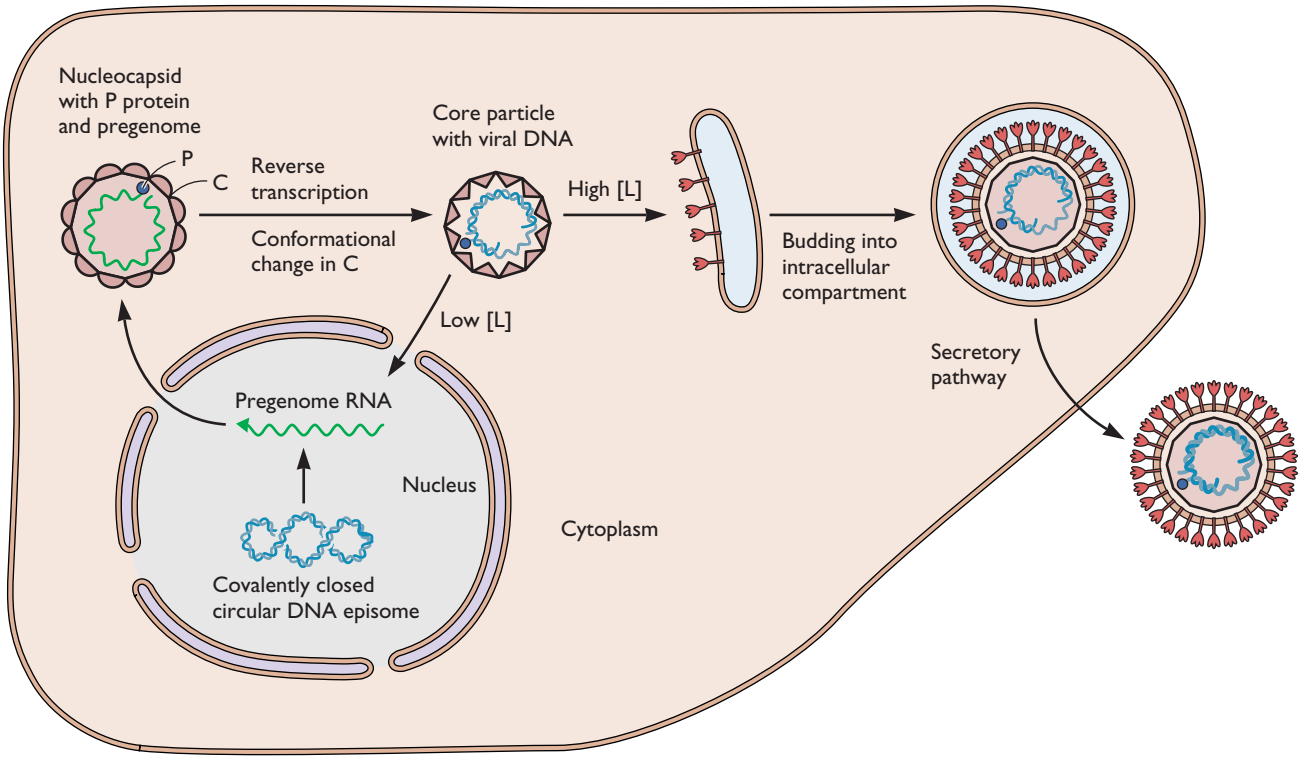
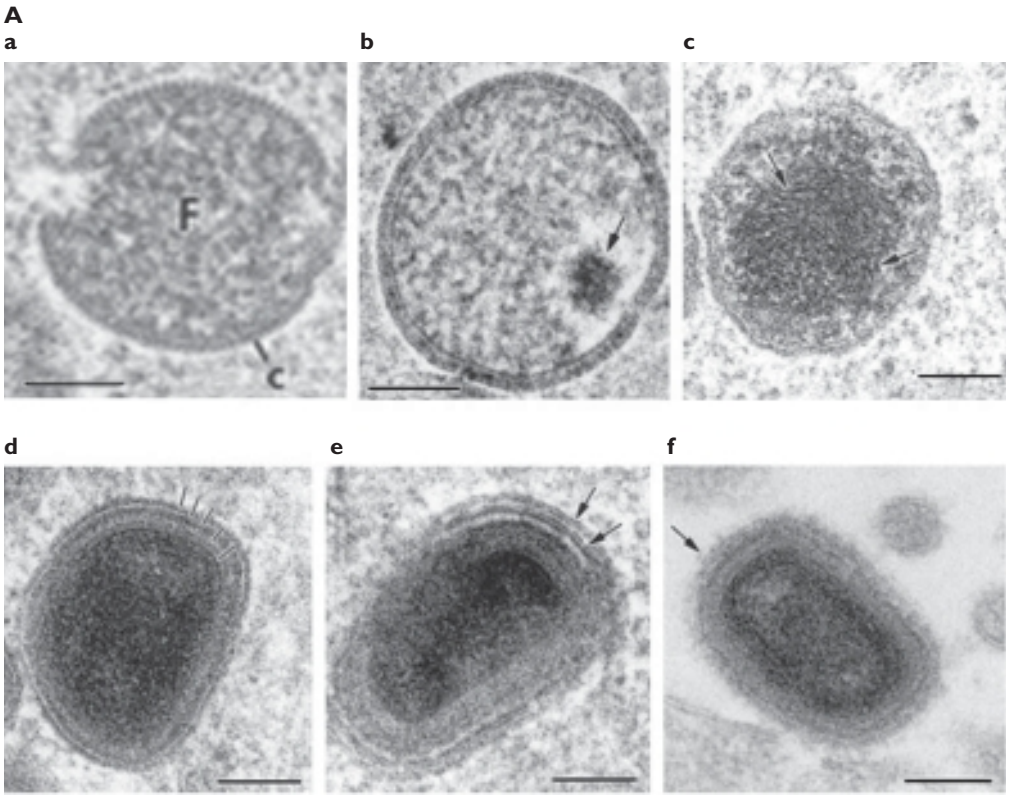


Figure 13.17 Model of hepatitis B virus envelopment. The pregenome RNA synthesized in infected cell nuclei (see Chapter 7) is exported to the cytoplasm, where it is incorporated into particles built from the capsid (C) protein. Reverse transcription to produce the DNA genome induces a conformational change in C protein that allows interaction of C with the large surface protein (L) inserted into internal membranes. Whether core particles containing DNA enter the nucleus or become enveloped by budding into compartments of the secretory pathway is determined by the concentration of the L protein. The L and middle (M) and small (S) envelope glycoproteins accumulate in membranes of the ER-Golgi intermediate compartment, into which subviral particles that contain only lipid and envelope proteins (primarily S) appear to bud. As the S, as well as the L, protein is required for envelopment, it is generally accepted that virions are also formed by budding into this same compartment of the secretory pathway. However, the results of recent experiments implicate the cellular proteins that participate in endocytic trafficking described previously in hepatitis B virus budding and release.

Finally, infectious particles leave the host cell by at least three distinct routes.

Vaccinia virus assembly is a complex process that includes the formation of several intermediates, such as crescents (see below) and immature virions, and major morphological rearrangements as infectious particles are formed (Fig. 13.18A). The assembly pathway was elucidated initially by electron microscopy in some of the earliest studies of vaccinia virus. Numerous viral proteins that participate in the various assembly reactions have been identified by genetic experiments (Table 13.4). Synthesis of viral DNA genomes and structural proteins takes place in discrete cytoplasmic domains termed viral factories. The first morphological sign of assembly is the appearance within viral factories of rigid, curved structures 10 to 15 nm thick (Fig. 13.18B). There has never been any doubt that these structures, termed crescents, contain at least one lipid bilayer. In contrast, the origin of this membrane, and whether a second bilayer is also present, have been subjects of much debate. The current consensus is that crescents contain a single lipid membrane that is derived from the ER membrane, but by a unique, perhaps virus-specific mechanism (Box 13.8). As the crescents enlarge, they retain their original curvature and therefore eventually form spheres surrounding viral macromolecules present in viral factories, including the DNA genome. Such immature virions then undergo major morphological transitions to form brick-shaped mature virions (Fig. 13.18B). This maturation process requires several distinct reactions. These include proteolytic cleavage of several structural proteins by viral protease(s) (Table 13.3), the action of the viral redox system (Chapter 12), and removal of at least one crescent-associated protein. These changes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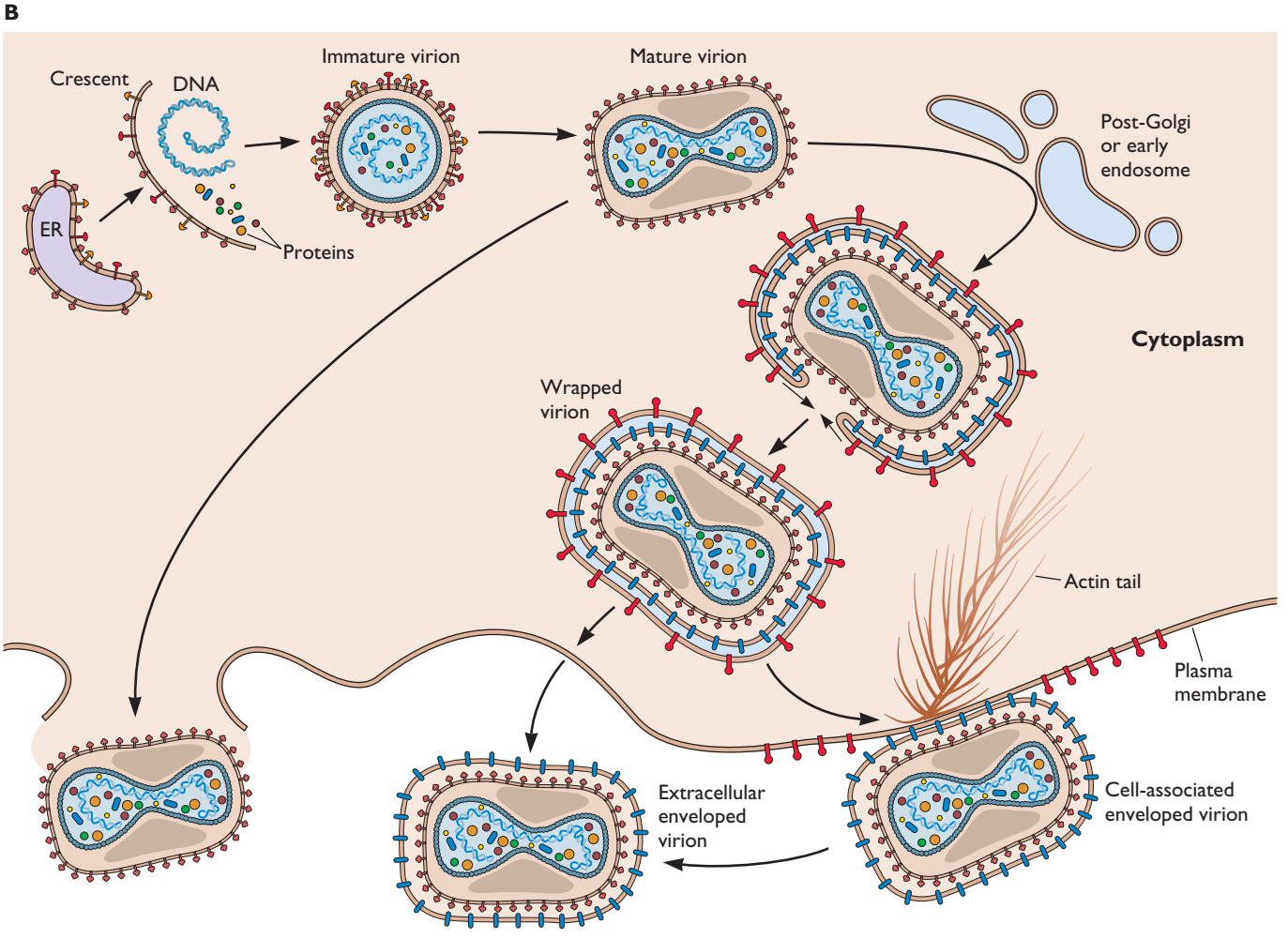

B
Crescent
DNA
Immature virion
Mature virion
Post-Golgi
or early
endosome
ER
Proteins
Cytoplasm
Wrapped
virion
Actin tail
Plasma
membrane
Extracellular
enveloped
virion
Cell-associated
enveloped virion

Table 13.4 Some proteins implicated in vaccinia virus assembly

Assembly reaction	Protein	Function/properties
Crescent formation	A14, A17	Essential for this step; integral membrane proteins; phosphorylated by F10; form a disulfide-bond-stabilized lattice
	D13	Imparts crescent curvature and rigidity; interacts with A17
	F10	Essential for ER membrane remodeling and appearance of crescents; dual-specificity protein kinase
Assembly of immature virion (IV)	Complex of 7 proteins, including F10	Association of viroplasm with crescents
	A32	Genome encapsidation; required for packaging DNA and I6
	I6	Genome encapsidation; binds specifically to terminal hairpins in DNA
Formation of mature virion (MV)	A4	Core assembly during morphogenesis; present in outer palisade layer of core wall
	A3	Formation of morphologically normal and transcriptionally active cores; proteolytically processed during morphogenesis
	G1	IV-to-MV transition; metalloprotease
	I7	IV-to-MV transition; cysteine protease, required for processing A3 and other proteins
	E10, A2.5, G4	IV-to-MV transition; thiol redox proteins (see Chapter 12)
Formation of wrapped virion	A27	Essential for this step: disulfide-bonded trimer bound to MV membrane
	B5, F13	Required for efficient wrapping; transmembrane proteins sorted to intracellular wrapping site(s)

resemble those that occur during assembly of herpesviruses and adenoviruses but are undoubtedly more complex.

The mature virus is released only upon lysis of the infected cell. However, a proportion of this population (that varies with the time of infection) becomes engulfed by the membranes of a second intracellular compartment, probably a *trans*-Golgi or early endocytic compartment, to form the wrapped virion (Fig. 13.18B). The mature virion is transported to the site(s) of wrapping via microtubules. The remodeling of organelle membranes to form the wrapped virion depends on a number of viral proteins that are present only in this type of particle (Table 13.4), and that appear to be sorted to wrapping sites via the secretory pathway. The mechanism(s) by which such proteins interact with mature virions to induce formation of the three membrane-containing wrapped virion is not known. This particle can be released from the cell as the two-membrane-containing extracellular enveloped virion following transport to the cell surface and fusion of its outer membrane with the plasma membrane (Fig. 13.18B). As the mature virion and the extracellular enveloped virion bind to different cell surface receptors,

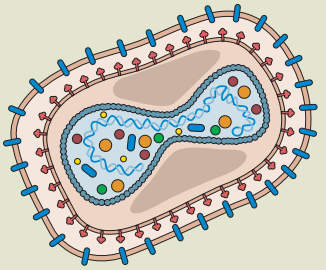
Figure 13.18 Vaccinia virus assembly and exocytosis. (A) Viral structures observed in infected cells. HeLa cells infected with vaccinia virus for 10 or 24 h were prepared for electron microscopy by quick freezing and negative staining while frozen. These procedures preserve fine structural detail. The following structures are shown. (a) Viral factory comprising a viral crescent (C) around a viroplasm focus (F). (b) Immature virions, in this example associated with DNA (arrows) entering via a pore in the particle. (c) Spherical, dense particles containing DNA-like material (arrows). (d) Intracellular mature virion. (e) Intracellular enveloped virion with the additional double membrane (arrows). (f) Extracellular enveloped virion, which carries an external fuzzy layer (arrow). Bars, 100 nm. Adapted from C. Risco et al., *J. Virol.* **76:**1839–1855, 2000, with permission. **(B)** Schematic model of assembly and exocytosis. Assembly begins with the formation of crescents by diversion of membrane from the ER. The viral D13 protein, which is associated with the outer leaflets of crescents, maintains the curvature and rigidity of the crescent membrane as it enlarges and eventually closes with the incorporation of viral DNA and proteins from viral factories. As the D13 protein is lost during the morphological transitions that form the brick-shaped mature virion, it is considered a scaffolding protein. The mature virion is released from infected cells only upon lysis. However, a significant proportion of these structures acquire additional membranes by wrapping, in membranes derived from a late or post-Golgi compartment to form the wrapped virion. This particle is transported to the plasma membrane, where fusion with this cellular membrane forms the cell-associated enveloped virus, which induces formation of actin tails. Adapted from B. Sodeik and J. Krijnse-Locker, *Trends Microbiol.* **10:**15–24, 2002, with permission.

BOX 13.8 DISCUSSION

How is the vaccinia virus crescent membrane formed? An unsolved mystery

It is simple to visualize how reorganization and fusion of internal cellular membranes can "wrap" structures in a double membrane, as during formation of wrapped virions of vaccinia virus (Fig. 13.18B). In contrast, it is not at all obvious how viral structures containing a single lipid bilayer, the crescent (and immature and mature virions), form by a nonbudding mechanism. This conundrum led to the early proposal that the crescent membrane is synthesized *de novo* from cellular lipids. No mechanism for such *de novo* assembly has been identified, and it is generally agreed that crescents are derived from preexisting cellular membranes.

There is accumulating evidence that the ER is the source of the crescent



membrane. For example, several of the major viral membrane proteins are inserted into the ER membrane *in vitro* or *in vivo*, and one (A9) is present near sites of assembly in tubular structures that contain the ER lumenal enzyme, protein disulfide isomerase. Furthermore, when a heterologous signal sequence was added to the N terminus of A9, the signal sequence was cleaved off and only the truncated protein was detected in immature and mature virions. As signal peptidase, which removes signal sequences, resides in the ER (see Chapter 12), this observation provides compelling support for the view that the ER membrane containing viral membrane proteins is the origin of the crescent membrane.

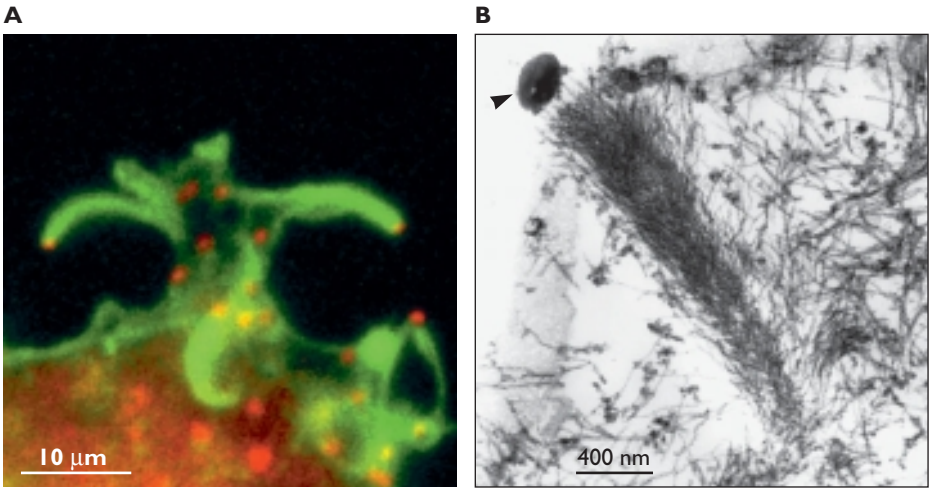
How a single lipid bilayer structure is produced from the ER remains an enigma.

Hussain, M., A. S. Weisberg, and B. Moss. 2006. Existence of an operative pathway from the endoplasmic reticulum to the immature poxvirus membrane. *Proc. Natl. Acad. Sci. USA* **103:**19506–19511.

the release of two types of infectious particle may increase the range of cell types that can be infected. A significant proportion of enveloped virions are not released following membrane fusion but, rather, remain attached to the host cell surface as cell-associated enveloped virions (Fig. 13.18B). The mechanisms of transport and egress that produce cell-associated virions are amazing processes that depend on major reorganization of components of the host cell cytoskeleton.

Wrapped virions initially travel from sites of assembly to the plasma membrane on microtubules, carried by the cellular motor protein kinesin. The interaction of these

Figure 13.19 Movement of vaccinia virus on actin tails. (A) Immunofluorescence micrograph of virus particles (red) at the ends of the cell surface projections containing actin tails (green). The coincidence of the tips of the projecting actin and viral particles gives yellow-orange signals (marked by white arrowheads), indicating that the particles are projected from the cell surfaces on the tips of actin tails. When infected cells are plated with uninfected cells, such actin-containing structures to which virus particles are attached can be seen extending from the former into the latter. **(B)** An electron micrograph of a virus particle attached (arrowhead) to an actin tail. From S. Cudmore et al., *Nature* **378:**636–638, 1995, with permission. Courtesy of S. Cudmore and M. Way, European Molecular Biology Laboratory.



particles with the motor depends on the viral A36 protein present in their outer membrane, which interacts with the light chain of the kinesin motor. Such active transport allows movement of the large wrapped virion to the cell periphery in less than 1 min (compared to an estimated 10 h that would be required by passive diffusion!). Remodeling of the dense layer of cortical actin that lies beneath the plasma membrane (Fig. 2.4) is also required to deliver these particles to the plasma membrane. This phenomenon is induced by a viral protein that modulates the cellular signaling pathway that regulates the dynamics of cortical actin. Actin remodeling is required for efficient release of wrapped virions, presumably in part because the movement of virus particles near the cell surface depends on the actin cytoskeleton.

The particles formed by fusion of wrapped virions with the plasma membrane remain cell associated because of a remarkable activity: they induce a further, and dramatic reorganization of the actin cytoskeleton just below the site of fusion. The number of typical actin stress fibers is significantly decreased, because the virus induces the formation of new, filamentous actin-containing structures. Each of these, which are termed actin tails, is in contact with a single virus particle (Fig. 13.19A and B). Viral particles attached to the tips of actin tails are propelled at an average speed of 0.18 nm/s, by polymerization of actin at the front end of the tail and its depolymerization at the back end. As the infection progresses, they can be seen on large microvilli induced by the actin tails (Fig. 13.19B). Remarkably, formation of actin tails in vaccinia virus-infected cells requires the same viral protein that allows transport of wrapped virions along microtubules, the A36 protein. This protein is phosphorylated at specific positions by the cellular tyrosine kinase Src, which plays an important role in regulation of actin dynamics in uninfected cells. Phosphorylation of A36 triggers its dissociation from kinesin and allows binding of cellular proteins that promote actin polymerization (Fig. 13.20).

The formation of vaccinia-actin tails is necessary for efficient spread of the virus, for mutants that cannot induce these structures form only small plaques on cultured cells. The viral particles attached to the outer surface of cellular projections containing actin tails can dissociate to become extracellular enveloped virions. However, such projections can extend from infected cells into neighboring uninfected cells, suggesting that they may also facilitate direct cell-to-cell spread of infectious particles.

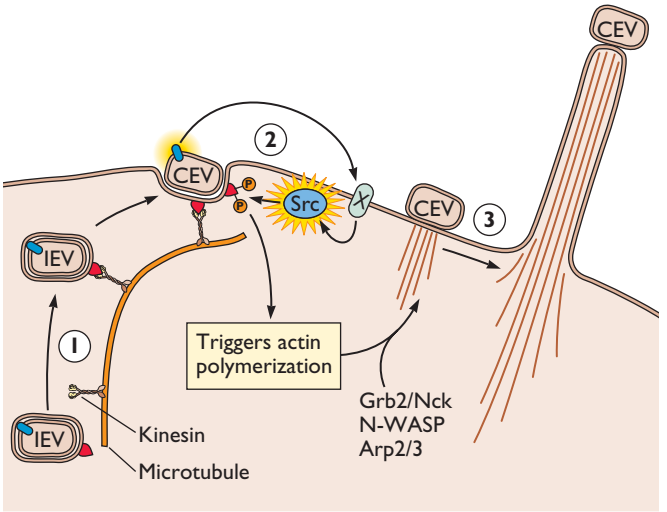


Figure 13.20 Model for the switch from microtubule- to actin-dependent transport of vaccinia virus particles. The A36R protein present in the outer membrane of wrapped virions binds to the light chain of the kinesin motor, which then transports the particles to the cell periphery. Remodeling of cortical actin by viral proteins allows close approach of the virions to the plasma membrane. Fusion of the outer membrane of wrapped virions with the plasma membrane releases cell-associated virions, which carry the B5R protein in their new outer membrane. This viral protein activates the cellular Src tyrosine kinase, presumably via interaction with one or more cellular membrane proteins (X). Src then phosphorylates the membrane-associated A36R protein, a modification shown by genetic experiments to be essential for formation of actin tails. Furthermore, A36 remains bound to kinesin in vaccinia virus-infected cells that lack Src or that are treated with inhibitors of this kinase. Phosphorylated A36R binds via adapter (Grb and Nck) and scaffolding (N-Wasp) to cellular proteins that induce actin polymerization. Such polymerization drives the formation of actin-tail containing protrusions that project cell-associated virions away from the host cell. Adapted from A. Hall, *Science* **306:**65–67, 2006, with permission.

Intranuclear Assembly

The problem of egress is especially acute for the enveloped herpesviruses, because the nucleocapsids assemble in the nucleus. The pathway by which the virus leaves the cell has been a topic of fierce controversy, centered on where and when the viral envelope is acquired. A large body of evidence now favors the less intuitive (and to some, inefficient) double-envelopment model summarized in Figure 13.21.

The first step in egress is exit of nucleocapsids from the nucleus, which is achieved not by transport through nuclear pore complexes but, rather, by a unique budding mechanism (Fig. 13.21). In the case of herpes simplex virus type 1, a subset of the tegument proteins, including VP16, associate with the nucleocapsid prior to budding. Late in infection, the dense meshwork of protein filaments that abuts the inner nuclear membrane (the nuclear lamina) is dramatically reorganized and perforated (Fig. 13.22), presumably to allow juxtaposition of the nucleocapsid and membrane. Such disruption of the nuclear

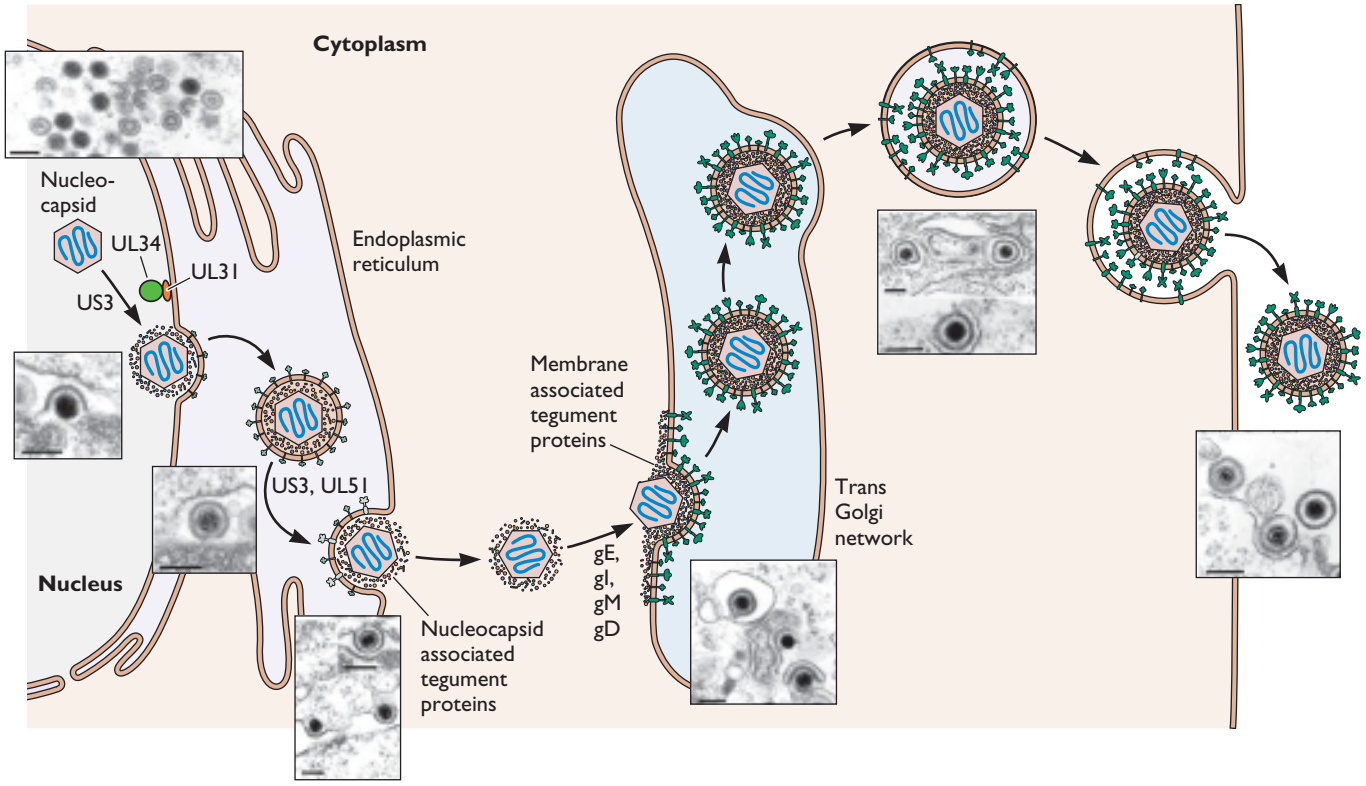


Figure 13.21 Pathway of herpesvirus egress. The mature nucleocapsid assembled within the nucleus (Fig. 13.5 and 13.10) initially acquires an envelope by budding through the inner nuclear membrane. Upon fusion with the outer nuclear membrane, this membrane is lost as unenveloped nucleocapsids are released into the cytoplasm. Some tegument proteins interact with the nucleocapsid in the cytoplasm, whereas others, including the UL11, UL46, and UL49 proteins, concentrate at sites of secondary envelopment. The latter are presumably localized at membranes of *trans*-Golgi compartments, by interactions with the cytoplasmic domains of viral glycoproteins, such as the binding of the UL11 and UL49 proteins to the cytoplasmic domains of gE and gD. The myristyolated UL11 protein accumulates at the membranes of *trans*-Golgi compartments and directs other tegument proteins to sites of secondary envelopment. The viral envelope is acquired upon budding of tegument-containing structures into compartments of the *trans*-Golgi network. Virions formed in this way are thought to be transported to the plasma membrane in secretory transport vesicles and released upon membrane fusion, as illustrated. Viral gene products implicated in specific reactions are indicated. The reactions are illustrated in the corresponding electron micrographs of cells infected by the alphaherpesvirus pseudorabies virus. Bar, 150 nm. Adapted from T. C. Mettenleiter, *J. Virol.* **76:**1537–1547, 2002, with permission. Courtesy of T. C. Mettenleiter, Federal Research Center for Virus Diseases of Animals, Insel Riems, Germany.

lamina requires the viral UL31 phosphoprotein and the UL34 transmembrane protein. When phosphorylated by the viral US3 protein kinase, UL31 and UL34 associate with one another at the inner surface of the inner nuclear membrane, and bind the proteins that form the lamina (lamins A/C and B) and cellular protein kinase C. This enzyme phosphorylates the lamins, while the US3 kinase phosphorylates the nuclear membrane protein emerin, which binds to the lamins and has been implicated in maintenance of nuclear integrity. These modifications are thought to disrupt the interactions that form the nuclear lamina. The mechanisms that then drive budding and fusion of the inner nuclear membrane are not understood, not least because the nucleocapsid component(s) that interacts with the membrane has not been identified. The deenvelopement reaction that subsequently releases nucleocapsids into the cytoplasm (Fig. 13.21) requires either the gB or the gH glycoproteins.

The second envelopment, in which particles acquire the virion envelope, takes place at the cytoplasmic surface of compartments of the *trans*-Golgi network. Viral membrane proteins, including those necessary for secondary envelopment (e.g., gD, gE/gI, gM, and the UL20 protein), are sorted to these cellular compartments via the

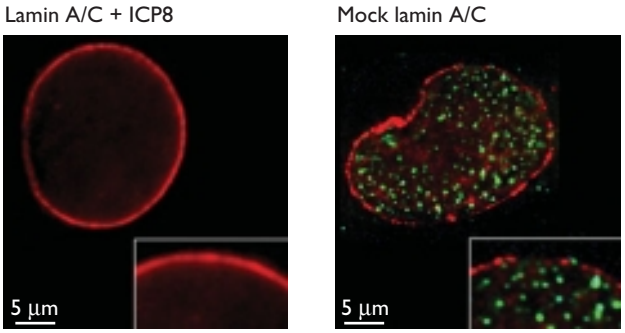


Figure 13.22 Disruption of the nuclear lamina in herpes simplex virus type 1- infected cells. Human cells mock-infected or infected with herpes simplex virus type 1 for 16 h were examined by indirect immunofluoresence. The cellular lamin A/C and viral 1CP8 proteins are in red and green, respectively. The insets show magnified regions of equal size. Adapted from M. Simpson-Holley et al., *J. Virol.* **79:**12840–12851, with permission. Courtesy of D. Knipe, Harvard University Medical School.

secretory pathway (see Chapter 12). Some tegument proteins accumulate at the sites of secondary envelopment, and are required for this step. Others associate with the nucleocapsid in the cytoplasm. These proteins include the US3 kinase described previously and the UL36 and UL37 proteins, which are required for transport of nucleocapsids through the cytoplasm. Once the nucleocapsid reaches the *trans*-Golgi network, interactions between these two classes of tegument protein must take place prior to envelopment. Nevertheless, the proteins that mediate such final assembly of the tegument have not yet been identified, nor have those that induce membrane budding and fusion. Some recent observations hint that such viral proteins may function via the cellular budding machinery that mediates the release of simpler enveloped viruses.

It is thought that enveloped virions released into the lumen of a *trans*-Golgi compartment travel to the plasma membrane in secretory vesicles, and are released upon fusion of such vesicles with that membrane (Fig. 13.21).

Maturation of Progeny Virions

Proteolytic Processing of Virion Proteins

The products of assembly of several viruses are noninfectious particles, often called **immature virions.** In all cases, proteolytic processing of specific proteins with which the particles are initially built converts them to infectious virions. The maturation reactions are carried out by virus-encoded enzymes, and take place late in assembly of particles, or following release of immature virions from the host cell. Proteolytic cleavage of virion proteins introduces an irreversible reaction into the assembly pathway, driving it in a forward direction. This modification can also make an important contribution to resolving the contradictory requirements of assembly and virus entry. One consequence of proteolytic processing is the exchange of covalent linkages between specific protein sequences for much weaker noncovalent interactions, which can be disrupted in a subsequent infection. A second is the liberation of a new N terminus and a new C terminus at each cleavage site, and hence opportunities for additional protein-protein contacts. Such changes in chemical bonding among virion proteins clearly facilitate virus entry, for the proteolytic cleavages that introduce them are necessary for infectivity. Accordingly, viral proteases and the structural consequences of their action are of considerable interest. Moreover, these enzymes are excellent targets for antiviral drugs, a property exemplified by the success of therapeutic agents that inhibit the human immunodeficiency virus type 1 protease.

Cleavage of Polyproteins

As we have seen, the protein shells of several viruses, including picornaviruses and many retroviruses, are assembled from polyproteins. The liberation of individual structural proteins is essential for formation of mature particles.

The alterations in the structure of the virus particle and their functional correlates are best understood for small RNA viruses, such as the picornavirus poliovirus. A single cleavage to liberate VP4 and VP2 from VP0 converts noninfectious provirions to mature infectious virions (Fig. 13.4). As the viral proteases are not incorporated into particles, VP0 cleavage may be catalyzed by a specific feature of the virion itself, with internal genomic RNA participating in the reaction. The structural changes induced by such maturation cleavage can be described in great detail, for the structures of mature virions and empty particles in which VP0 have been determined at high resolution. Cleavage of VP0 allows the extensive internal structures of the particle (Fig. 4.13C), to be established in its final form. Cleavage of VP0 therefore allows additional protein-protein interactions that make important contributions to the stability of the virion.

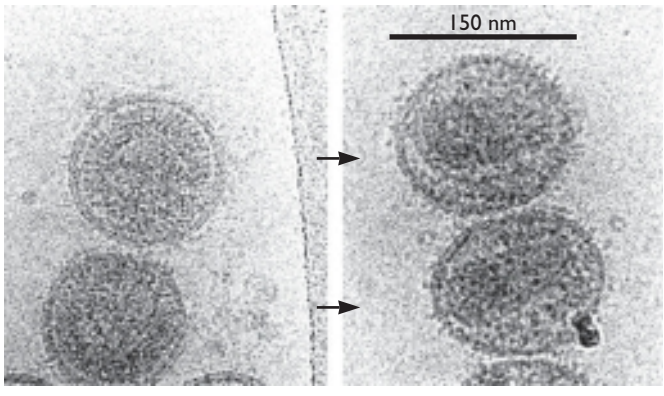
Cleavage of VP0 to VP4 and VP2 is also necessary for release of the RNA genome into a new host cell. The conformational transitions that mediate entry of the genome following attachment of the virus to its receptor are not fully understood. However, many alterations that impair receptor binding and entry map to just those regions of the capsid proteins that participate in the structures that adopt their final organization only upon VP0 cleavage. Cleavage of VP0 therefore not only further stabilizes the virion, but also "spring-loads" it for the conformational

transitions that take place during entry and release of the genome.

Following release of most retrovirus particles, the Gag polyprotein is processed by the viral protease, concomitant with substantial morphological and conformational rearrangements (Fig 13.23; Box 13.9). However, the mature, infectious particles of members of the *Spumaretrovirinae*, which resemble hepadnaviruses in some aspects of their infectious cycles, contain uncleaved Gag.

Processing of Gag plays an essential part in the mechanisms by which most infectious retroviruses are assembled and released. As we have seen, interactions among Gag polyproteins, and of their NC and MA domains with the viral RNA and the plasma membrane, respectively, build and organize assembling retrovirus particle. Efficient and orderly assembly also depends on "spacer" peptides that are removed during proteolysis. Furthermore, the membrane-binding signal of MA is exposed when MA is part of Gag, but is blocked by a C-terminal α-helix of MA in the mature protein. It is therefore very unlikely that retrovirus particles could be constructed correctly from mature Gag proteins. Indeed, alterations that increase the catalytic activity of the viral protease inhibit budding and production of infectious particles, indicating that premature processing of the polyproteins is detrimental to assembly. On the other hand, the covalent connection of the virion proteins that is so necessary during assembly is incompatible with release of the virion core following fusion of the viral envelope with the membrane of a new host cell. Such covalent linkage also precludes efficient activity of virion enzymes, which are incorporated as Gag-Pol proteins. In some virions, including those of Moloney murine leukemia virus, the protease also removes a short C-terminal segment of the cytoplasmic tail of the TM envelope protein to activate the fusionogenic activity of TM. The retroviral proteases that sever such connections therefore are absolutely necessary for production of infectious virions, even though they are dispensible for assembly.

Figure 13.23 Morphological rearrangement of retrovirus particles upon proteolytic processing of the Gag polyprotein. These two cryo-electron micrographs show the maturation of human immunodeficiency virus type 1 virions. **(Left)** The immature particles contain a Gag polyprotein layer below the viral membrane and its external spikes. **(Right)** Processing of Gag converts such particles to mature virions with elongated internal capsids. Courtesy of T. Wilk, European Molecular Biology Laboratory.



The retroviral proteases belong to a large family of enzymes with two aspartic acid residues at the active site (aspartic proteases). The viral and cellular members of this family are similar in sequence, particularly around the active site, and are also similar in three-dimensional structure. All aspartic proteases contain an active site formed between two lobes of the protein, each of which contributes a catalytic aspartic acid. The retroviral proteases are homodimers, in which each monomer corresponds to a single lobe of their cellular cousins. Consequently, the active site is formed only upon dimerization of two identical subunits. This property undoubtedly helps avoid premature activity of the protease within infected cells, in which the low concentration of their polyprotein precursors mitigates against dimerization. Indeed, dimerization of the protease appears to be rate limiting for maturation of virions. Fusion of the protease to the NC domain of Gag also inhibits dimerization. Consequently, synthesis of the protease as part of a polyprotein precursor not only allows incorporation of the enzyme into assembling virions, but also contributes to regulation of its activity. These properties raise the question of how the protease is activated, a step that requires its cleavage from the polyprotein. Polyproteins containing the protease (e.g., made in bacteria) possess some activity, sufficient to liberate fully active enzyme at a very low rate *in vitro*. It is therefore thought that such activity of the polyproteins initially releases protease molecules within the particle. Furthermore, it has been shown, using Gag-Pol proteins yielding distinguishable cleavage products, that the initial proteolytic cleavages are intramolecular. The high local concentrations of protease molecules within the assembling particle would facilitate subsequent dimerization of protease molecules to form the fully active enzyme.

Cleavage of Precursor Proteins

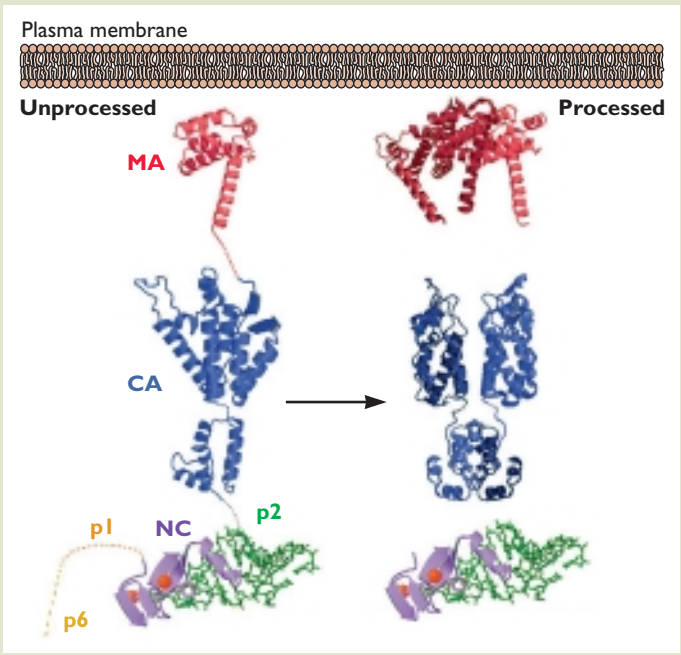
Like its retroviral counterpart, the adenoviral protease converts noninfectious particles to infectious virions, in this case by cleavage at multiple sites within six virion proteins (Fig. 13.8). Although the adenoviral enzyme does not process polyprotein precursors, the cleavage of so many proteins also appears to alter protein-protein interactions necessary for virion assembly, in preparation for early steps in the next

BOX 13.9 DISCUSSION

Model for refolding of the human immunodeficiency virus type 1 CA protein on proteolytic processing of Gag

The model for the radial organization of the human immunodeficiency virus type 1 Gag polyprotein (left) is based on cryo-electron micrographs like that in Fig. 13.3. The three-dimensional structures of the processed proteins, the MA trimer (red), the CA dimer (blue), and monomeric NC (violet) bound to the SL3 packaging signal (green), shown on the right, are derived from high-resolution structures discussed in this and preceding chapters.

In the X-ray crystal structure of the N-terminal portion of mature CA (right), the charged N terminus is folded back into the protein by a β hairpin formed by amino acids 1 to 13 and forms a buried salt bridge with the carboxylate of Asp51. The lack of a charged N terminus prior to cleavage of CA from MA, and the steric difficulties of burying the N terminus of CA attached to an MA extension (left), indicate that the β hairpin and buried salt bridge can form only after proteolytic cleavage. Furthermore, the viral protease recognizes the cleavage site between MA and CA in an extended conformation. As the N-terminal β hairpin of mature CA forms a CA-CA interface, it has been proposed that proteolytic cleavage and the consequent refolding of the N terminus of CA facilitate the rearrangements to form the conical core during maturation of virus particles. The inhibition of core assembly and formation of infectious viral particles *in vivo* by alteration of amino acids in this interface support this model.



Conformational changes in segments of Gag during assembly may regulate proteolytic processing. For example, efficient cleavage to liberate human immunodeficiency virus type 1 NC depends on the binding of NC to RNA, at least *in vitro*. Figure courtesy of T. L. Stemmler and W. Sundquist, University of Utah.

von Schwedler, U. K., T. L. Stemmler, V. Y. Klishko, S. Li, K. H. Albertine, D. R. Davis, and W. I. Sundquist. 1998. Proteolytic refolding of the HIV-1 capsid protein amino-terminus to facilitate viral core assembly. *EMBO J.* **17:**1555–1568.

infectious cycle. The enzyme is a cysteine protease containing an active-site cysteine and two additional cysteines, all highly conserved. One mechanism by which its activity is regulated is by interaction with a small peptide, a product of cleavage of the virion protein pVI, or with pVI itself (Fig. 13.24). The pVI peptide binds covalently via a disulfide bond to the proteases both *in vitro* and in virions to increase the catalytic efficiency of the enzyme over 1,000-fold. Its binding site is not located close to the active site of the protease, suggesting that pVI peptide induces or stabilizes an active-site conformation that is optimal for catalysis.

Other Maturation Reactions

Newly assembled virus particles appear to undergo few maturation reactions other than proteolytic processing. However, the trimming of certain oligosaccharides, or formation of disulfide bonds, is required in some cases. Moreover, a surprising extracellular assembly process has been identified recently (Box 13.10).

Terminal sialic acid residues are removed from the complex oligosaccharides added to the envelope HA and NA glycoproteins of influenza A virus during their transit to the plasma membrane. The influenza A virus receptor is sialic acid, which is specifically recognized by the HA protein. Consequently, newly synthesized virus particles have the potential to aggregate with one another, and with the surface of the host cell, by binding of an HA molecule on one particle to a sialic acid present in an envelope protein of another particle, or in cell surface proteins. Such aggregation is observed when the viral neuraminidase is inactivated. The neuraminidase eliminates such binding of newly synthesized virions to one

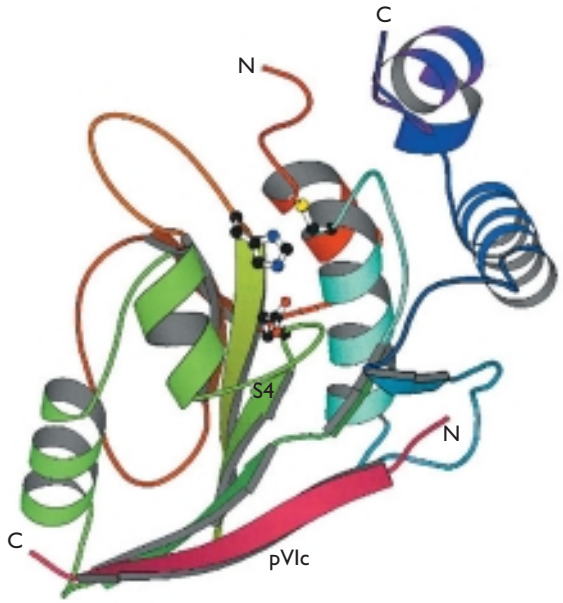


Figure 13.24 Three-dimensional structure of the human adenovirus type 2 protease bound to the pVI peptide. This structure, determined by X-ray crystallography of the complex and shown as a ribbon diagram, allowed identification of the active site of the enzyme (at which side chains are shown) by comparison with the secondary and tertiary structures of cellular cysteine proteases. This structure closely resembles that of papain, suggesting that the adenoviral protease employs the same catalytic mechanism. The β-strand formed by the activating pVIc peptide (deep red) is covalently linked to the enzyme by a disulfide bond, but also makes many noncovalent interactions. Adapted from J. Ding et al., *EMBO J.* **15:**1778–1783, 1995, with permission. Courtesy of W. Mangel, Brookhaven National Laboratory.

another and to cell surface proteins. The activity of this enzyme, which removes terminal sialic acid residues from oligosaccharide chains, is essential for effective release of progeny virus particles from the surface of a host cell. This property has been exploited to develop new drugs (e.g., Tamiflu) designed specifically to inhibit the viral neuraminidase.

The capsids of nonenveloped papillomaviruses, which are built from 72 pentamers of the major structural protein L1, are stabilized by intermolecular disulfide bonds between specific L1 cysteine residues. This protein does not travel the secretory pathway, raising the question of how such cysteines become oxidized. When the human papillomavirus type 16 (or 18) L1 and the minor capsid (L2) proteins are made in mammalian cells, they assemble to form particles that are less stable and less infectious than mature capsids, and that lack disulfide bonds. Such bonds form spontaneously at a low rate, when immature particles are incubated 37°C, and more quickly in the presence of oxidizing agents. Disulfide bond formation is accompanied by increased stability and infectivity, and the appearance of more regularly structured particles. Papillomaviruses are thought to be released slowly during natural infections, as the outer layers of the epithelia in which they replicate are shed. It is therefore likely that newly assembled capsids are exposed to an oxidizing environment for a considerable period (several days) prior to release.

Cell-to-Cell Spread

The raison d'être of all progeny virions is to infect a new host cell in which the infectious cycle can be repeated. Many viruses are released as free particles by the mechanisms described in preceding sections, and must travel within the host until they encounter a susceptible cell. The new host cell may be an immediate neighbor of that originally infected, or a distant cell reached via the circulatory or nervous systems of the host. Virions are designed to withstand such intercellular passage, but they are susceptible to several host defense mechanisms that can destroy virus particles (Volume II, Chapter 4). Localized release of virus particles only at points of contact between an infected cell and its uninfected neighbor(s) can minimize exposure to these host defense mechanisms. Furthermore, some viruses can spread from one cell to another by mechanisms that circumvent the need for release of progeny virions into the extracellular environment.

In some cases, viruses can be transferred directly from an infected cell to its neighbors (Box 13.11), a strategy that avoids exposure to host defense mechanisms targeted against extracellular virus. Such cell-to-cell spread, which is defined operationally as infection that still occurs when released virus particles are neutralized by addition of antibodies, depends on the viral fusion machinery. In the case of herpes simplex virus type 1, the glycoproteins gB, gH, and gL, which promote fusion during entry, are required, as is gD. The latter protein binds to the cell surface protein nectin-1, which is localized to cell-cell junctions. Two additional proteins are also essential for efficient cell-to-cell spread, but have no known role in entry of extracellular particles: mutant viruses that lack the gE or gI genes form only small plaques when transfer of free virus particles from one cell to another is prevented. They are also defective for both lateral spread of infection in polarized epithelial cells and spread of infection from an axon terminal to an uninfected neuron in animals. Such cell-to-cell spread of herpesviruses is thought to occur at specialized junctions, such as tight junctions of epithelial cells, and sites of synaptic contact between individual neurons. Experiments with herpes simplex virus gE mutants in polarized cells demonstrate that in the absence of gE, virions do not accumulate at lateral surfaces with tight junctions, and mutations that

BOX 13.10 EXPERIMENTS

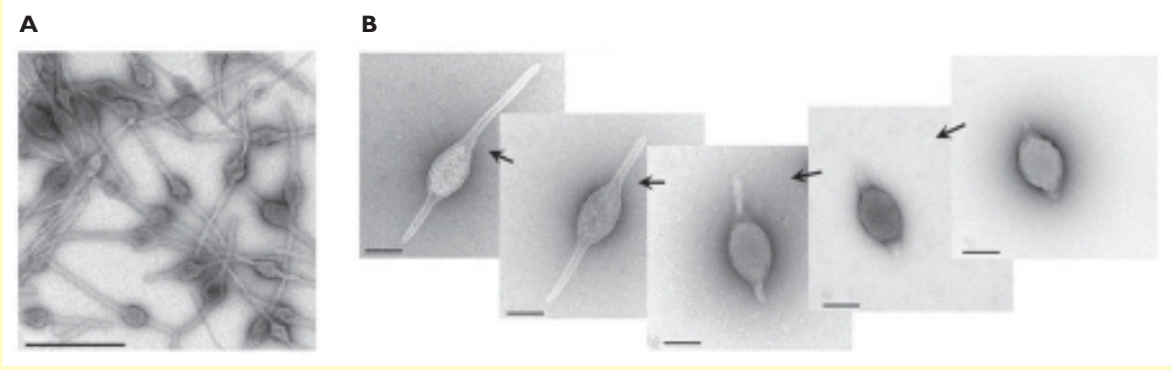
A notable example of virus maturation: extracellular assembly of specific structures

Acidianus two-tailed virus was discovered in an acidic hot spring (pH 1.5, 85 to 93°C) at Pozzuoli, Italy, where it replicates in the thermophilic archaeon *Acidianus convivator*. The virus particles isolated from this source have a lemon-shaped body with filamentous tails of different length protruding from each end **(A)**. However, when the virus replicated in host cells grown in culture at 75°C, the released particles lacked such tails **(B)**. Remarkably, tails formed over 1 week when such particles were incubated at 75°C in the **absence** of host cells **(B, right to left)**. Moreover, this extracellular assembly reaction was complete in less than 1 h when particles were incubated at the temperatures optimal for host cell growth, 85 to 90°C.

Although the morphological changes that accompany maturation of virus particles are well documented (see the text), *Acidianus* two-tailed virus represents the first example of extracellular assembly. This property implies that the tailless particles released from host cells contain all the components and information necessary for tail assembly.

Häring, M., G. Vestergaard, R. Rachel, L. Chen, R. A. Garrett, and D. Prangishvili. 2005. Independent virus development outside a host. *Nature* **436:**1101–1102.

Electron micrographs of *Acidianus* two-tailed virus particles isolated from a hot spring (A) or released from host cells infected in culture at 75°C and maintained in cell-free medium at 75°C for 0, 2, 5, 6, and 7 days (B, right to left). Scale bars, 0.5 μm (A) and 0.1 μm (B). From M. Häring et al., *Nature* **436:**1101–1102, 2005, with permission. Courtesy of David Prangishvili, Institut Pasteur, Paris, France.



block trafficking of gE from the *trans*-Golgi network to cell junctions impair cell-to cell spread. Exactly how gE-gI glycoprotein oligomers promote cell-to-cell spread via these specialized junctions is not yet clear.

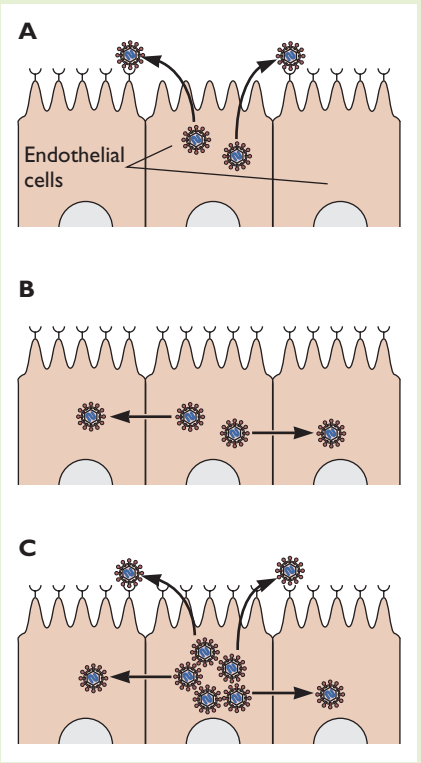
Recent studies indicate that direct cell-to-cell spread is the predominant mechanism for transmission of human immunodeficiency virus type 1 and other retroviruses. Specialized intercellular structures called **virological synapses** assemble when an infected cell contacts an uninfected neighbor. Virological synapses form at lipid raft regions of the plasma membrane that are enriched in cholesterol and sphingomyelin (see Chapter 12), and also the sites of release of viral particles by budding into the extracellular milieu. The viral Env protein, as well as the CD4 and CxCr4 coreceptors, accumulate in virological synapses, and Env-CD4 interactions are required for intercellular transfer of human immunodeficiency virus type 1. This mode of transmission is some 2 to 3 orders of magnitude more efficient than infection via entry of extracellular virions. It depends on formation of stable filopodia between uninfected and infected cells as a result of strong association of the viral Env protein of released virus particles with its receptor on the uninfected cells. Virus particles then travel toward the uninfected cell along the outer surface of the filopodial bridge.

Persistent measles virus (a paramyxovirus) infection of the brain is associated with subacute sclerosing panencephalitis (Volume II, Appendix A, Fig. 14). Spread of this virus between neurons occurs by a mechanism different from that in nonneuronal tissue: little infectious virus can be recovered from brain tissue of patients with this disease, although the genomic RNA and viral proteins are present. Indeed, budding of virus particles does not take place from the surfaces of infected mouse or human neurons in culture, which contain nucleocapsids accumulating at presynaptic membranes. Nor does spread of measles virus between cultured neurons require the viral receptors. Rather, cell-cell contact and the fusion protein are necessary. In neurons, measles virus therefore spreads without release and attachment to infected cells of free

BOX 13.11 BACKGROUND

Extracellular and cell-to-cell spread

Many viruses spread from one host cell to another as extracellular virions released from an infected cell **(A)**. Such extracellular dissemination is necessary to infect another naive host. Some viruses, notably alphaherpesviruses and some retroviruses, can also spread from cell to cell without passage through the extracellular environment **(B)** and can therefore spread by both mechanisms **(C)**.



virus particles: rather, it spreads from cell to cell, perhaps through synapses.

There are other examples of more radical mechanisms of transfer. In astrocyctes (supporting cells of the central nervous system), measles virus spreads by inducing the formation of syncytia, sheets of neighboring cells fused to one another (Fig. 13.25A). Certain cell types infected by human immunodeficiency virus type 1 also form syncytia when they would not normally do so (Fig. 13.25B). Perhaps most unusual is the direct transfer of vaccinia virus from an infected cell to its neighbors, by the novel actin-containing structures induced by the virus, as described above.

The production of "decoys," noninfectious particles released in large quantities, is one alternative strategy to avoid host defense mechanisms during transmission. The vast majority of particles detected in hepatitis B virus-infected humans are empty. Another strategy would be to disguise virus particles with normal products of a host cell. Some viral envelopes retain cellular proteins, such as major histocompatibility complex class II proteins and the adhesion receptor Icam-1, in their membranes. The latter protein substantially increases the infectivity of human immunodeficiency virus type 1 particles. However, the importance of such a "wolf in sheep's clothing" strategy for the spread of a virus from one cell to another in the host has yet to be documented.

Perspectives

The assembly of even the simplest virus is a complex process, in which multiple reactions must be completed in the correct sequence and coordinated in such a way that the overall pathway is irreversible. These requirements for efficient production and release of stable structures must be balanced with the fabrication of virus particles primed for ready disassembly at the start of a new infectious cycle. The integration of information collected by the application of structural, imaging, biochemical, and genetic methods of analysis has allowed an outline of the dynamic processes of assembly, release, and maturation for many viruses. Despite the considerable structural diversity of virions, the repertoire of mechanisms for successful completion of the individual reactions is limited. Furthermore, we can identify common mechanisms that ensure that assembly proceeds efficiently and irreversibly, or that resolve the apparent paradox of great particle stability during assembly and release but facile disassembly at the start of the next infectious cycle. These mechanisms include high concentrations of virion components at specific sites within the infected cell, and proteolytic cleavage of virion proteins at one or more steps in the production of infectious particles. Indeed, for some smaller viruses, the structural changes that accompany the production of infectious virions from noninfectious precursor particles can be described in atomic detail. Such information has revealed unanticipated relationships between structures that stabilize virions, and interactions that prime them for conformational rearrangements during entry.

On the other hand, the pathways for assembly, production, and release of even the simplest virions cannot be fully described. These reactions are difficult to study in infected cells, and even the simplest proved more difficult to reconstitute *in vitro* than originally anticipated. The latter property emphasizes the crucial contributions to virus assembly that can be made by cellular proteins that assist

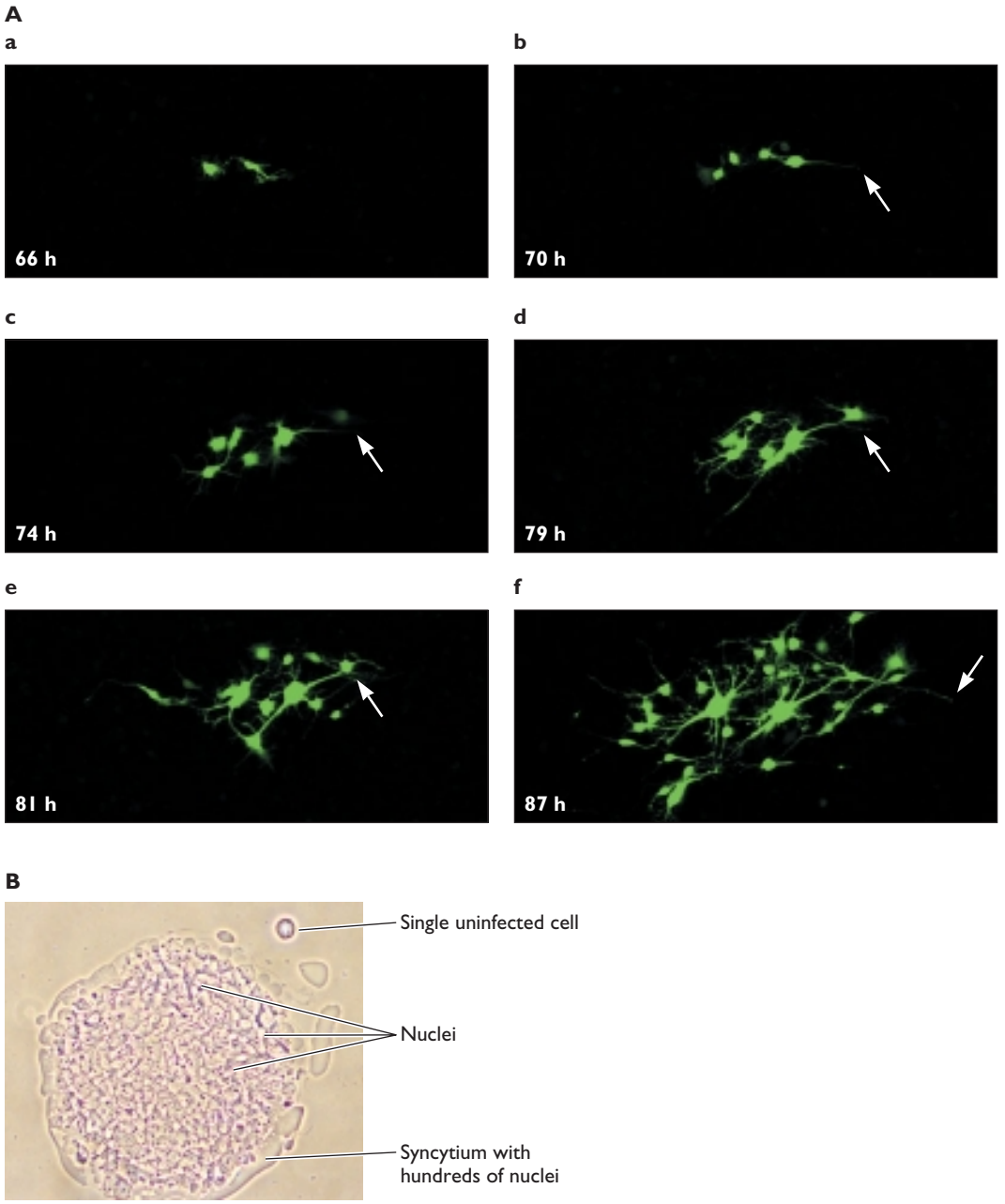


Figure 13.25 Formation of syncytia. (A) Cell-to-cell spread by measles virus. Human astrocytoma cells were infected at low multiplicity with a recombinant measles virus carrying a green fluorescent protein-coding sequence in its genome. The autofluorescence of this protein identifies infected cells (a) and allows spread of the virus to be monitored in living cells. With increasing time, the virus spreads to cells neighboring those initially infected and can be clearly seen in the processes connecting the cells that become infected (b to f). The arrows point to an extended astrocyte process of a newly infected cell (b), the weak autofluorescence of a nucleus of a cell in a very early phase of infection (c), the nucleus from the same cell 5 and 7 h later (d and e, respectively), and an extended astrocytic process issuing from the cell shown in panels d and e (f). From W. P. Duprex et al., *J. Virol.* **73:**9568–9575, 1999. Courtesy of W. D. Duprex, Queen's University, Belfast, United Kingdom. **(B)** Syncytia formed by human immunodeficiency virus type 1 in a T-cell line. The photograph shows a large syncytium of SupT1 cells (a $CD4^+$ T-lymphotropic cell line) that had been infected with a viral vector that expresses the *env* gene. Large quantities of this Env protein accumulate at the cell surfaces mediating fusion. A single cell is indicated for comparison. Courtesy of Matthias Schnell, Philip McKenna, and Joseph Kulkosky, Thomas Jefferson University School of Medicine, Philadelphia, PA.

protein folding and oligomerization (chaperones), or that covalently modify virion proteins. Historically, assembly reactions have received less attention than mechanisms of viral gene expression or replication of viral genomes. However, the development of new structural and imaging methods, coupled with the experimental power and flexibility provided by modern molecular biology, has revitalized investigation of the crucial processes of assembly, release, and maturation of virus particles. This renaissance has been further stimulated by the success of therapeutic agents designed to inhibit virus-specific reactions crucial for the production of infectious particles. Consequently, the advances in our understanding of assembly and egress illustrated in this chapter seem certain to continue at an accelerated pace.

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APPENDIX

Structure, Genome Organization, and Infectious Cycles

Adenoviruses

Family *Adenoviridae*

Genus	Type species
Mastadenovirus	Human adenovirus C
Aviadenovirus	Fowl adenovirus A

Human serotypes are very widespread in the population. Infection by these viruses is often asymptomatic but can result in respiratory disease in children (members of subgroup B and C), conjunctivitis (members of subgroup B and D), and gastroenteritis (subgroup F serotypes 40 and 41). Human adenoviruses 40 and 41 are the second leading cause (after rotaviruses) of infantile viral diarrhea. These viruses share capsid morphology and linear double-stranded DNA genomes, but the members of the two genera differ in size, organization, and coding sequences. The *Mastadenovirinae* comprise 51 human adenoviruses and adenoviruses of other mammals, including mice, sheep, and dogs, and some are oncogenic in rodents. Study of human adenovirus transformation of cultured cells has provided fundamental information about mechanisms that control progression through the cell cycle and oncogenesis. Characteristic features of replication of these viruses include precise temporal control of viral gene expression and an unusual mechanism of initiation of viral DNA synthesis (protein priming). Mastadenoviral genomes also include genes transcribed by cellular RNA polymerase III.

Figure 1 Structure and genome organization of human adenovirus type 5. (A) Virion structure. The electron micrograph shows a negatively stained human adenovirus type 5 particle (courtesy of M. Bisher, Princeton University, Princeton, NJ). Bar = 50 nm. **(B) Genome organization.** The DNA genome length is 36 to 38 kbp. Green and tan arrows represent RNA polymerase II and III transcription products, respectively. Hatched lines show splicing of the major late (ML) tripartite leader. Ori, origin of replication.

Figure 2 Single-cell reproductive cycle of human adenovirus type 2. The virus attaches to a permissive human cell via interaction between the fiber and (with most serotypes) the coxsackie-adenovirus receptor on the cell surface. The virus enters the cell via endocytosis **(1** and **2)**, a step that depends on the interaction of a second virion protein, penton base, with a cellular integrin protein (red cylinder). Partial disassembly takes place prior to entry of particles into the cytoplasm **(3)**. Following further uncoating, the viral genome associated with core protein VII is imported into the nucleus **(4)**. The host cell RNA polymerase II system transcribes the immediate-early E1A gene **(5)**. Following alternative splicing and export of E1A mRNAs to the cytoplasm **(6)**, E1A proteins are synthesized by the cellular translation machinery **(7)**. These proteins are imported into the nucleus **(8)**, where they regulate transcription of both cellular and viral genes. The larger E1A protein stimulates transcription of the viral early genes by cellular RNA polymerase II **(9a)**. Transcription of the VA genes by host cell RNA polymerase III also begins during the early phase of infection **(9b)**. The early pre-mRNA species are processed, exported to the cytoplasm **(10)**, and translated **(11)**. These early proteins include the viral replication proteins, which are imported into the nucleus **(12)** and cooperate with a limited number of cellular proteins in viral DNA synthesis **(13)**. Replicated viral DNA molecules can serve as templates for further rounds of replication **(14)** or for transcription of late genes **(15)**. Some late promoters are activated simply by viral DNA replication, but maximally efficient transcription of the major late transcription unit (Fig. 1, ML) requires the late IVa2 and L4 proteins. Processed late mRNA species are selectively exported from the nucleus as a result of the action of the E1B 55-kDa and E4 Orf6 proteins **(16)**. Their efficient translation in the cytoplasm **(17)** requires the major VA RNA, VA RNA-I, which counteracts a cellular defense mechanism, and the late L4 100-kDa protein. The latter protein also serves as a chaperone for assembly of trimeric hexons as they and the other structural proteins are imported into the nucleus **(18)**. Within the nucleus, capsids are assembled from these proteins and the progeny viral genomes to form noninfectious immature virions **(19)**. Assembly requires a packaging signal located near the left end of the genome, as well as the IVa2 and L4 22/33-kDa proteins. Immature virions contain the precursors of the mature forms of several proteins. Mature infectious virions are formed **(20)** when these precursor proteins are cleaved by the viral L3 protease, which enters the virion core. Progeny virions are released **(21)**, usually upon destruction of the host cell via mechanisms that are not well understood.

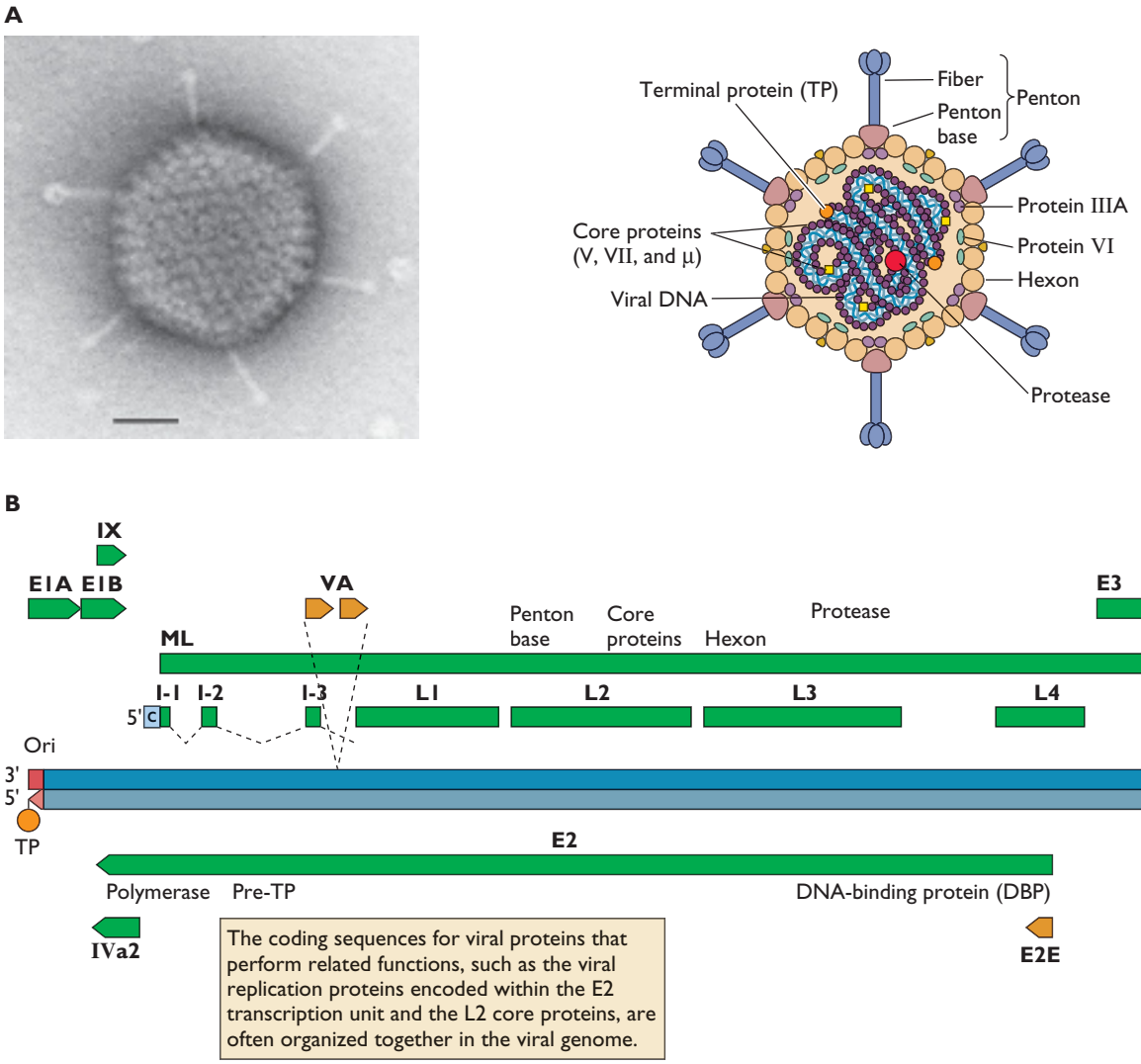

Figure 1 Structure and genome organization of human adenovirus type 5.

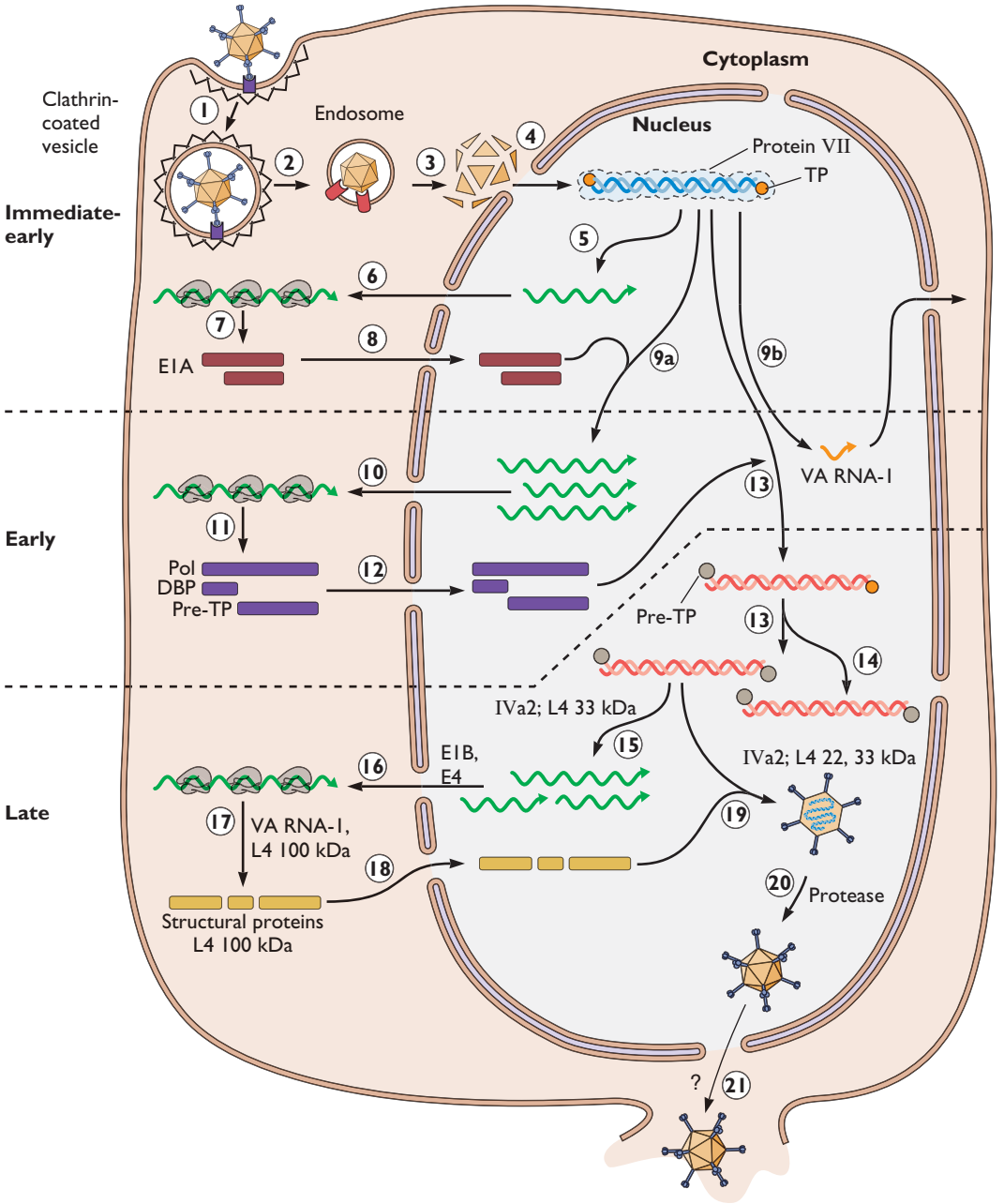

Figure 2 Single-cell reproductive cycle of human adenovirus type 2.

Hepadnaviruses

Family *Hepadnaviridae*

Genus	Type species
Orthohepadnavirus	Human hepatitis B virus
Avihepadnavirus	Duck hepatitis B virus

The hepadnaviruses all show very narrow host specificity and marked tropism for liver tissue. Hepadnaviruses can replicate following inoculation of primary hepatocytes with virus-containing serum, but most hepadnaviruses cannot be propagated in established cell lines. However, replication can be initiated by transfection of liver cell lines with cloned viral DNA. Hepadnaviruses replicate via an RNA intermediate and, like the retroviruses, encode a reverse transcriptase. Both families are included in the group called **retroid viruses.** Natural infections may be acute or persistent, depending on host age, inoculum dose, and other (undefined) factors that influence the host immune response. Approximately 5% of the world's population has been infected with human hepatitis B virus; 250 million to 300 million are persistently infected. Persistent infection with the orthohepadnaviruses but not the avihepadnaviruses confers an increased risk for hepatocellular carcinoma.

Figure 3 Structure and genome organization of orthohepadnaviruses. (A) Virion structure. The electron micrograph shows negatively stained woodchuck hepatitis virus, a mammalian hepadnavirus related to human hepatitis B virus (courtesy of W. Mason and T. Gales, Fox Chase Cancer Center, Philadelphia, PA). **(B) Genome organization**. The (−) strand of the human hepatitis virus DNA genome is 3,227 nucleotides long.

Figure 4 Single-cell reproductive cycle of hepatitis B virus. The virion attaches to a susceptible hepatocyte **(1)** through recognition of a cell surface receptor(s) that has yet to be identified. The mechanism of virus uptake **(2)** is also unknown, and repair of the gapped (+) DNA strand is accomplished **(3)** by as yet unidentified enzymes. The DNA is translocated to the nucleus **(4)**, where it is found in a covalently closed circular form called CCC DNA. The (−) strand of such CCC DNA is the template for transcription by cellular RNA polymerase II **(5)** of a longer-than-genome-length RNA called the pregenome and shorter, subgenomic transcripts (Fig. 3B), all of which serve as mRNAs. Viral mRNAs are transported from the nucleus **(6)**. Subgenomic viral mRNAs encoding the viral envelope protein are translated by ribosomes bound to the endoplasmic reticulum (ER) **(7)**, and the proteins destined to become surface antigens (HBsAg) in the viral envelope enter the secretory pathway. The pregenome RNA is translated to produce capsid protein **(8)** and, at low efficiency, the 90-kDa P protein **(9),** which possess reverse transcriptase activity. P then binds to a specific site, the packaging signal, at the 5′ end of its own transcript, where viral DNA synthesis is eventually initiated. Concurrently with capsid formation, the RNA-P protein complex is packaged **(10)** and priming of DNA replication occurs from a tyrosine residue on the polymerase **(11)**. Following synthesis of a few nucleotides, there is a template exchange in which the 3′ end of the pregenome is engaged by the polymerase and DNA synthesis continues to the 5′ end of this RNA template **(12)**. At early times after infection, the DNA is transported to the nucleus **(13)**, where the process is repeated, resulting in the eventual accumulation of 10 to 30 molecules of CCC DNA and a concomitant increase in viral mRNA concentrations. At later times, and possibly as a conseqence of the accumulation of sufficient envelope proteins, mature DNA-containing nucleocapsids acquire envelopes as they bud into the ER **(14)**, where viral maturation is completed. Progeny enveloped virions are released from the cell by exocytosis **(15)**.

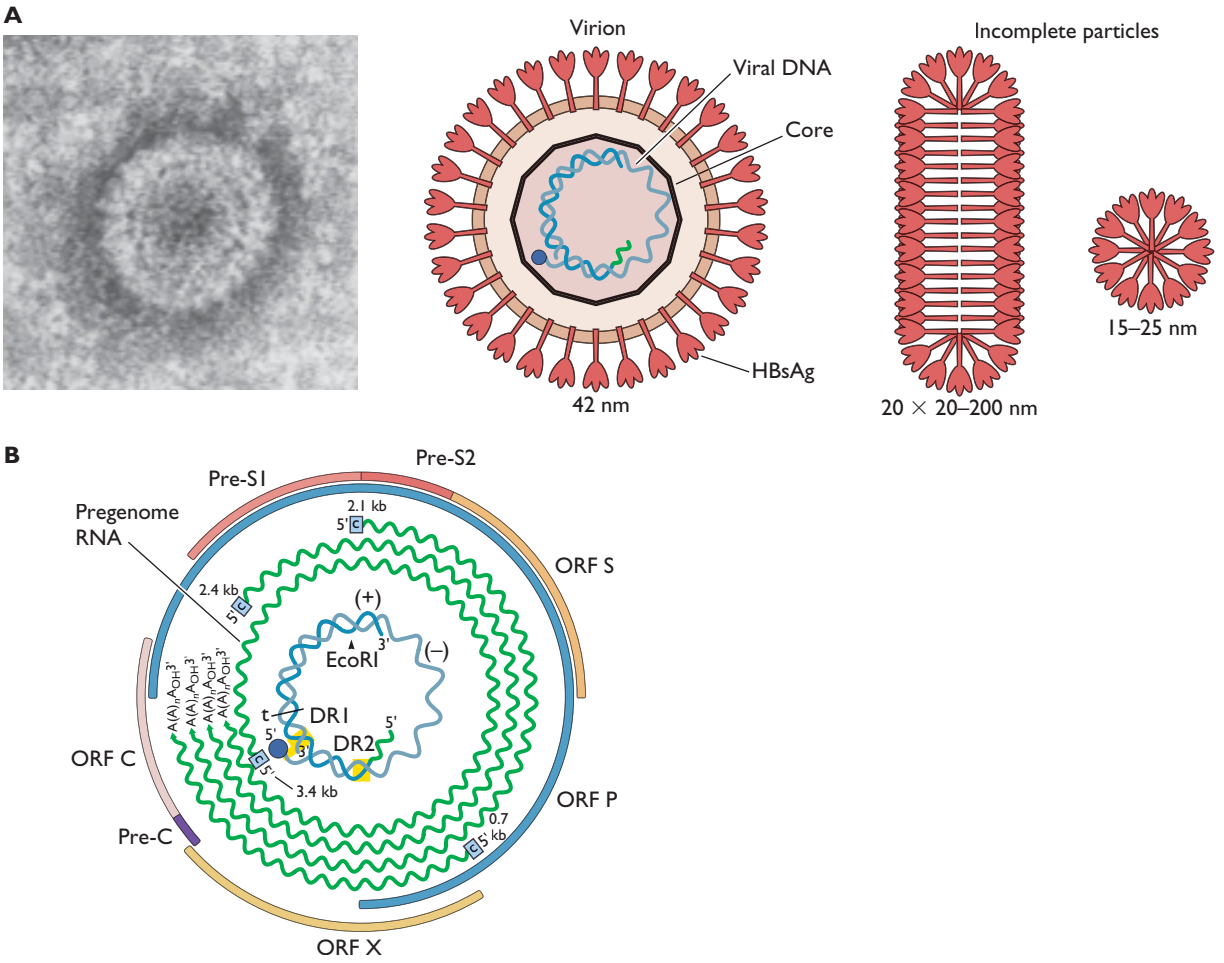

Figure 3 Structure and genome organization of orthohepadnaviruses.

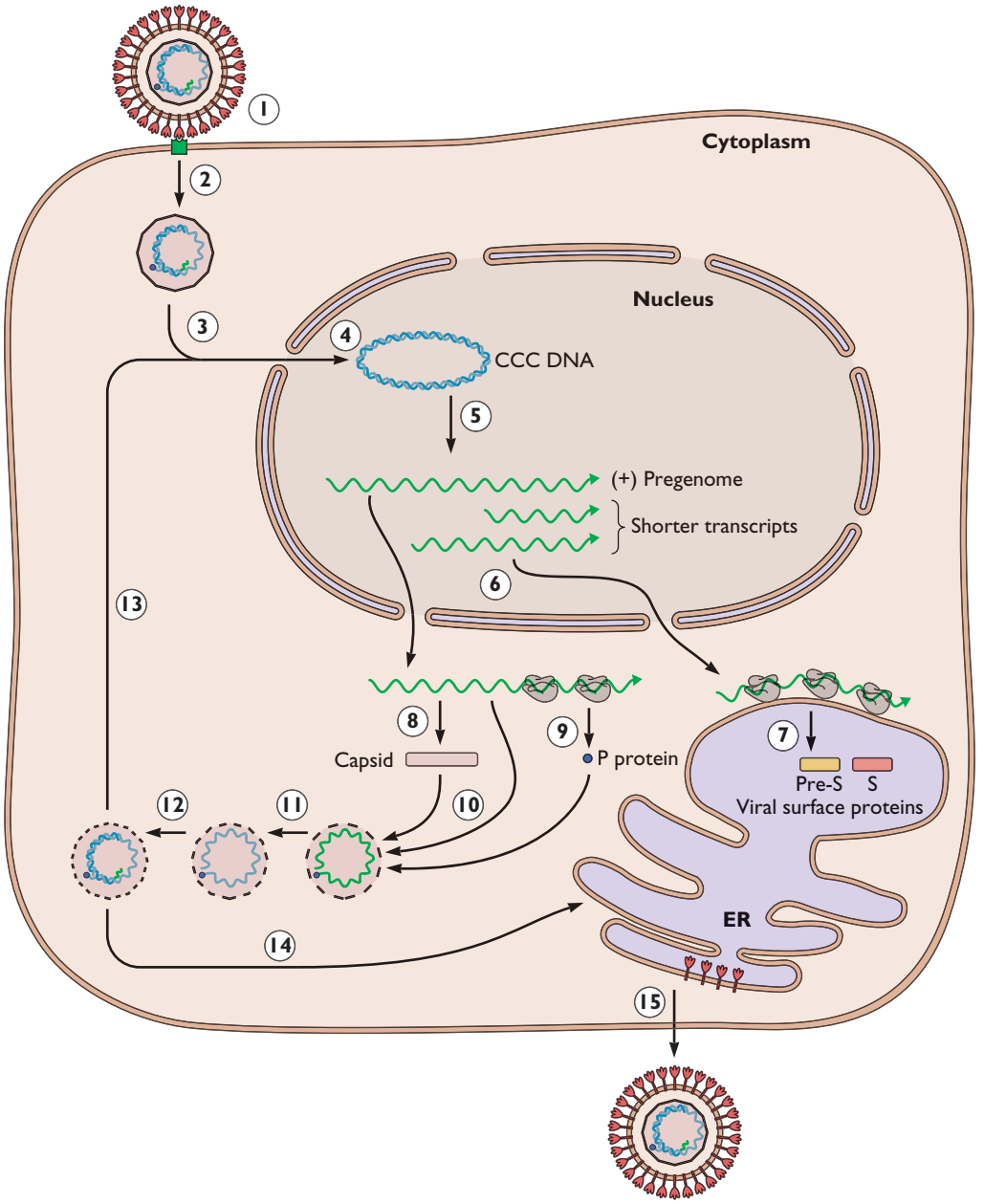

Figure 4 Single-cell reproductive cycle of hepatitis B virus.

Family *Herpesviridae*

Genus	Type species
Subfamily *Alphaherpesvirinae*	
Simplexvirus	Human herpes simplex virus type 1
Varicellovirus	Varicella-zoster virus
Subfamily *Betaherpesvirinae*	
Cytomegalovirus	Human cytomegalovirus
Muramegalovirus	Murine cytomegalovirus 1
Roseolovirus	Human herpesvirus 6
Subfamily *Gammaherpesvirinae*	
Lymphocryptovirus	Epstein-Barr virus
Rhadinovirus	Herpesvirus saimiri
Undefined subfamily: channel catfish virus, oyster herpesvirus	

The family *Herpesviridae* comprises over 120 viruses that infect a wide range of vertebrates and at least one invertebrate (the oyster). While some herpesviruses have broad host ranges, most are restricted to infection of a single species and spread in the population by direct contact or aerosols. The hallmark of herpesvirus infections is the establishment of a lifelong, latent infection that can reactivate to cause one or more rounds of disease. Many herpesvirus infections are not apparent, but if the host's immune defenses are compromised, infections can be devastating. Humans are the natural hosts of eight different herpesviruses: herpes simplex virus types 1 and 2, varicella-zoster virus, Epstein-Barr virus, human cytomegalovirus, and human herpesviruses 6, 7, and 8 (the last of these is also known as Kaposi's sarcoma-associated herpesvirus). Agricultural pathogens include the alphaherpesviruses (pseudorabies virus, bovine herpes virus type 1, equine herpesvirus type 1, and avian Marek's disease virus types 1 and 2) and the gamma herpesviruses (bovine herpesvirus 4 and equine herpesvirus 2).

Figure 5 Structure and genome organization of alphaherpesviruses. (A) Virion structure. The electron micrograph shows a negatively stained pseudorabies viron (courtesy of T. Mettenleiter, Federal Research Center for Virus Diseases of Animals, Insel Riems, Germany). **(B) Genome organization.** The herpes simplex virus type 1 genome can "isomerize" or recombine via the inverted repeat sequences (TRL and IRL, or IRS and TRS) such that all populations consist of four equimolar isomers in which the UL and US sequences are inverted with respect to each other. There are at least 84 open reading frames in this genome, as well as three replication origins (Ori). The approximate locations of some genes are noted. Genes encoding related functions have the same color shading to illustrate their dispersed distribution in herpesviral genomes.

Figure 6 Single-cell reproductive cycles of herpes simplex virus type 1. (A) Productive infection. Virions bind to the extracellular matrix (heparan sulfate or chondroitin sulfate proteoglycans) via gB and gC **(1)**. Another viral membrane protein (gD) interacts with a second cellular receptor (nectin-1) **(2)**. Viral and plasma membrane fusion is then mediated by viral membrane glycoproteins (gD, gB, gH, and gL) **(3)**. On membrane fusion, tegument proteins and the nucleocapsid are released into the cytoplasm **(4)**. Viral nucleocapsids attach to microtubules and are transported to the nucleus **(5a)**. Certain tegument proteins are transported to the nucleus **(5b)**. Others, such as Vhs, remain in the cytoplasm **(6)**. Viral nucleocapsid docks at the nuclear pore **(7)**, releasing DNA into the nucleus. VP16 **(8)** interacts with host transcription proteins to stimulate transcription of immediate-early genes by host cell RNA polymerase II. Some immediate-early mRNAs are spliced and transported to the cytoplasm **(9)** where they are translated. The immediate-early proteins (α proteins) are transported to the nucleus, where they activate transcription of early genes and regulate transcription of immediate-early genes **(10)**. Early-gene transcripts, which are rarely spliced, are transported to the cytoplasm **(11)** where they are translated. The early proteins (β proteins) function primarily in DNA replication and production of substrates for DNA synthesis. Some β proteins are transported to the nucleus **(12)**, and some function in the cytoplasm. Viral DNA synthesis is initiated from viral origins of replication **(13)**. DNA replication and recombination produces long, concatemeric DNA, the template for late-gene expression **(14)**. Most late mRNAs are not spliced, but nevertheless are transported to the cytoplasm **(15)**, where they are translated. Late proteins (γ proteins) are primarily virion structural proteins and additional proteins needed for virus assembly and particle egress. Some γ proteins are made on, and inserted into, membranes of the rough endoplasmic reticulum **(16a)**. Many of these membrane proteins are modified by glycosylation. Some precursor viral membrane proteins are thought to be localized both to the outer and inner nuclear membranes, as well as membranes of the endoplasmic reticulum **(16b)**. The precursor glycoproteins are also transported to the Golgi apparatus for further modification and processing **(16c)**. Mature glycoproteins are transported to the plasma membrane of the infected cell **(16d)**. Some γ proteins are transported

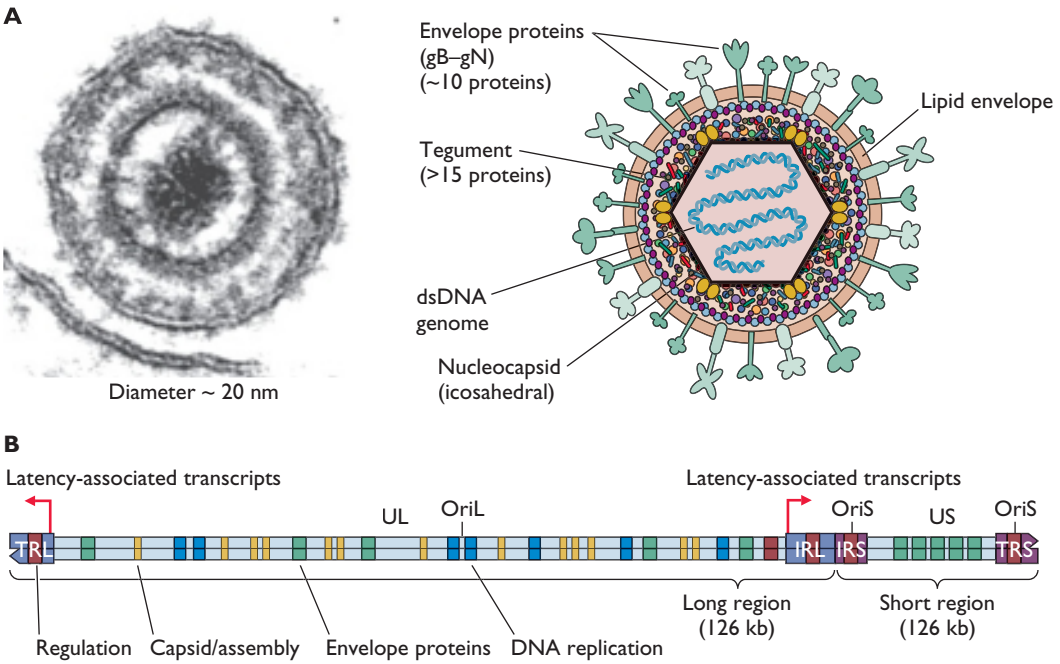

Figure 5 Structure and genome organization of alphaherpesviruses.

to the nucleus for assembly of the nucleocapsid and DNA packaging, while some remain in the cytoplasm **(17)**. Newly replicated viral DNA is packaged into nucleocapsids **(18)**. DNA-containing nucleocapsids, together with some tegument proteins, bud from the inner nuclear membrane into the perinuclear lumen, acquiring an envelope thought to contain precursors to viral membrane proteins **(19).** Immature enveloped virions fuse with the outer nuclear membrane from within **(20)**, leaving behind viral membrane proteins while releasing the nucleocapsid into the cytoplasm. This structure is transported to a late *trans*-Golgi compartment or an endosome that contains mature viral membrane proteins **(21)**. Tegument proteins added in the nucleus remain with the nucleocapsid, and others are added in the cytoplasm. As nucleocapsids bud into the late Golgi-endosome compartment, they acquire an envelope containing mature viral envelope proteins and the complete tegument layer (secondary envelopment **[22]**). The enveloped virus particle then buds into a vesicle **(23)** that is transported to the plasma membrane for release by exocytosis **(24)**. **(B) Latent infection.** Latent infection occurs primarily in neurons found in ganglia of the peripheral nervous system. In the simplest model, the initiation of latent infection occurs as in **steps 1 to 7** of the productive infection (A), except that the viral DNA circularizes in the nucleus and is wrapped around nucleosomes. It is unclear if tegument proteins are transported into the nucleus during the establishment of the latent infection **(8)**. The latent genome is transcriptionally silent, and only a single pre-mRNA is produced from the latency-associated transcript (LAT) promoter **(9)**. Low-level or sporadic transcription of immediate-early and early genes can occur, but is not sufficient to initiate a productive infection. The LAT RNA is spliced, and a stable intron in the form of a lariat, called the 2-kb LAT, is maintained in the nucleus **(10)**. The spliced LAT mRNA is transported to the cytoplasm, where several small open reading frames (e.g., OrfO and OrfP) may be translated into proteins **(11)**. The function of LAT RNAs and the production of LAT proteins are controversial. After months to years, and in the absence of viral tegument and IE proteins, cellular proteins produced in response to changes in neuronal physiology induced by trauma, hormonal changes, and other stressful conditions render some latently infected neurons permissive for viral replicaton. The entire genome is then transcribed and replicated as during productive infection, and progeny virions are produced as shown in panel A.

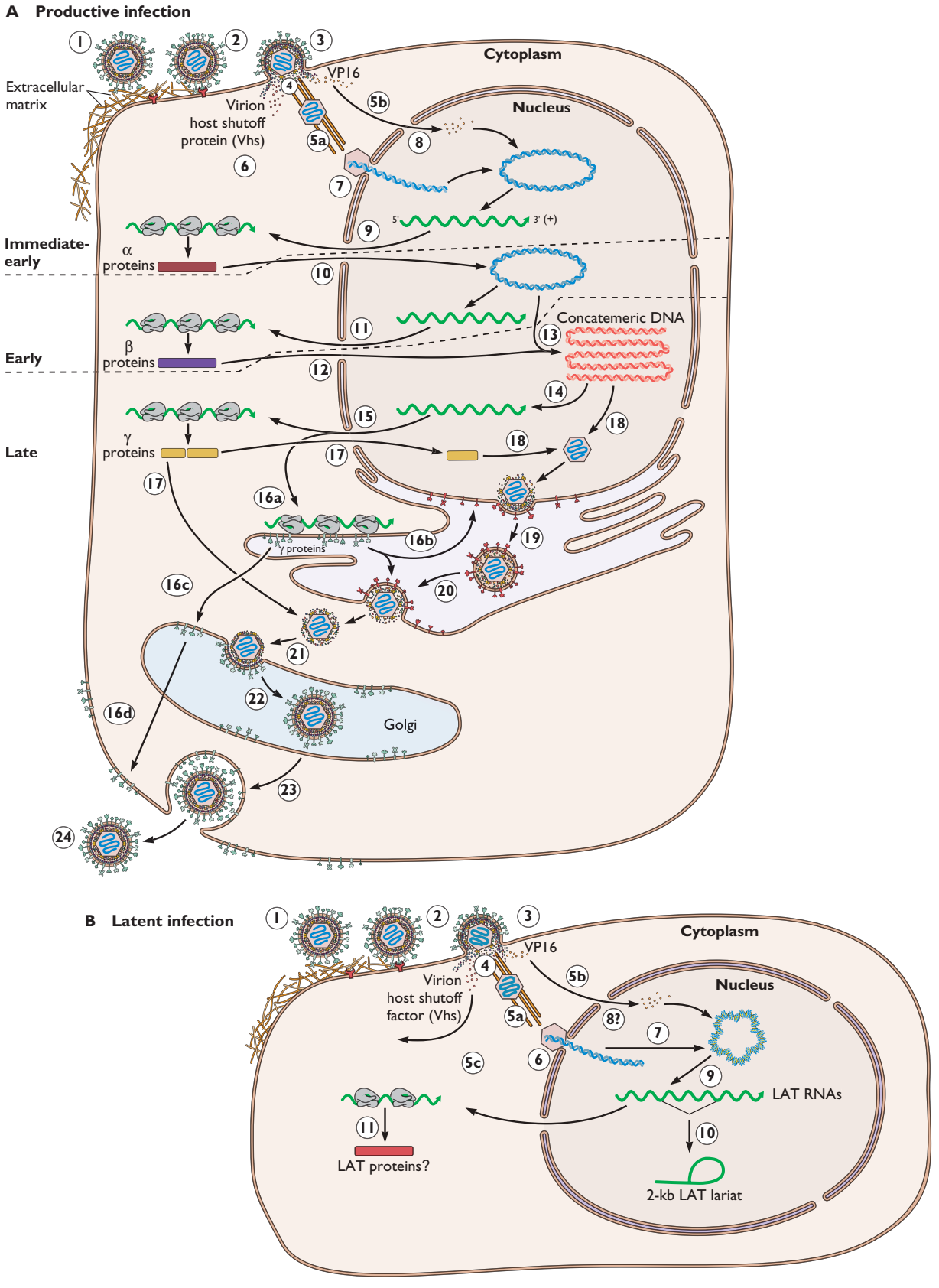

Figure 6 Single-cell reproductive cycles of herpes simplex virus type 1.

Orthomyxoviruses

Family *Orthomyxoviridae*

Genus	Type species
Influenza A virus	A/PR/8/34(H1N1)
Influenza B virus	B/Lee/40
Influenza C virus	C/California/78
Thogotovirus	Thogoto virus
Isavirus	Infectious salmon anemia virus

Influenza viruses are the causative agents of a highly contagious and often serious acute respiratory illness. Influenza viruses are unusual among RNA viruses in that all viral RNA synthesis occurs in the cell nucleus. Initiation of viral mRNA synthesis with a capped primer derived from host cell mRNA was first observed in cells infected with influenza viruses. The viral genomes undergo extensive reassortment and variation, and are expressed via a remarkable panoply of unusual strategies, including RNA splicing, overlapping reading frames, and leaky scanning.

Figure 7 Structure and genomic organization of the orthomyxovirus influenza A virus. (A) Virion structure. The electron micrograph shows negatively stained influenza A virus particles (courtesy of P. Palese, Mount Sinai School of Medicine, New York, NY). **(B) Genome organization.** The (−) strand RNA genome comprises eight segments, each of which encodes at least one viral protein as shown. Some fraction of the (+) strand mRNA of the smallest genomic RNA segments, 7 and 8, is spliced by host cell enzymes, allowing the production of two proteins from each. The NS1 (nonstructural) protein is so named because it is not incorporated into virus particles. An accessory protein with proapoptotic activity, PB1-F2, is produced from the PB1 RNA, by translation of an overlapping open reading frame.

Figure 8 Single-cell reproductive cycle of influenza A virus. The virion binds to a sialic acid-containing cellular surface protein or lipid and enters the cell via receptor-mediated endocytosis **(1)**. Upon acidification of the vesicle, the viral membrane fuses with the membrane of the vesicle, releasing the eight viral nucleocapsids into the cytoplasm (for simplicity, only one is shown) **(2)**. The viral nucleocapsids containing (−) strand genomic RNA, multiple copies of the NP protein, and the P proteins are transported into the nucleus **(3)**. The (−) strand RNAs are copied by virion RNA polymerase into viral mRNA, using the capped 5′ ends of host pre-mRNAs (or mRNAs) as primers to initiate synthesis **(4)**. The mRNAs are transported to the cytoplasm **(5)**, following splicing in the case of the mRNAs encoding NEP and M2 **(6)**. The mRNAs specifying the viral membrane proteins (HA, NA, and M2) are translated by ribosomes bound to the endoplasmic reticulum (ER) **(7)**. These proteins enter the host cell's secretory pathway, where HA and NA are glycosylated. All other mRNAs are translated by ribosomes in the cytoplasm **(8** and **9)**. The PA, PB1, PB2, and NP proteins are imported into the nucleus **(10a)**, where they participate in the synthesis of full-length (+) strand RNAs **(11)** and then of (−) strand genomic RNAs **(12)**, both of which are synthesized in the form of nucleocapsids. Some of the newly synthesized (−) strand RNAs enter the pathway for mRNA synthesis **(13)**. The M1 protein and the NS1 protein are transported into the nucleus **(10b)**. Binding of the M1 protein to newly synthesized (−) strand RNAs shuts down viral mRNA synthesis and, in conjunction with the NEP protein, induces export of progeny nucleocapsids to the cytoplasm **(14)**. The HA, NA, and M2 proteins are transported to the cell surface **(15)** and become incorporated into the plasma membrane **(16).** The virion nucleocapsids associated with the M1 protein **(17)** and the NEP protein **(18)** are transported to the cell surface and attach to regions of the plasma membrane that contain the HA, NA, M1, and M2 proteins. Assembly of virions is completed at this location by budding from the plasma membrane **(19).**

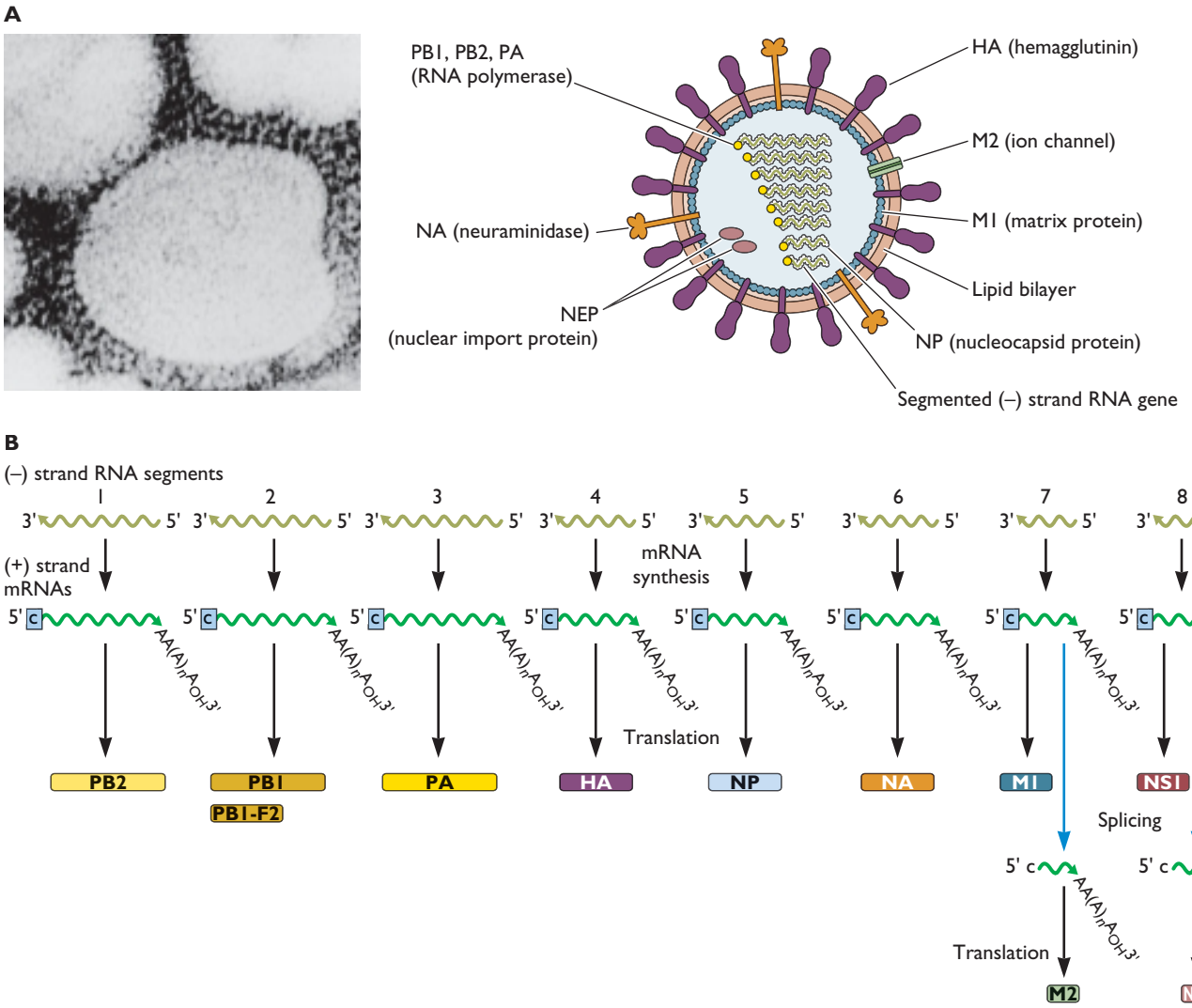

Figure 7 Structure and genomic organization of the orthomyxovirus influenza A virus.

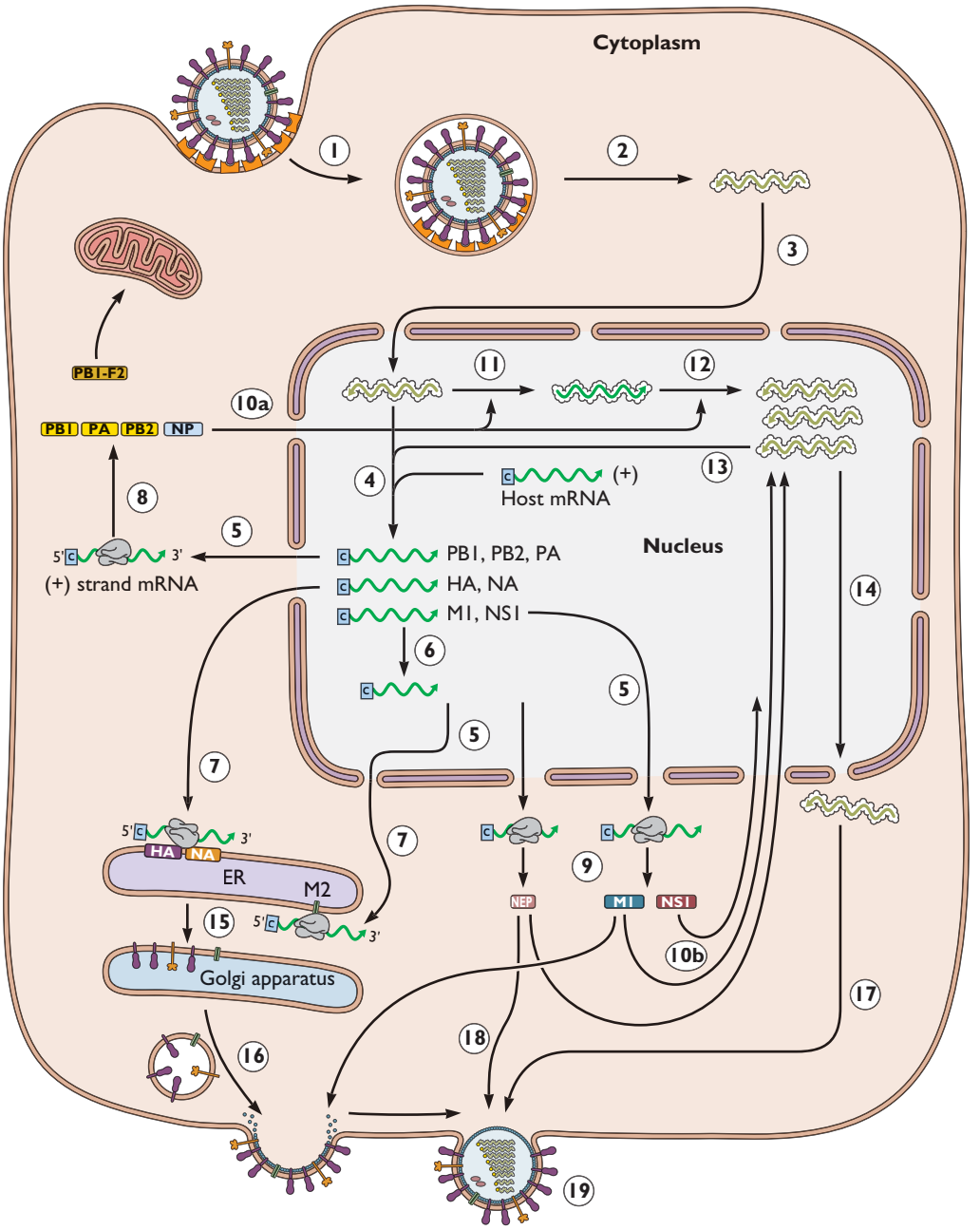

Figure 8 Single-cell reproductive cycle of influenza A virus.

Parvoviruses

Family *Parvoviridae*

Genus	Type species
Subfamily *Densovirinae* (3 genera; insect viruses)	
Subfamily *Parvovirinae*	
Parvovirus	Minute virus of mice
Erythrovirus	Human B19 virus
Dependovirus	Human adeno-associated viruses

Members of the family *Parvoviridae* are among the smallest of the DNA animal viruses. They are of particular interest because of their unique genomic DNA structure and mechanism of replication. Most parvoviruses, such as the well-studied minute virus of mice, can propagate autonomously, although they require the host cell to go through S phase in order to replicate. Replication of dependoviruses requires a helper, adenovirus or herpesvirus, to induce S phase and to provide components that promote dependovirus replication. These viruses can establish a latent infection during which their DNA is integrated into the host cell genome in an inactive state, to be activated upon subsequent infection with a helper. Because of its ability to persist and its lack of pathogenicity, human adeno-associated virus has been developed as a potential vector for gene therapy.

Figure 9 Structure and genome organization of adeno-associated virus (AAV). (A) Virion structure. The electron micrograph shows AAV4 (courtesy of Mavis Agbandje-McKenna, University of Florida, Gainesville). The three-dimensional structure of canine parvovirus is from cryo-electron microscopy with the icosahedral two-, three-, and fivefold axes indicated. The illustration was prepared by Agbandje-McKenna and obtained from N. Muzyczka and K. I. Berns, *in* D. M. Knipe et al. (ed.), *Fields Virology*, 4th ed. (Lippincott Williams & Wilkins, Philadelphia, PA, 2001), with permission. The capsid comprises 60 protein subunits, primarily (~90%) VP3. Virions contain (+) or (−) single-stranded DNA in separate particles. **(B) Genome organization.** The best-characterized DNA genome, that of AAV 2, comprises ca. 4,600 nucleotides and includes terminal repeats (TR) of 145 nucleotides, the first 125 of which form a palindromic sequence. The TR is required in *cis* for genome replication, transcription, and encapsidation, and plays a role in integration into the host DNA during establishment of a latent infection. Use of multiple initiation codons and alternative splicing results in synthesis of multiple Rep (tan bars) and structural proteins (purple bars), respectively. ORF, open reading frame. Adapted from R. M. Linden and K. Berns, p. 68–84, *in* S. Faisst and J. Rommelaere (ed.), *Contributions to Microbiology*, vol. 4, *Parvoviruses: from Molecular Biology to Pathology and Therapeutic Uses* (S. Karger, Basel, Switzerland, 2000), with permission.

Figure 10 Single-cell reproductive cycle. (A) Latent infection. Heparan sulfate proteoglycans are the primary cell surface receptors for AAV2. However, the processes of adsorption **(1)**, uncoating **(2)**, and entry of the DNA into the nucleus **(3)** are poorly understood for all *Parvoviridae*. In the absence of helper virus, some replication of the single-stranded genome occurs prior to integration **(4)** to produce a double-stranded DNA template for transcription from the p5 promoter **(5)**. Translation of this transcript produces Rep proteins **(6)** that are required to both facilitate integration **(7)** and suppress further transcription from all three promoters. The junction with cellular DNA is usually within the terminal repeat (TR) or near a Rep-binding site within the p5 promoter. Neither the actual site in the viral DNA nor the cellular sequence at the junction is unique. However, 70 to 100% of the integration events occur within a region of several hundred nucleotides in chromosome 19q13.3-qter. **(8)**. In most latently infected cells the viral DNA is integrated as a tandem (head-to-tail) array of several genome equivalents. This unexpected arrangement indicates that the mechanism of integration is likely to be distinct from that of viral DNA replication [see (B), step 7]. The integrated viral genome(s) can remain dormant through many cell cycles. Upon superinfection with a helper virus, a productive infection is initiated; molecular details of this process have not yet been elucidated. **(B) Productive infection.** Upon coinfection with a helper virus **(1)**, AAV undergoes a productive infection. With an adenovirus helper, this response is dependent on the expression of early genes E1A, E1B, E4, and E2A **(2)**, which induce S phase and the concomitant production of cellular DNA replication proteins needed for viral DNA synthesis **(3)**. The adenovirus EIA transcriptional activator also induces transcription from the p5 promoter **(4)**, leading to the production of Rep78/68 mRNA and proteins **(5)**. These proteins then function as powerful transcriptional activators (rather than repressors as in latency), and induce transcription from both the p5 and p19 promoters **(6a)**. Viral DNA is replicated by a single-strand displacement mechanism that is initiated by recognition of the terminal resolution site (*trs*) by the Rep78/68 proteins, which remain linked covalently to the DNA through subsequent steps of DNA synthesis **(6b)**. A very large number of replicating forms (ca. 10^6 double-stranded genomes/cell) can be produced within a short time **(7)**. The capsid proteins produced in the cytoplasm **(8)** self-associate in the nucleus during virion assembly **(9)**. As with the adenovirus helper, progeny virions are released **(10)**, usually upon destruction of the cell. The (+) or (−) strand genomes are encapsidated in equal numbers in progeny virions.

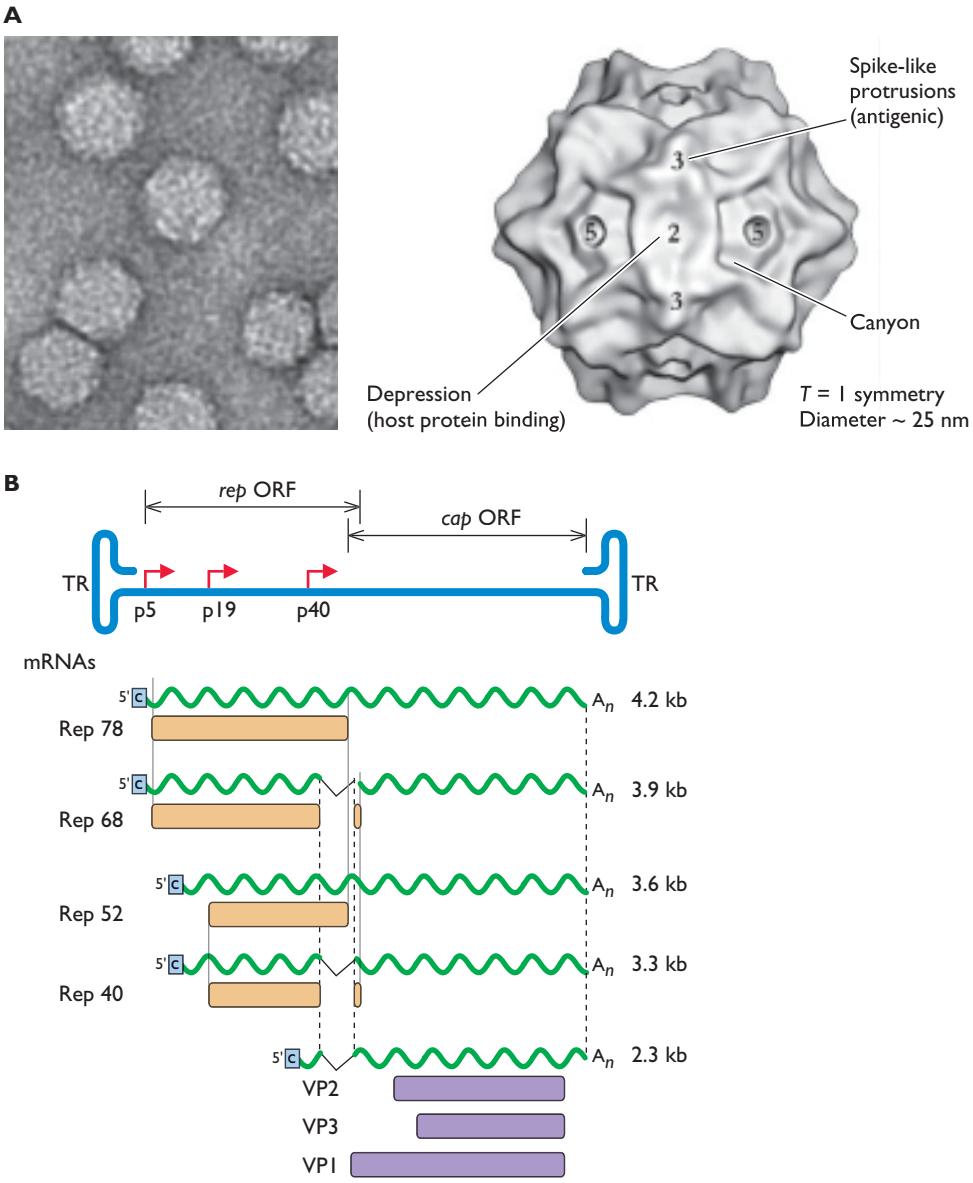

Figure 9 Structure and genome organization of adeno-associated virus (AAV).

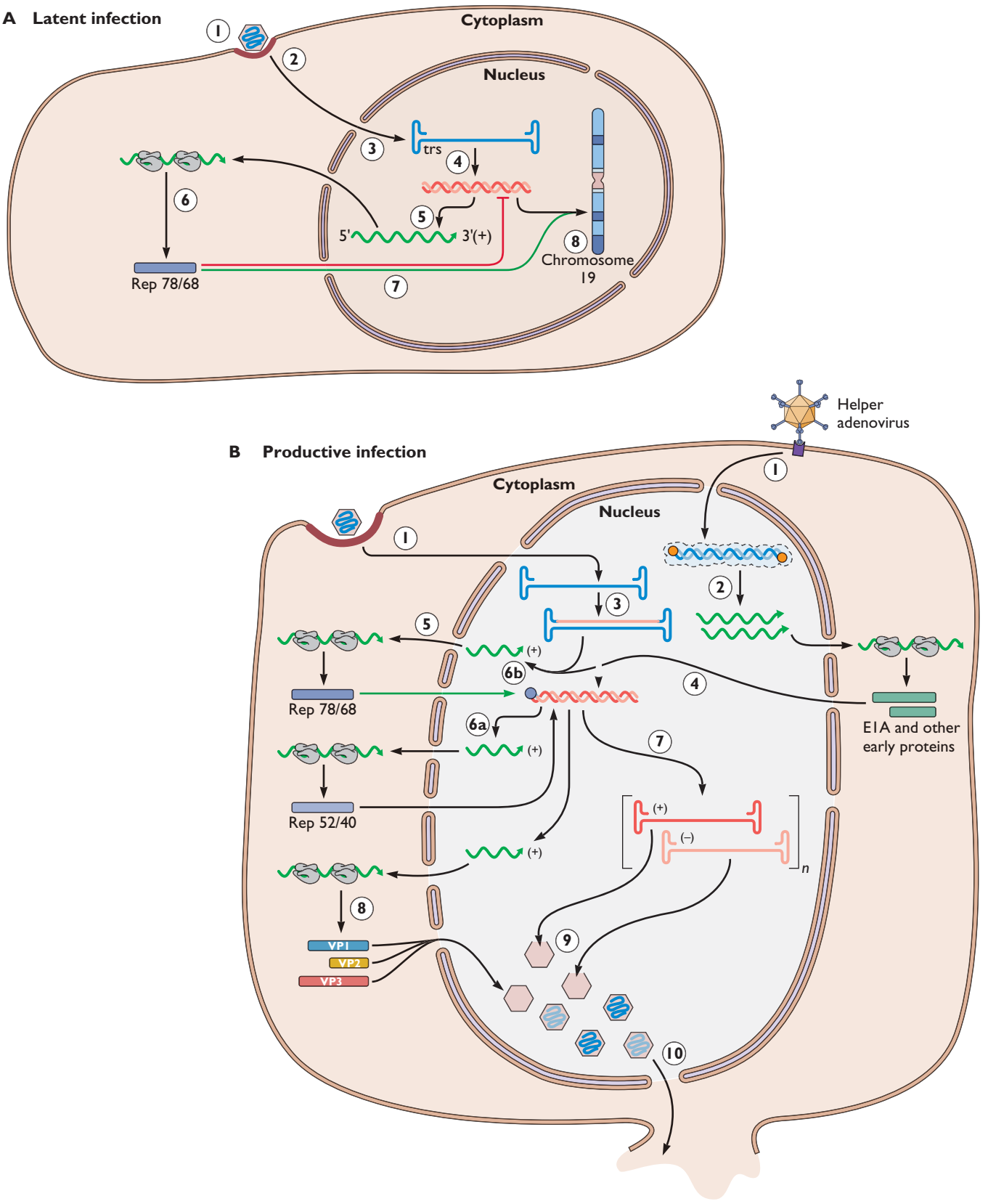

Figure 10 Single-cell reproductive cycle.

Picornaviruses

Family *Picornaviridae*

Genus	Type species
Enterovirus	Poliovirus
Rhinovirus	Human rhinovirus A
Cardiovirus	Encephalomyocarditis virus
Aphthovirus	Foot-and-mouth disease virus
Hepatovirus	Hepatitis A virus
Parechovirus	Human parechovirus
Erbovirus	Equine rhinitis B virus
Kobuvirus	Aichi virus
Teschovirus	Porcine teschovirus 1

The family *Picornaviridae* includes many important human and animal pathogens, such as poliovirus, hepatitis A virus, foot-and-mouth disease virus, and rhinovirus. Because they cause serious disease, poliovirus and foot-and-mouth disease viruses are the best-studied picornaviruses. These two viruses have had important roles in the development of virology. The first animal virus discovered, in 1898, was foot-and-mouth disease virus. The plaque assay was developed using poliovirus, and the first RNA-dependent RNA polymerase discovered was poliovirus $3D^{pol}$. Polyprotein synthesis was discovered in experiments with poliovirus-infected cells, as was translation by internal ribosome entry. The first infectious DNA clone of an animal RNA virus was that of the poliovirus genome, and the first three-dimensional structures of animal viruses determined by X-ray crystallography were those of poliovirus and rhinovirus.

Figure 11 Structure and genomic organization. (A) Virion structure. The electron micrograph shows negatively stained poliovirus (courtesy of N. Cheng and D. M. Belnap, National Institutes of Health, Bethesda, MD). The capsid consists of 60 structural units (each made up of a single copy of VP1, VP2, VP3, and VP4, colored blue, yellow, red, and green, respectively) arranged in 12 pentamers. One of the icosahedral faces has been removed in the diagram to illustrate the locations of VP4 and the viral RNA. **(B) Genome organization.** Polioviral RNA is shown with the VPg protein covalently attached to the 5′ end. The genome is of (+) polarity and encodes a polyprotein precursor. The polyprotein is cleaved during translation by two virus-encoded proteases, $2A^{pro}$ and $3C^{pro}$, to produce structural and nonstructural proteins, as indicated.

Figure 12 Single-cell reproductive cycle. The virion binds to a cellular receptor **(1)**; release of the poliovirus genome occurs from within early endosomes located close (within 100 to 200 nm) to the plasma membrane **(2)**. The VPg protein, depicted as a small orange circle at the 5′ end of the virion RNA, is removed, and the RNA associates with ribosomes **(3)**. Translation is initiated at an internal site 741 nucleotides from the 5′ end of the viral mRNA, and a polyprotein precursor is synthesized **(4)**. The polyprotein is cleaved during and after its synthesis to yield the individual viral proteins **(5)**. Only the initial cleavages are shown here. The proteins that participate in viral RNA synthesis are transported to membrane vesicles **(6)**. RNA synthesis occurs on the surfaces of these infected-cell-specific membrane vesicles. The (+) strand RNA is transported to these membrane vesicles **(7)**, where it is copied into double-stranded RNAs **(8)**. Newly synthesized (−) strands serve as templates for the synthesis of (+) strand genomic RNAs **(9)**. Some of the newly synthesized (+) strand RNA molecules are translated after the removal of VPg **(10)**. Structural proteins formed by partial cleavage of the P1 precursor **(11)** associate with (+) strand RNA molecules that retain VPg to form progeny virions **(12)**, which are released from the cell upon lysis **(13)**.

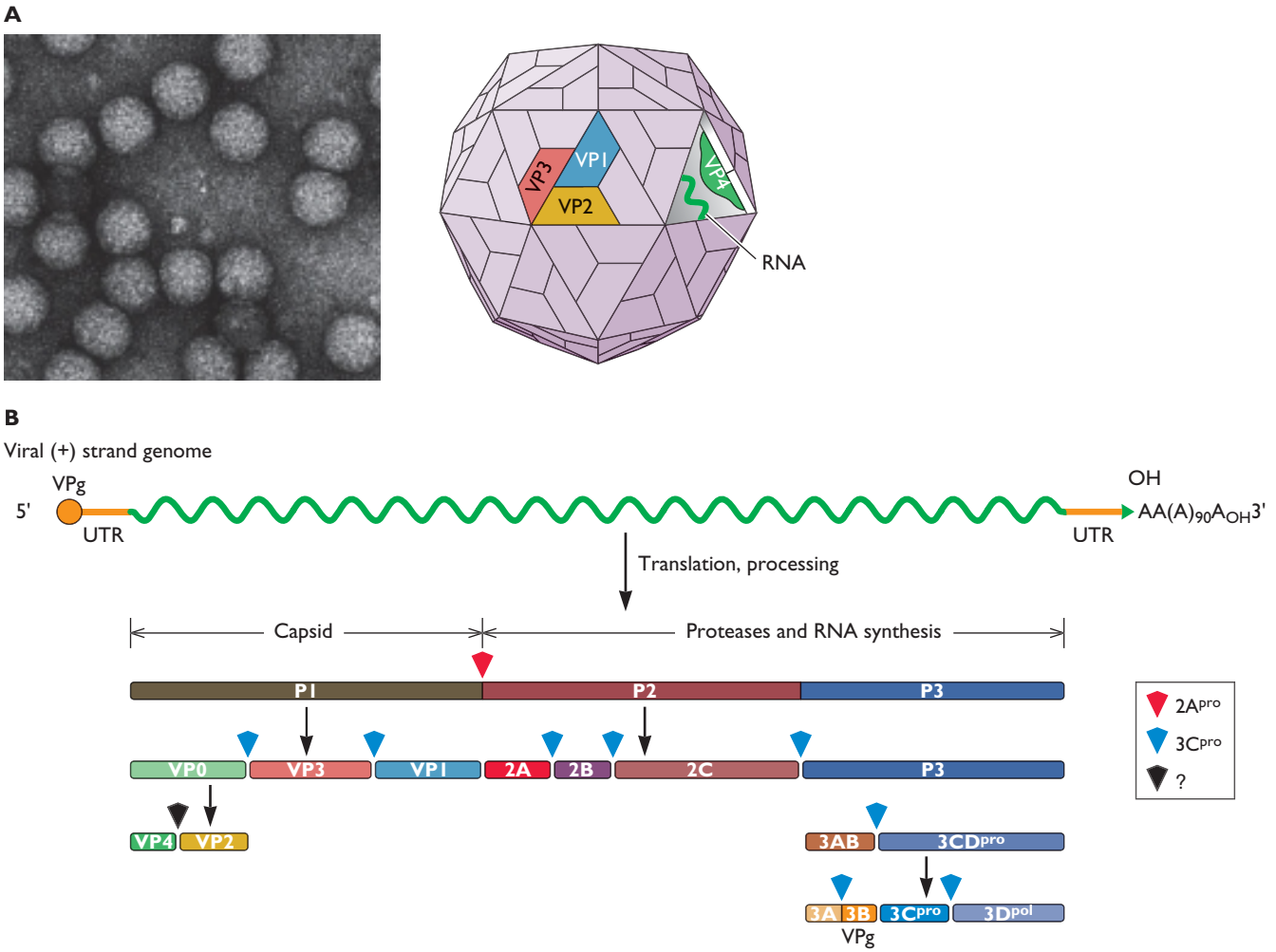

Figure 11 Structure and genomic organization.

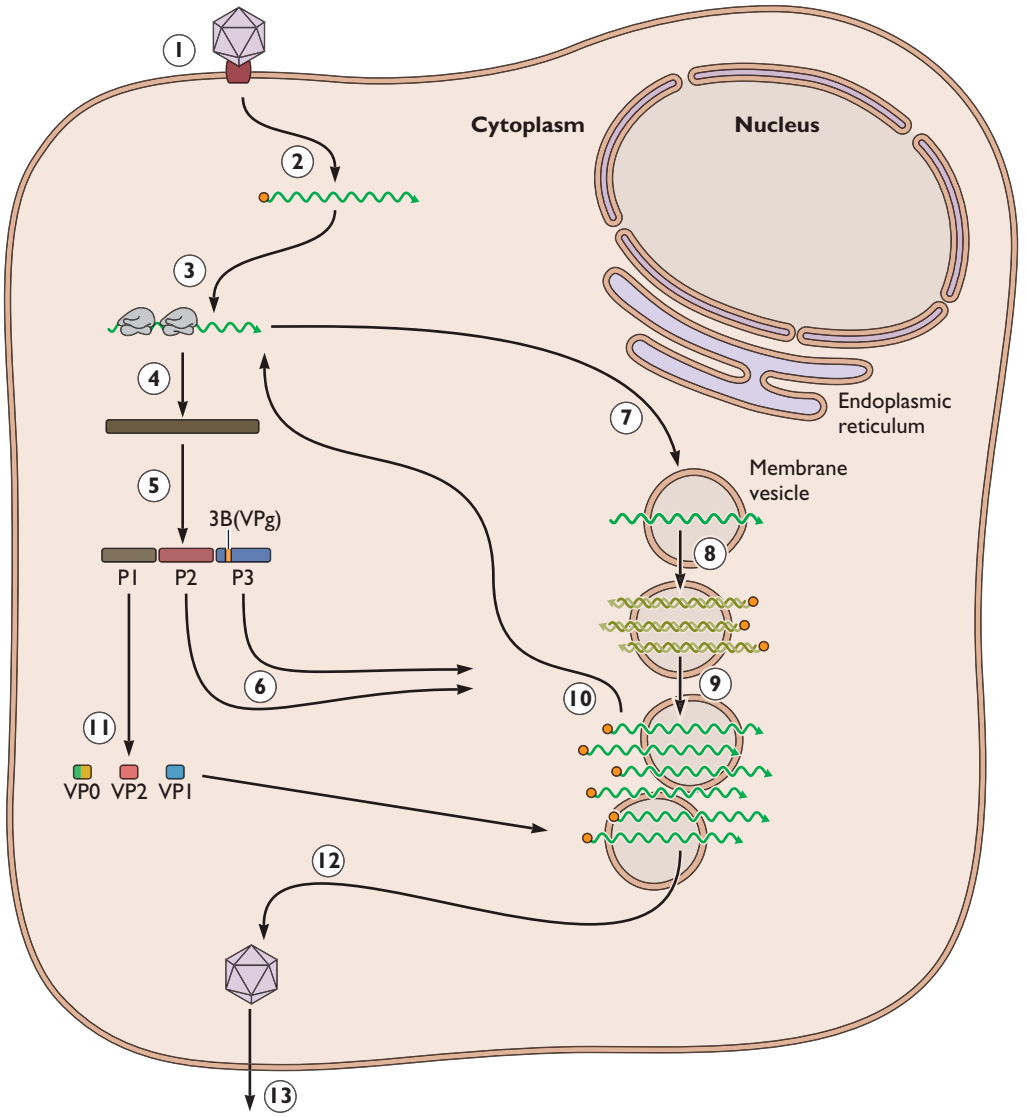

Figure 12 Single-cell reproductive cycle.

Polyomaviruses

Family *Polyomaviridae*

Genus	Type species
Polyomavirus	Simian virus 40

The family *Polyomaviridae* includes mouse polyomaviruses, simian virus 40, and two human viruses, JC and BK viruses, which were isolated from a patient with progressive multifocal leukoencephalopathy and an immunosuppressed recipient of a kidney transplant, respectively. Under some conditions, mouse polyomavirus infection of the natural host results in formation of a wide variety of tumors (hence the name). A characteristic property of the members of this family is an ability to transform cultured cells or to induce tumors in animals. Investigation of such transforming activity has provided much information about mechanisms of oncogenesis, including the discovery of the cellular tumor suppressor protein p53. These viruses, particularly simian virus 40, have also been important in elucidation of cellular mechanisms of transcription and its regulation. For example, the simian virus 40 enhancer was the first member of this class of regulatory sequences to be identified.

Figure 13 Structure and genome organization. (A) Virion structure. The electron micrograph shows negatively stained simian virus 40 virions (from F. A. Andered et al., *Virology* **32:**511–523, 1967, with permission). The double-stranded DNA genome is organized into approximately 25 nucleosomes by the cellular core histones. One molecule of either VP2 or VP3, which possess a common C-terminal sequence, is associated with each VP1 pentamer. **(B) Genome organization.** The 5,243-bp simian virus 40 genome showing locations of the origin of viral DNA synthesis (Ori) and of the early and late mRNAs. The late mRNA species generally contain additional open reading frames in their 5′-terminal exons, such as that encoding leader protein 1 (LP1).

Figure 14 Single-cell reproductive cycle of simian virus 40. The virion attaches to permissive monkey cells upon binding of VP1 to a major histocompatibility complex (MHC) class I molecule on the surface. The virion is then endocytosed in caveolae **(1** and **2)**, transported to the endoplasmic reticulum, and enters that organelle **(3)**. Subsequently, it is transported to the nucleus and uncoated by unknown mechanisms **(4)**. The viral genome packaged by cellular nucleosomes is found within the nucleus **(5)**. The early transcription unit is transcribed by host cell RNA polymerase II **(6)**. After alternative splicing and export to the cytoplasm **(7)**, the early mRNAs are translated to produce the early proteins LT and sT **(8)**. The former is imported into the nucleus **(9)**, where it binds to the simian virus 40 origin of replication to initiate DNA synthesis **(10)**. Apart from LT, all components needed for viral DNA replication are provided by the host cell. As they are synthesized, daughter viral DNA molecules associate with cellular nucleosomes to form the viral nucleoproteins often called minichromosomes. LT also stimulates transcription of the late gene from replicated viral DNA templates **(11)**. Processed late mRNAs are exported to the cytoplasm **(12)**, and translated to produce the virion structural proteins VP1, VP2, and VP3 **(13)**. These structural proteins are imported into the nucleus **(14)** and assemble around viral minichromosomes to form progeny virions **(15)**. Virions are released by an unknown mechanism **(16)**.

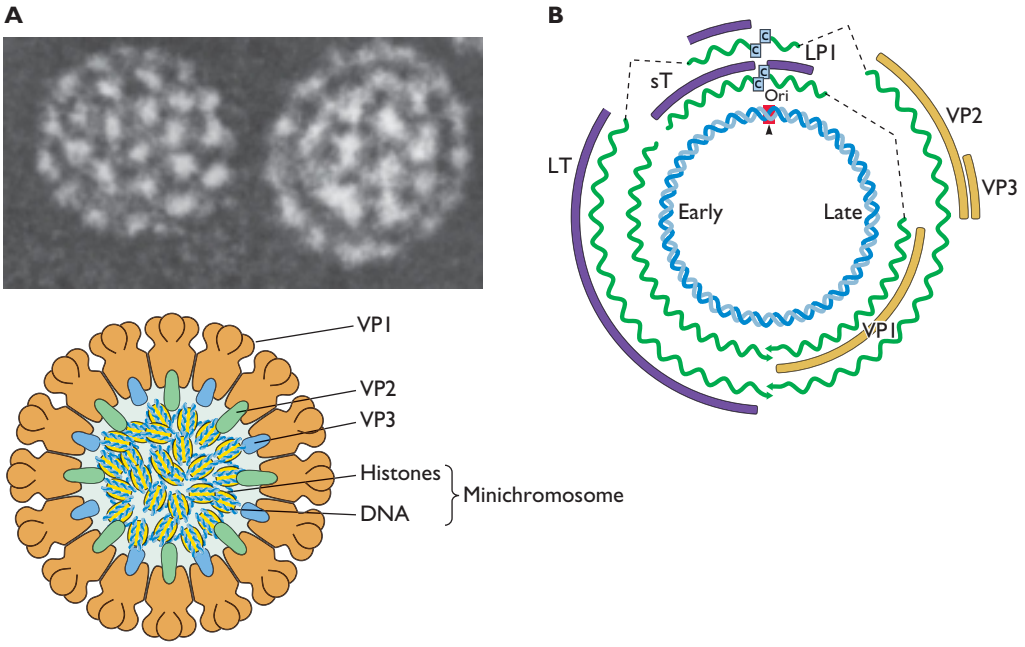

Figure 13 Structure and genome organization.

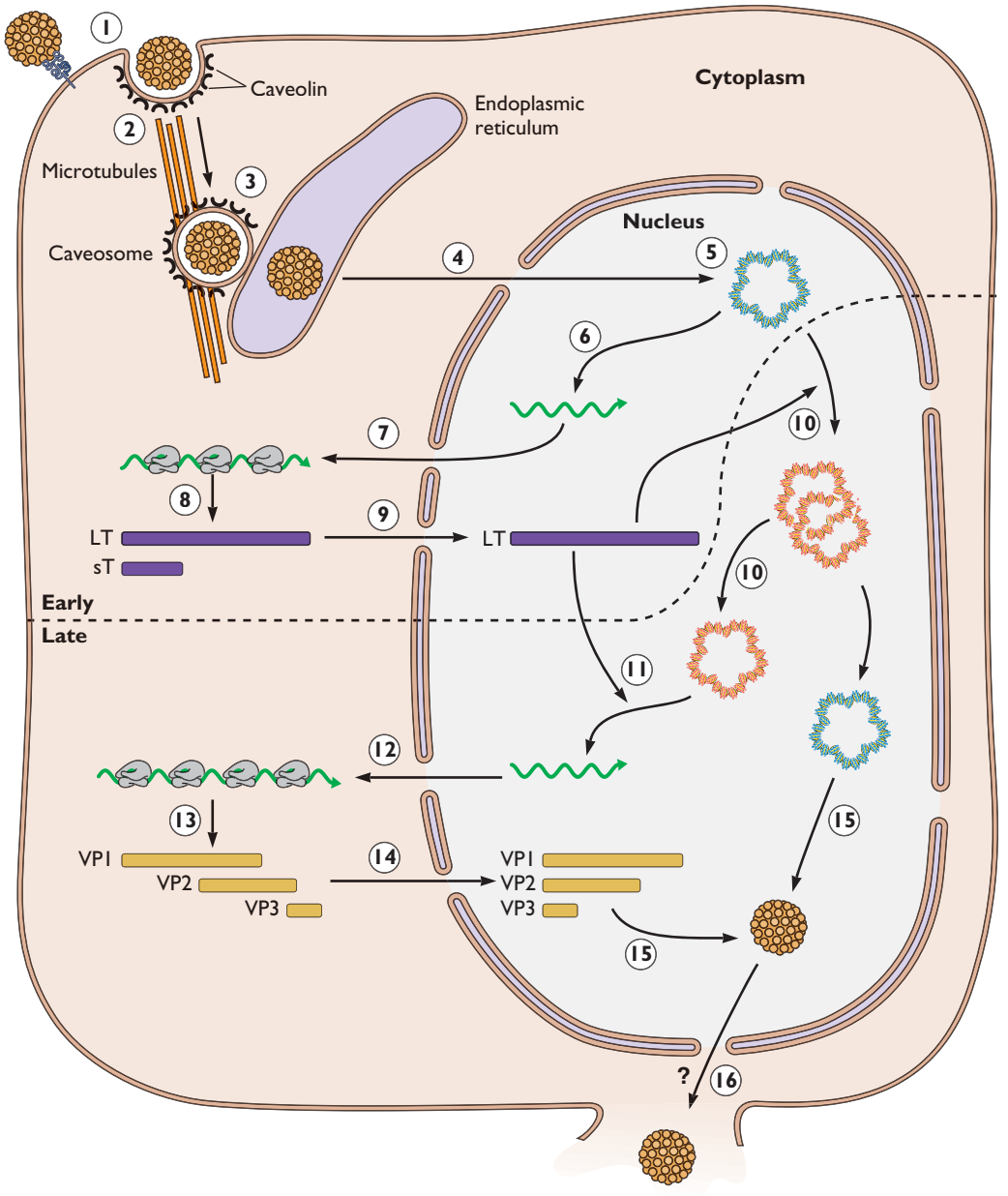

Figure 14 Single-cell reproductive cycle of simian virus 40.

Poxviruses

Family *Poxviridae*

Genus	Type species
Subfamily *Chordopoxvirinae*	
Orthopoxvirus	Vaccinia virus
Parapoxvirus	Orf virus
Avipoxvirus	Fowlpox virus
Capripoxvirus	Sheeppox virus
Leporipoxvirus	Myxoma virus
Suipoxvirus	Swinepox virus
Molluscipoxvirus	Molluscum contagiosum virus
Yabapoxvirus	Yaba monkey tumor virus
Subfamily *Parvovirinae*	
Three genera of viruses that infect insects	

Poxviruses infect most vertebrates and invertebrates, causing a variety of diseases of veterinary and medical importance. The best-known poxviral disease is smallpox, a devastating human disease that has been eradicated by vaccination. The origins of modern vaccinia virus, the virus used in smallpox virus vaccine, are obscure, but this virus is widely studied as a model poxvirus in the laboratory. Myxoma virus, which causes an important disease of domestic rabbits, was described in 1896. Rabbit fibroma virus, which was first described by Shope in 1932, was the first virus proven to cause tissue hyperplasia. The genomes of poxviruses are large DNA molecules that include genes for all proteins needed for DNA synthesis and production of viral mRNAs. These viruses replicate in the cytoplasm and are minimally dependent on the host cell.

Figure 15 Structure and genome organization of the poxvirus vaccinia virus. (A) Virion structure. The electron micrograph shows the mature virion in cross section (courtesy of David J. Vaux, Sir William Dunn School of Pathology, Oxford University, Oxford, United Kingdom). **(B) Genome organization.** Shown are details for the 191-kb genome of the Copenhagen strain of vaccinia virus, with open reading frames in a small section of the genome. This genome includes ~185 unique protein-coding sequences. Those that encode structural proteins and essential enzymes are clustered in the center; those that affect virulence, host range, or immunomodulation are predominantly near the ends.

Figure 16 Single-cell reproductive cycle of vaccinia virus. After fusion of viral and plasma membranes, or fusion following endocytosis, the viral core is released into the cytoplasm **(1)**. Early mRNAs are synthesized **(2)** and are translated by the cellular protein-synthesizing machinery **(3)**. Some early proteins have sequence similarity to cellular growth factors, and can induce proliferation of neighboring host cells following their secretion **(4).** Other early proteins counteract host immune defense mechanisms. Some early viral proteins induce a second uncoating reaction in which the viral genome is released from the core in a nucleoprotein complex **(5)**, and others mediate replication of the genome **(6)**. Newly synthesized viral DNA molecules can serve as templates for additional cycles of genome replication **(7)** and for transcription of viral intermediate genes **(8)**. Transcription of intermediate genes requires viral initiation proteins, which are products of early genes, and a cellular protein (Vitf2), which relocates from the infected cell nucleus to the cytoplasm. Proteins made upon translation of intermediate mRNAs **(9)** include those necessary for transcription of late genes **(10)**. Late mRNAs are translated to produce virion structural proteins, virion enzymes, and other essential proteins that are needed early in subsequent infections and must be incorporated into virus particles during assembly. Assembly of progeny particles begins in specialized perinuclear sites, termed viral factories, that form upon viral DNA synthesis. These sites contain cellular membranes, probably derived from the endoplasmic reticulum, which are initially reorganized by specific viral proteins to form crescents **(12)**, the precursor to spherical DNA-containing particles, called **immature virions (13)**. Upon proteolysis and release from viral factories, these particles then mature into brick-shaped intracellular mature virions **(14)**, which are released only upon cell lysis **(15)**. However, these particles can acquire a second, double membrane from a *trans*-Golgi or early endosomal compartment to form intracellular **wrapped virions (16)**. The latter particles move to the cell surface on microtubules where fusion with the plasma membrane forms **cell-associated virions (17)** that induce actin polymerization for direct transfer to surrounding cells **(18)** or dissociate from the membrane as the **extracellular virion**. Association of the extracellular virion with a host cell in the next cycle of infection is thought to result in rupture of the outer membrane, giving rise to the **mature virion (MV).**

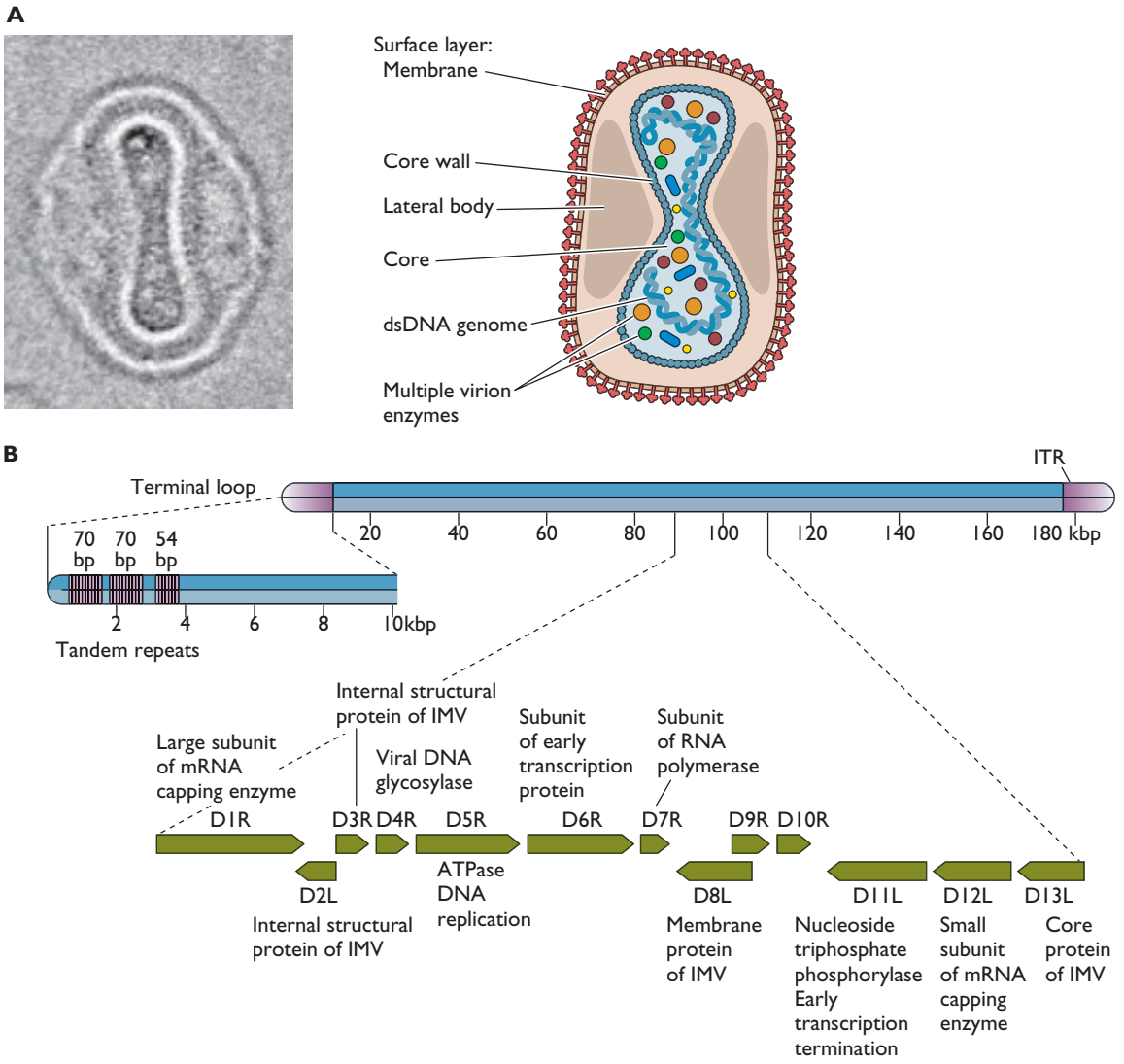

Figure 15 Structure and genome organization of the poxvirus vaccinia virus.

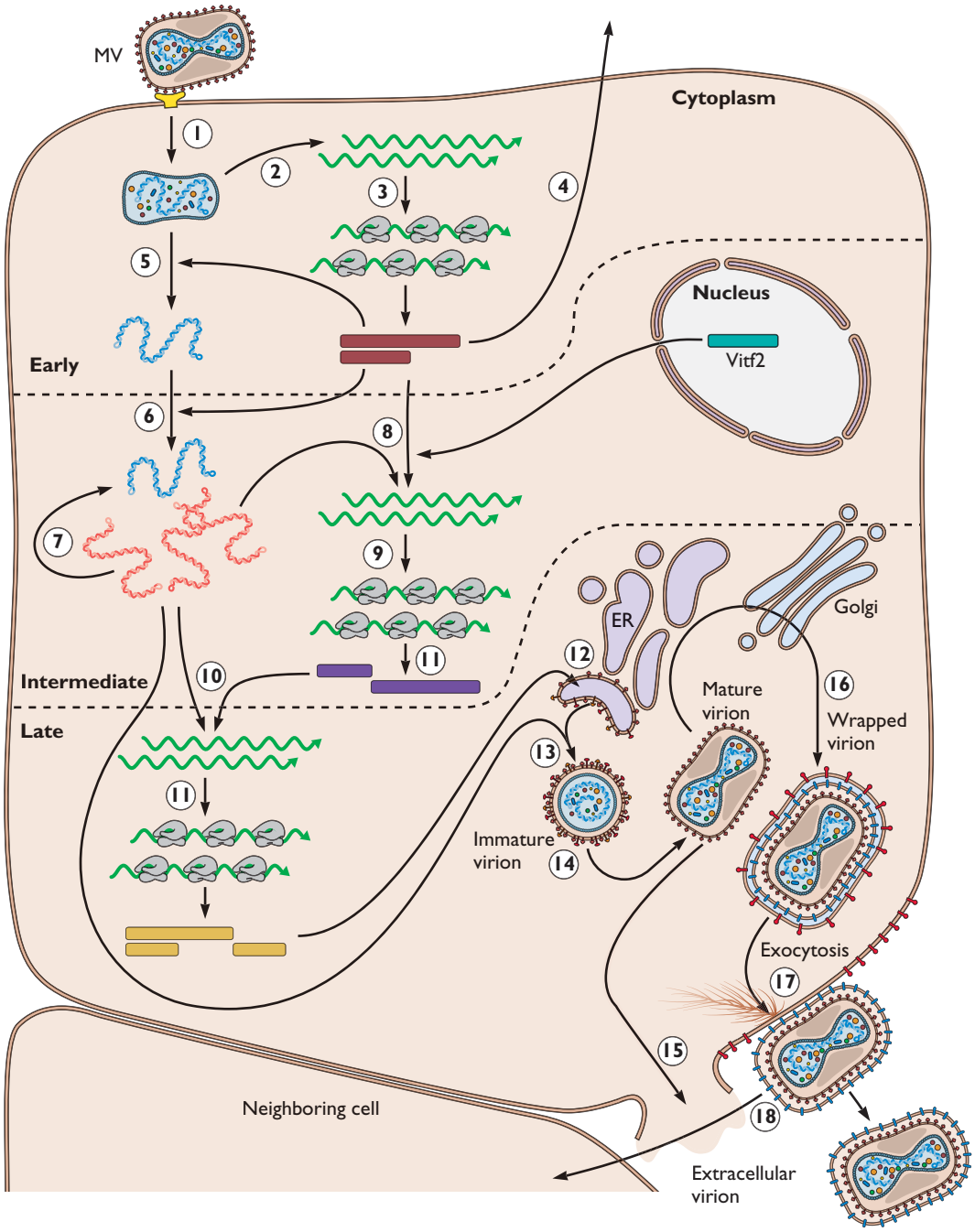

Figure 16 Single-cell reproductive cycle of vaccinia virus.

Family *Reoviridae*

Genus	Type species
Orthoreovirus	Mammalian orthoreovirus
Orbivirus	Bluetongue virus
Rotavirus	Rotavirus A
Coltivirus	Colorado tick fever virus
Aquareovirus	Aquareovirus A
Cypovirus	Cypovirus 1
Fijivirus	Fijivirus 1
Phytoreovirus	Rice dwarf virus
Oryzavirus	Rice ragged stunt virus
Seadornavirus	Banna virus
Idnoreovirus	Idnoreovirus 1
Mycoreovirus	Mycoreovirus 1

Reoviridae is one of six families of viruses with double-stranded RNA genomes. Included in this family are the human pathogens rotaviruses and Colorado tick fever virus. Reoviruses are the best studied of all the double-stranded RNA viruses. Some of the first *in vitro* research on RNA synthesis was done using reoviruses, and the 5′-terminal cap structure of mRNA was discovered in studies of reovirus mRNAs.

Figure 17 Structure and genomic organization of an orthoreovirus. (A) Virion structure. Electron micrograph of negatively stained reovirus particles, courtesy of S. McNulty, Queen's University, Belfast, United Kingdom. The locations of six virion proteins are indicated on the three-dimensional reconstructions. **(B) Genome organization.** The double-stranded genome comprises 10 segments, named according to size: large (L), medium (M), and small (S). The S1 RNA encodes two proteins: σ1s protein is translated from a second initiation codon in a different reading frame from σ1. Two proteins are also produced from the M3 RNA: protein μNSC is produced by translation at a second initiation codon in the same reading frame as μNS.

Figure 18 Single-cell reproductive cycle of orthoreovirus. The virion **(1)** or a proteolytic derivative **(3)** called the infectious subviral particle (ISVP) **(4)** binds to the same cellular receptor **(2** and **5)** and enters the cell via receptor-mediated endocytosis **(6)**. Under some conditions (e.g., in the lumen of the intestine) the ISVP may be produced by extracellular proteolysis. In endosomes and lysosomes, the virion undergoes acid-dependent proteolytic cleavage **(7)** and can then penetrate the vacuolar membrane **(8** and **9)**. However, the entry of ISVPs may not depend on endocytosis **(10)**. Synthesis of 10 capped viral mRNAs begins within the core particle **(11)**, which is derived from the ISVP. These mRNAs are translated and associate with newly synthesized viral proteins **(12)** to form RNase-sensitive subviral particles in which reassortment may occur **(13)**. Each of the 10 mRNAs is a template for (−) strand RNA synthesis, leading to the production of an RNase-resistant subviral particle that contains 10 double-stranded RNAs **(14)**. Viral mRNAs produced within subviral particles **(15)** are used for the synthesis of viral proteins and the assembly of additional virus particles. In the final steps of capsid assembly, preformed complexes of outer capsid proteins are added to subviral particles **(16)**. Mature virus particles are released from the cell by lysis **(17)**.

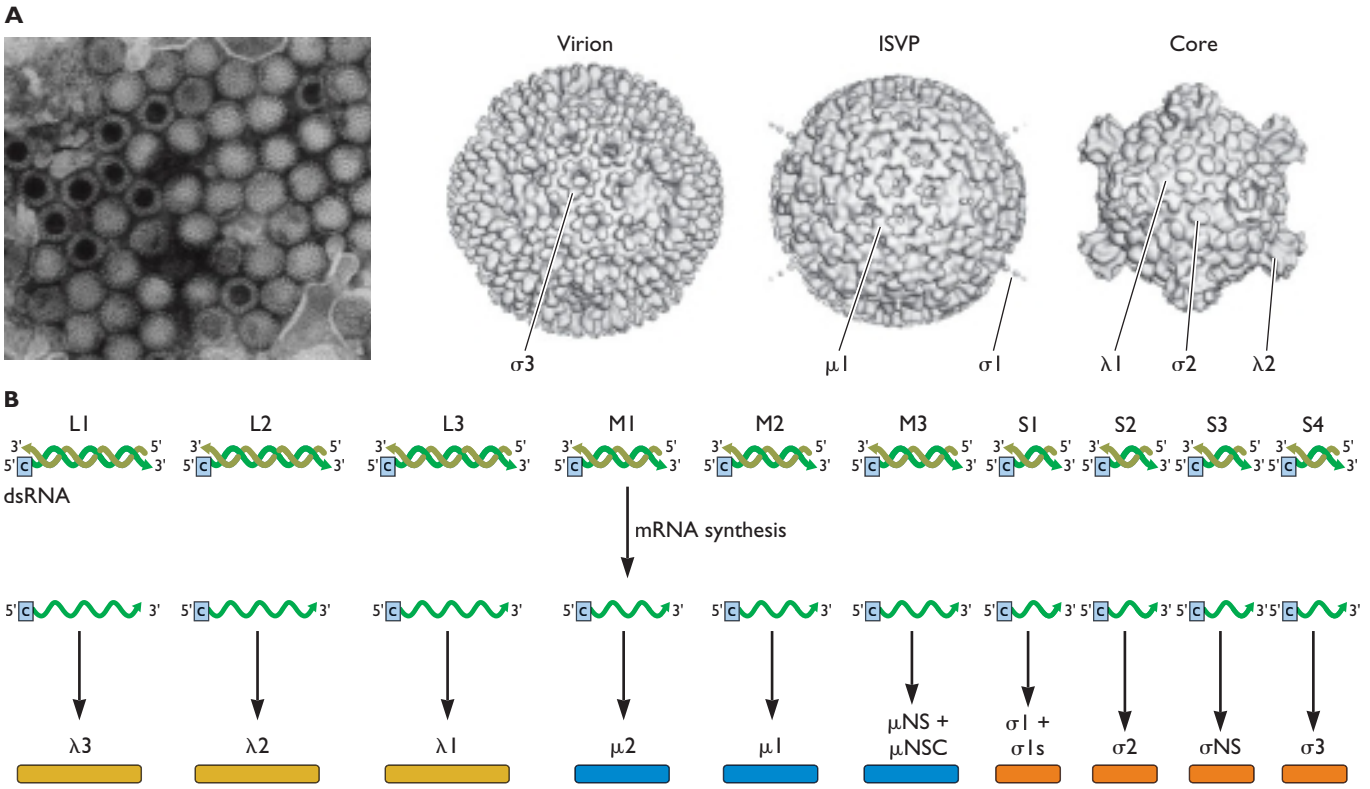

Figure 17 Structure and genomic organization of an orthoreovirus.

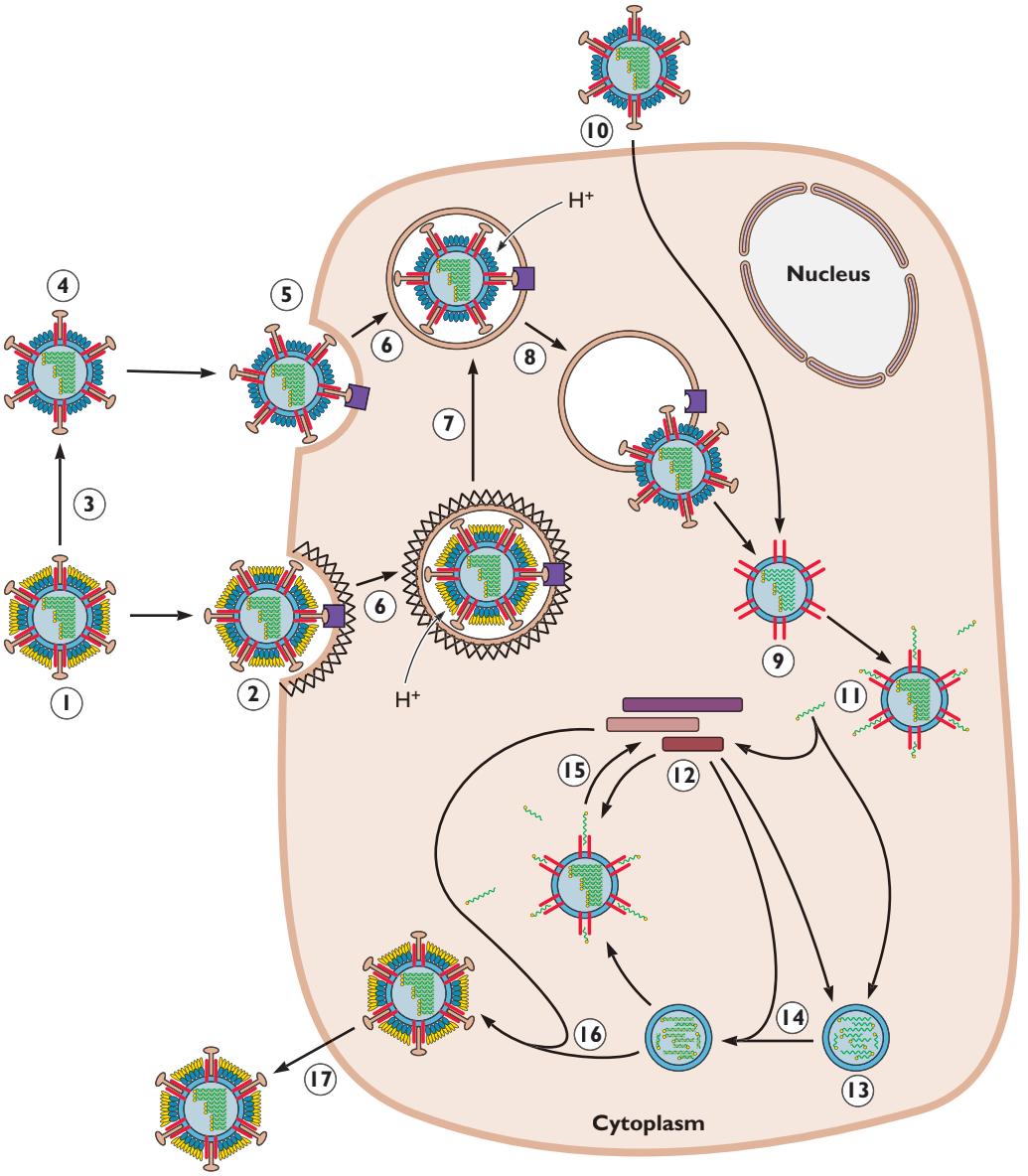

Figure 18 Single-cell reproductive cycle of orthoreovirus.

Retroviruses

Family *Retroviridae*

Genus	Type species
Subfamily *Alphaherpesviridae*	
Alpharetrovirus	Avian leukosis virus
Betaretrovirus	Mouse mammary tumor virus
Gammaretrovirus	Murine leukemia virus
Deltaretrovirus	Bovine leukemia virus
Epsilonretrovirus	Walleye dermal sarcoma virus
Subfamily *Orthoretrovirinae*	
Lentivirus	Human immunodeficiency virus type 1
Subfamily *Spumavirinae*	
Spumavirus	Chimpanzee foamy virus

Retrovirus particles contain the enzyme reverse transcriptase, which mediates synthesis of a double-stranded DNA copy of the viral RNA genome. Although once thought to be unique to this family, similar enzymes are now known to be encoded in other viral genomes (i.e., hepadnaviruses and caulimoviruses), and the term **retroid viruses** has been coined to include these families. Retrovirus particles contain a second enzyme, integrase, that catalyzes the insertion of the viral DNA into essentially random sites in host DNA. The retroviruses can be propagated as integrated elements (called proviruses) that are transmitted in the germ line or as exogenous infectious agents. They infect a wide range of animal hosts and can cause cancer by multiple mechanisms. Some retroviruses, i.e., alpha-, beta-, and gammaretroviruses, have **simple** genomes that encode only the three genes common to all retroviruses—*gag*, *pol*, and *env*. All of the others have more **complex** genomes, which include auxiliary or accessory genes that encode nonstructural proteins that affect viral gene expression and/or pathogenesis.

Figure 19 Structure and genomic organization. (A) Virion structure. The electron micrograph shows a negatively stained alpharetrovirus, Rous sarcoma virus (courtesy of R. Katz and T. Gales, Fox Chase Cancer Center, Philadelphia, PA). Envelope protein projections are not visible in this image. **(B) Genome organization.** (Left) A retrovirus with a simple genome (avian leukosis virus [ALV]), here denoted "Simple retrovirus." Proviral genes are encoded in different reading frames (indicated by different horizontal positions) and are also overlapping. Colored boxes delineate open reading frames; LTR, long terminal repeats that include transcription signals. Origins of RNA and protein products are shown below. U, sequences unique to the 5′ (U5) or 3′ (U3) RNA ends. (Right) A retrovirus with a more complex genome, here denoted "Complex retrovirus" and illustrated with the lentivirus human immunodeficiency virus type 1 (HIV-1). Proviral genes are located in all three reading frames, as indicated by the overlaps. Human immunodeficiency virus type 1 mRNAs fall into one of three classes. The first type is an unspliced transcript of 9.1 kb, identical in function to that of the simple retrovirus shown at the left. The second type comprises singly spliced mRNAs (average length, 4.3 kb) that result from splicing from a 5′ splice site upstream of *gag* to any one of a number of 3′ splice sites near the center of the genome. One of these mRNAs specifies the Env polyprotein precursor, as illustrated for the simple retrovirus. The others specify the human immunodeficiency virus type 1 accessory proteins. The third type comprises a complex class of mRNAs (average length, 1.8 kb) derived by multiple splicing from 5′ and 3′ splice sites throughout the genome. They include mRNAs that specify the regulatory proteins Tat and Rev and are the first to accumulate after infection.

Figure 20 Single-cell reproductive cycle of a simple retrovirus. The virus attaches by binding of the viral envelope protein to specific receptors on the surface of the cell **(1)**. The identities of receptors are known for several retroviruses. The viral core is deposited into the cytoplasm **(2)** following fusion of the virion and cell envelopes. Entry of some beta- and gammaretroviruses may involve endocytic pathways. The viral RNA genome is reverse transcribed by the virion reverse transcriptase (RT) **(3)** within a subviral particle. The product is a linear double-stranded viral DNA with ends that are shown juxtaposed in preparation for integration. Viral DNA and integrase (IN) protein gain access to the nucleus with the help of intracellular trafficking machinery or, in some cases, by exploiting nuclear disassembly during mitosis **(4)**. Integrative recombination, catalyzed by IN, results in site-specific insertion of the viral DNA ends, which can take place at virtually any location in the host genome **(5)**. Transcription of integrated viral DNA (the **provirus**) by host cell RNA polymerase II **(6)** produces full-length RNA transcripts, which are used for multiple purposes. Some full-length RNA molecules are exported from the nucleus and serve as mRNAs **(7)**, which are translated by cytoplasmic ribosomes to form the viral Gag and Gag-Pol polyprotein precursors **(8)**. Some full-length RNA molecules are destined to become encapsidated as progeny viral genomes **(9)**. Other full-length RNA molecules are spliced within the nucleus **(10)** to form mRNA for the Env polyprotein precursor proteins. Env mRNA is translated by ribosomes bound to the endoplasmic reticulum (ER) **(11)**. The Env proteins are transported through

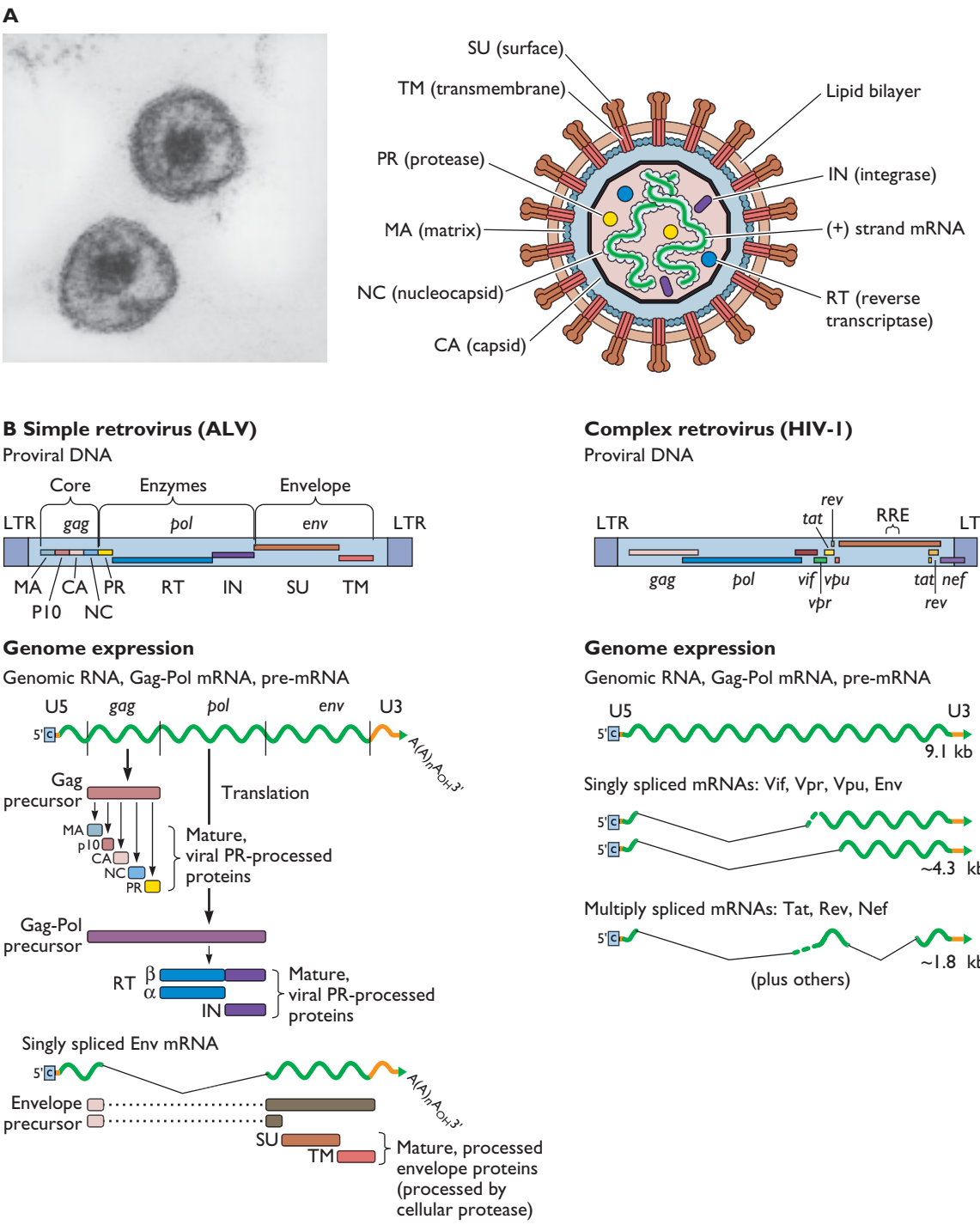

Figure 19 Structure and genomic organization.

the Golgi apparatus **(12)**, where they are glycosylated and cleaved by cellular enzymes to form the mature SU-TM complex. Mature envelope proteins are delivered to the surface of the infected cell **(13)**. Virion components (viral RNA, Gag and Gag-Pol precursors, and SU-TM) assemble at budding sites **(14)** with the help of *cis*-acting signals encoded in each that exploit intracellular vesicular trafficking machinery. Type C retroviruses (e.g., alpharetroviruses and lentiviruses) assemble at the inner face of the plasma membrane, as illustrated. Other types (A, B, and D) assemble on internal cellular membranes. The nascent virions bud from the surface of the cell **(15)**. Maturation (and infectivity) requires the action of the virus-encoded protease (PR), which is itself a component of the core precursor polyprotein. During or shortly after budding, PR cleaves at specific sites within the Gag and Gag-Pol precursors **(16)** to produce the mature virion proteins. This process causes a characteristic condensation of the virion cores.

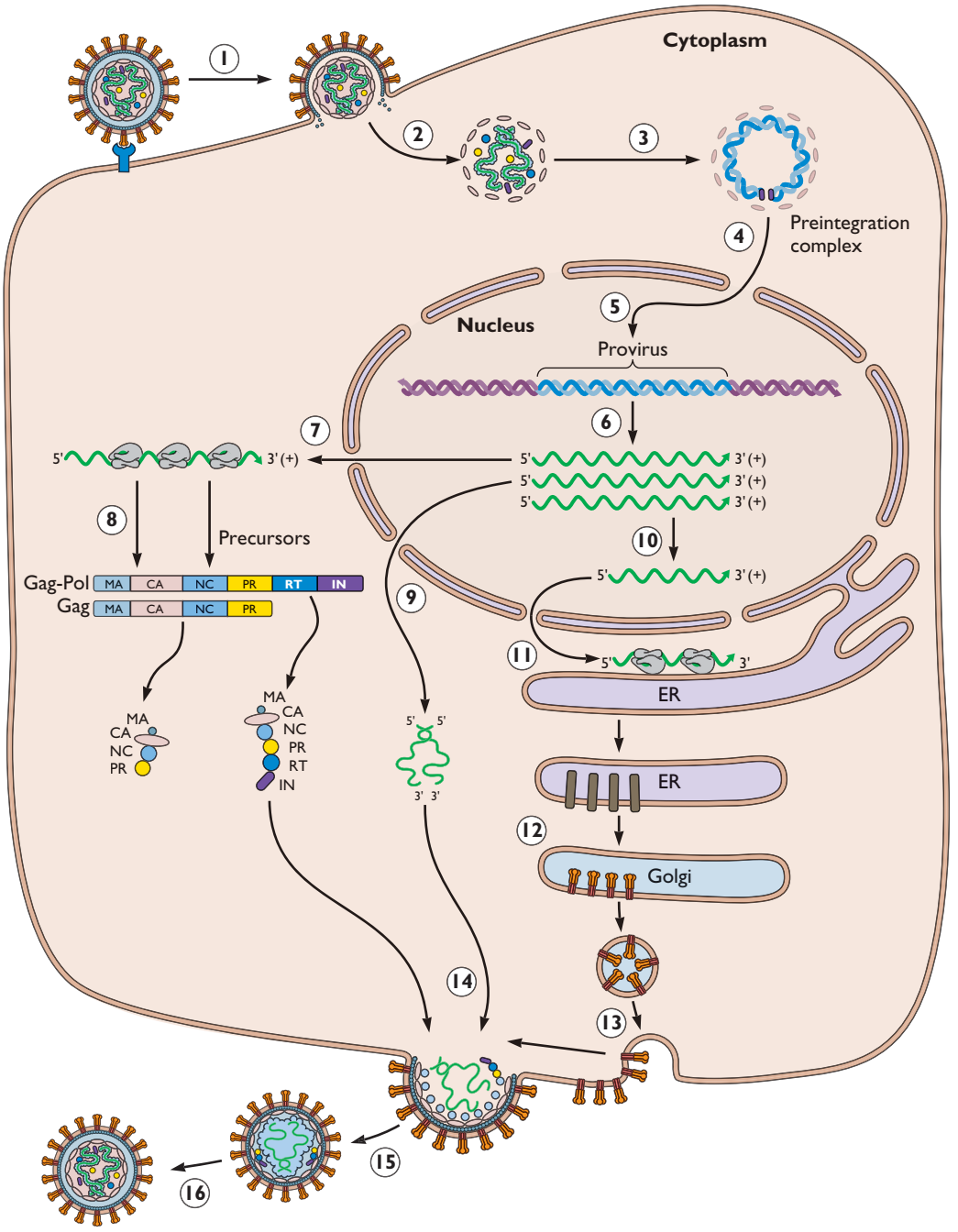

Figure 20 Single-cell reproductive cycle of a simple retrovirus.

Rhabdoviruses

Family *Rhabdoviridae*

Genus	Type species
Vesiculovirus	Vesicular stomatitis virus Indiana
Lyssavirus	Rabies virus
Ephemerovirus	Bovine ephemeral fever virus
Novirhabdovirus	Infectious hematopoietic necrosis virus
Cytorhabdovirus	Lettuce necrotic yellows virus
Nucleorhabdovirus	Potato yellow dwarf virus

Among the 175 known rhabdoviruses are the causative agents of rabies, one of the oldest recognized infectious diseases, and economically important diseases of fish. The host range of these viruses is very broad: they infect many vertebrates, invertebrates, and plants. The genome of vesicular stomatitis virus has been a model for the replication and expression of viral genomes that consist of a single molecule of (–) strand RNA. The first RNA-dependent RNA polymerase discovered in a virus particle was that of vesicular stomatitis virus.

Figure 21 Structure and genomic organization of vesicular stomatitis virus. (A) Virion structure. The electron micrograph shows negatively stained vesicular stomatitis virus (courtesy of J. Rose, Yale University School of Medicine, New Haven, CT). **(B) Genome organization.** The (–) strand RNA is the template for synthesis of leader RNA and five monocistronic mRNAs (capped and polyadenylated) encoding the five viral proteins.

Figure 22 Single-cell reproductive cycle. The virion binds to a cellular receptor and enters the cell via receptor-mediated endocytosis **(1)**. The viral membrane fuses with the membrane of the endosome, releasing the helical viral nucleocapsid **(2)**. This structure comprises (–) strand RNA coated with nucleocapsid protein molecules and a small number of L and P protein molecules, which catalyze viral RNA synthesis. The (–) strand RNA is copied into five subgenomic mRNAs by the L and P proteins **(3)**. The N, P, M, and L mRNAs are translated by free cytoplasmic ribosomes **(4)**, while G mRNA is translated by ribosomes bound to the endoplasmic reticulum **(5)**. Newly synthesized N, P, and L proteins participate in viral RNA replication. This process begins with synthesis of a full-length (+) strand copy of genomic RNA, which is also in the form of a ribonucleoprotein containing the N, L, and P proteins **(6)**. This RNA in turn serves as a template for the synthesis of progeny (–) strand RNA in the form of nucleocapsids **(7)**. Some of these newly synthesized (–) strand RNA molecules enter the pathway for viral mRNA synthesis **(8)**. Upon translation of G mRNA, the G protein enters the secretory pathway **(9)**, in which it becomes glycosylated and travels to the plasma membrane **(10)**. Progeny nucleocapsids and the M protein are transported to the plasma membrane **(11** and **12)**, where association with regions containing the G protein initiates assembly and budding of progeny virions **(13)**.

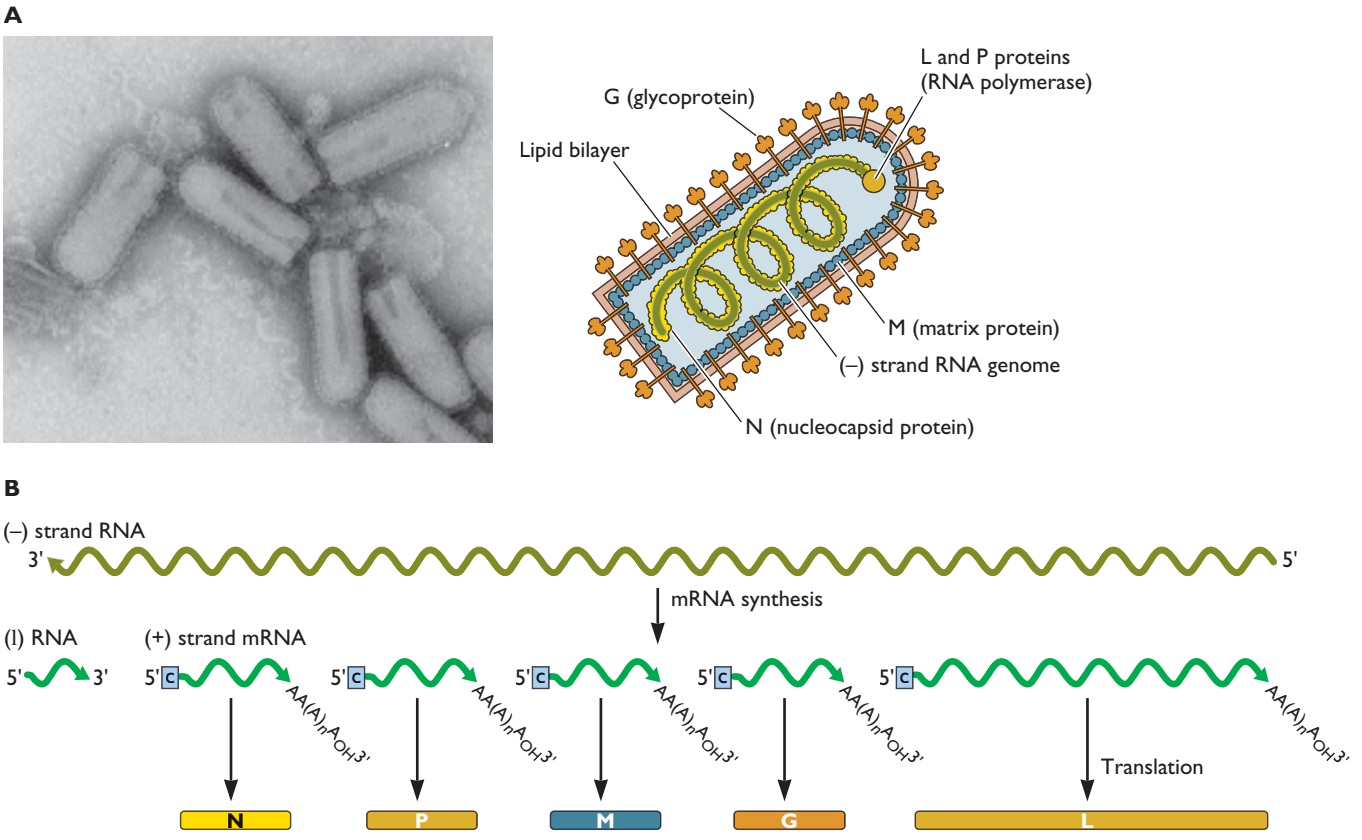

Figure 21 Structure and genomic organization of vesicular stomatitis virus.

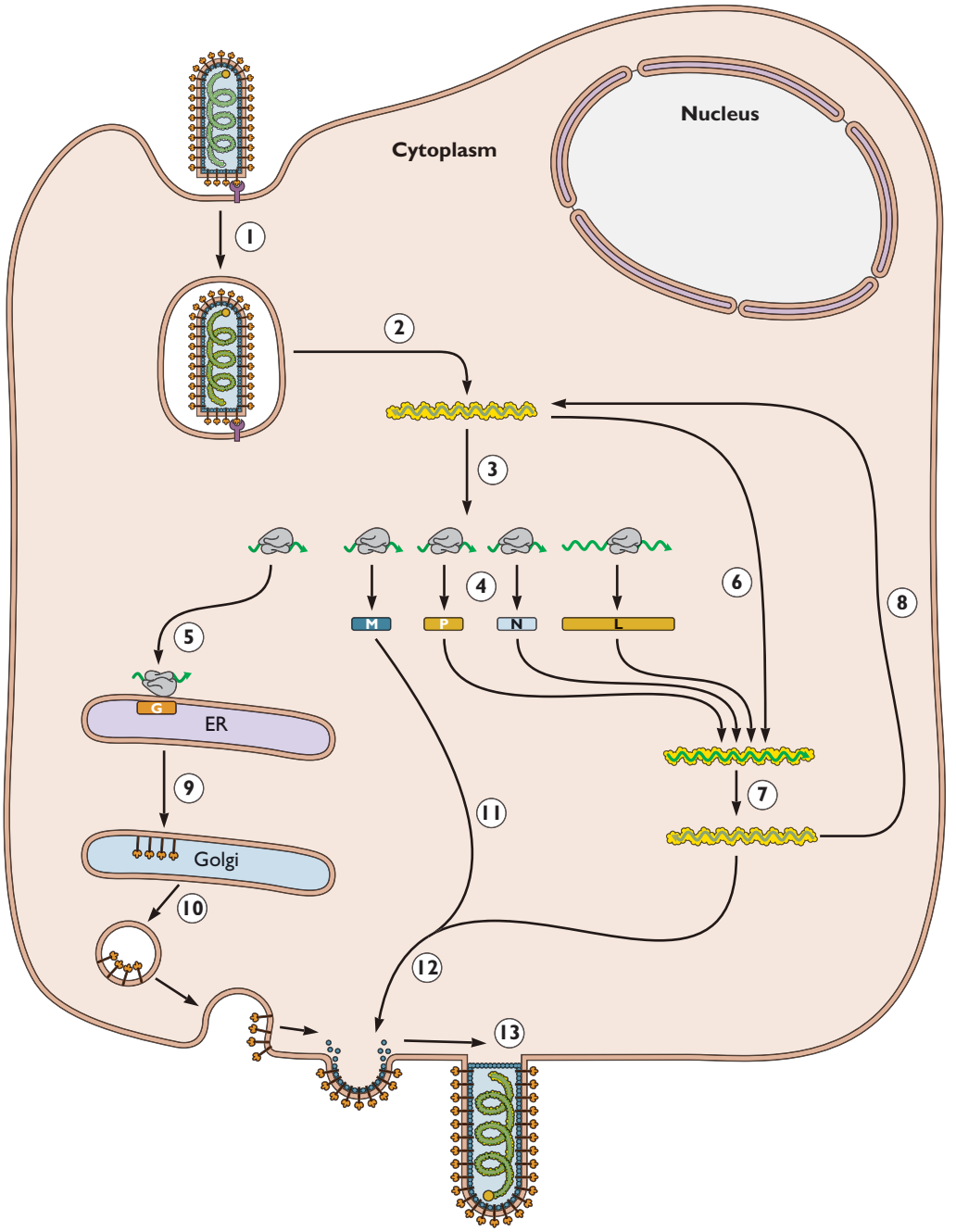

Figure 22 Single-cell reproductive cycle.

Togaviruses

Family *Togaviridae*

Genus	Type species
Alphavirus	Sindbis virus
Rubivirus	Rubella virus

Members of the *Togaviridae* are responsible for two very different kinds of human disease. The alphaviruses are all transmitted by arthropods and cause encephalitis, arthritis, and rashes in humans. Rubella virus is the agent of a mild rash disease that can also cause congenital abnormalities in the fetus when acquired by the mother early in pregnancy. Because these virions have a lipid envelope, they have been important models for studying the synthesis, posttranslational modification, and localization of membrane glycoproteins.

Figure 23 Structure and genomic organization. (A) Virion structure. The cryo-electron micrograph shows the alphavirus Ross River virus (courtesy of N. Olson, Purdue University, West Lafayette, IN). The three-dimensional image reconstructions (courtesy of B. V. V. Prasad, Baylor College of Medicine, Houston, TX) show the intact Sindbis virus (yellow, with glycoprotein spikes visible), its nucleocapsid (blue), and a cross section of the particle (green), and illustrate the relationship among the spike glycoproteins (S), the lipid membrane (M), the capsid (C), and the viral RNA genome (RNA). **(B) Genome organization.** The first two-thirds of togavirus genomic RNA, which is of (+) polarity and carries a 5′ cap, is translated to produce the polyproteins P123 and P1234. The latter is the precursor of the RNA polymerase. For some alphaviruses, the P1234 polyprotein is produced by translational suppression of a stop codon located at the end of the nsP3 coding region. The proteins encoded in the 3′-terminal one-third of the genome are produced from a subgenomic mRNA that is copied from a full-length (−) strand RNA intermediate. The subgenomic mRNA encodes the structural proteins.

Figure 24 Single-cell reproductive cycle. The virion binds to a cellular receptor and enters the cell via receptor-mediated endocytosis **(1)**. Upon acidification of the vesicle, viral RNA is uncoated **(2)** and translated to form the polyprotein P1234. Sequential cleavage of this polyprotein at different sites produces RNA polymerases with different specificities. **(3)** These viral enzymes then copy (+) strands into full-length (−) and (+) strands **(4)** and catalyze synthesis of the subgenomic mRNA **(5)**. This mRNA is translated by free cytoplasmic ribosomes to produce the capsid protein **(6)**; proteolytic cleavage to liberate the capsid protein exposes a hydrophobic sequence of PE2 that induces the ribosomes to associate with the endoplasmic reticulum (ER) **(7)**. As a result, the PE2-6K-E1 polyprotein enters the secretory pathway. The glycoproteins are transported to the cell surface (**8** and **9**). The capsid protein and (+) strand genomic RNA assemble to form capsids **(10)** that migrate to the plasma membrane and associate with viral glycoproteins **(11)**. The capsid acquires an envelope by budding at this site **(12)**, and mature virions are released **(13).**

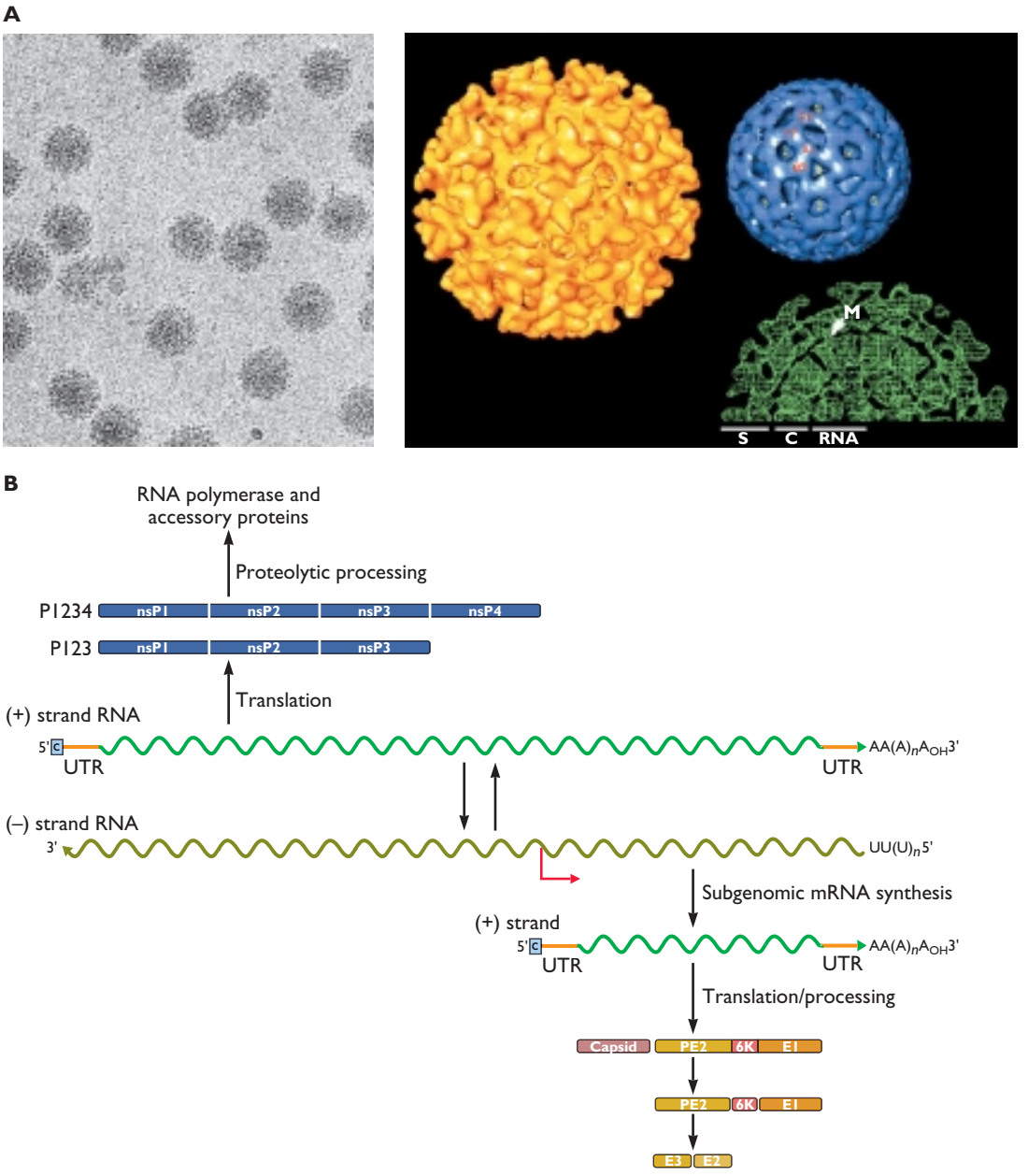

Figure 23 Structure and genomic organization.

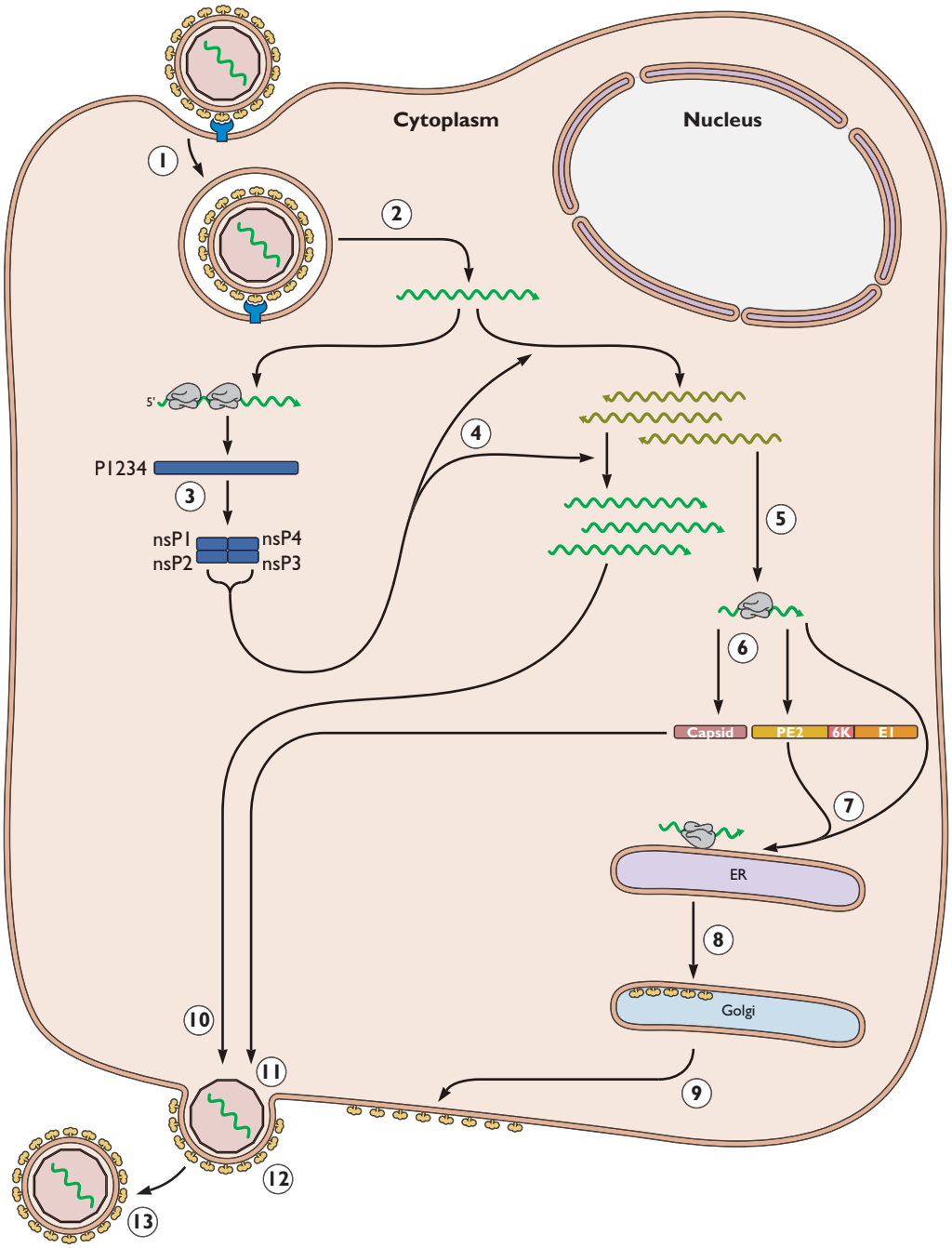

Figure 24 Single-cell reproductive cycle.

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Glossary

Absolute efficiency of plating The plaque titer divided by the number of virus particles in the sample. *(Chapter 2)*

Acylation Posttranslational addition of saturated or unsaturated fatty acids to a protein. *(Chapters 9 and 12)*

Allele specific Complementing only a specific change; refers to suppressor mutations. *(Chapter 3)*

Alternative splicing Splicing of different combinations of exons in a pre-mRNA, generally leading to synthesis of mRNAs with different protein-coding sequences. *(Chapter 10)*

Ambisense Producing mRNAs from both (−) strand genomic RNA and the complementary (+) strand; refers to viral genomes. *(Chapter 6)*

Amphipathic Having both hydrophilic and hydrophobic portions. *(Chapter 2)*

Aneuploid Abnormal in chromosome morphology and number. *(Chapter 2)*

Apical surface The specialized surface of a epithelial cell exposed to the environment. Also called apical domain. *(Chapters 2, 9, and 12)*

Asymmetric unit The unit from which capsids or nucleocapsids of a virus particle are built. Also called protomer or structural unit. *(Chapter 4)*

Attenuated An infection in which normally severe symptoms or pathology are mild or inconsequential; a state of reduced virulence. *(Chapter 1)*

Bacteriophages Viruses that infect bacteria; derived from the Greek word *phagein*, meaning "to eat." *(Chapter 1)*

Basal lamina A thin layer of extracellular matrix bound tightly to the basolateral surface of cells; the basal lamina is linked to the basolateral membrane by integrins. *(Chapter 2)*

Basolateral surface The nonspecialized surface of an epithelial cell that contacts an internal basal lamina or adjacent or underlying cells in the tissue. Also called basolateral domain. *(Chapters 2, 9, and 12)*

Burst The yield of viruses from one cell. *(Chapter 2)*

Capping The addition of m^7G via a 5′-5′ phosphodiester bond to the 5′ ends of cellular and viral transcripts made in eukaryotic cells. *(Chapter 10)*

Capsid The outer shell of viral proteins that surrounds the genome in a virus particle. *(Chapters 1, 3, and 4)*

Cap snatching Cleavage of cellular RNA polymerase II transcripts by a viral endonuclease to produce capped primers for viral mRNA synthesis. *(Chapter 10)*

Caveolae Flask-shaped invaginations of the plasma membrane of many types of cells that contain the protein caveolin and are rich in lipid rafts; caveolae internalize membrane components, extracellular ligands, bacterial toxins, and some animal viruses. *(Chapter 5)*

Centrosome An organelle that is the main microtubule-organizing center. *(Chapter 5)*

Chaperone A protein that facilitates the folding of other polypeptide chains, the assembly of multimeric proteins, or the formation of macromolecular assemblies (e.g., chromatin). Also called molecular chaperone. *(Chapters 4, 12, and 13)*

Chemokines Small proteins that attract and stimulate cells of the immune defense; produced by many cells in response to infection. Also called chemotactic cytokines. *(Chapter 5)*

Coactivator A protein that stimulates transcription by RNA polymerase II without binding to a specific DNA sequence; generally interacts with sequence-specific transcriptional activators. *(Chapter 8)*

Codon Three contiguous bases in an mRNA template that specify the amino acids incorporated into protein. *(Chapter 11)*

Complementation The ability of gene products of two different, nonreplicating mutants to interact functionally in the same cell to permit viral replication. *(Chapter 3)*

Constitutive splicing Splicing of a pre-mRNA that removes all introns and joins all exons. *(Chapter 10)*

Constitutive transport elements Sequences in certain unspliced viral mRNAs that direct export from the nucleus by host cell proteins. *(Chapter 10)*

Continuous cell lines Cultures of a single cell type that can be propagated indefinitely in culture. *(Chapter 2)*

Copy choice A mechanism of recombination in which an RNA polymerase first copies the 3′ end of one parental strand and then exchanges one template for another at the corresponding position on a second parental strand. *(Chapters 6 and 7)*

Coreceptor A cell surface molecule that is required, in addition to the receptor, for entry of virus particles into cells. *(Chapter 5)*

Core promoter The minimal set of DNA sequences required for accurate initiation of transcription by RNA polymerase II. *(Chapter 8)*

Culling Removing and destroying diseased or potentially exposed animals to prevent further spread of infection. *(Chapter 1)*

Cytopathic effects The morphological changes induced in cells by viral infection. *(Chapter 2)*

Cytoskeleton The intracellular structural network composed of actin filaments, microtubules, and intermediate filaments. *(Chapter 2)*

Defective interfering RNAs Subgenomic RNAs that replicate more rapidly than full-length RNA and therefore compete for the components of the RNA synthesis machinery and interfere with the replication of full-length RNAs. *(Chapter 6)*

Deletion mutation Loss of one or more bases in a nucleic acid. *(Chapter 3)*

Diploid cell strains Cell cultures that consist of a homogeneous population of a single type and that can divide up to 100 times before dying. *(Chapter 2)*

Eclipse period The phase of viral infection during which the viral nucleic acid is uncoated from its protective shell and no infectious virus can be detected inside cells. *(Chapter 2)*

Elongation Stepwise incorporation of ribodeoxynucleoside monophosphates or deoxynucleoside monophosphates into the 3′-OH end of the growing RNA or DNA chain in the 5′ → 3′ direction. *(Chapter 6)*

Endemic Having a disease pattern typical of a particular geographic area; persisting in a population for a long period without reintroduction of the causative virus from outside sources. *(Chapter 1)*

Endogenous proviruses Proviruses that enter the germ line at some point in the history of an organism and are thereafter inherited in normal Mendelian fashion by every cell in that organism and by its progeny. *(Chapter 7)*

Endosome A vesicle that transports molecules from the plasma membrane to the cell interior. *(Chapter 5)*

Enhancer A DNA sequence containing multiple elements that can stimulate RNA polymerase II transcription over long distances, independently of orientation or location relative to the site of transcriptional initiation. *(Chapter 8)*

Envelope The host cell-derived lipid bilayer carrying viral glycoproteins that forms the outer layer of many virus particles. *(Chapter 4)*

Epidemic A pattern of disease characterized by rapid and sudden appearance of cases spreading over a wide area. *(Chapter 1)*

Epitope A short contiguous sequence or unique conformation of a macromolecule that can be recognized by the immune system; also called an antigenic determinant; a T-cell epitope is a short peptide recognized by a particular T-cell receptor, while a B-cell epitope is recognized by the antigen-binding domain of antibody and is part of an intact protein. *(Chapter 2)*

Exons Blocks of noncontiguous coding sequences (generally short) present in many cellular and viral pre-mRNAs. *(Chapter 10)*

Foci Clusters of cells that are derived from a single progenitor and share properties, such as unregulated growth, that cause them to pile up on one another. *(Chapter 2)*

Fusion peptide A short hydrophobic amino acid sequence (20 to 30 amino acids) that is thought to insert into target membranes to initiate fusion. *(Chapter 5)*

Fusion pore An opening between two lipid bilayers formed by the action of fusion proteins; it allows exchange of material across membranes. *(Chapter 5)*

Glycoforms The total set of forms of a protein that differ in the number, location, and nature of oligosaccharide chains. *(Chapter 12)*

Glycoprotein A protein carrying covalently linked sugar chains (oligosaccharides). *(Chapter 4)*

G_0 state A state in which the cell has ceased to grow and divide and has withdrawn from the cell cycle. Also called resting state. *(Chapter 9)*

Half-life The time required for decay to half of the original value. *(Chapter 10)*

Helical symmetry The symmetry of regularly wound structures defined by the relationship $P = \mu \times \rho$, where P = pitch of the helix, μ = the number of structural units per turn, and ρ = the axial rise per unit. *(Chapter 4)*

Helper virus A virus that provides viral proteins needed for the replication of a coinfecting defective virus. *(Chapter 6)*

Hemagglutination A method for measuring virus concentrations via the linking of multiple red blood cells by virus particles, resulting in a lattice. *(Chapter 2)*

Heterogeneous nuclear RNAs Nuclear precursors to mRNAs that are larger than mRNAs and heterogeneous in size. *(Chapter 10)*

Homologous recombination The exchange of genetic information between any pair of related DNA sequences. *(Chapter 9)*

Host range A listing of species and cells (hosts) that are susceptible to and permissive for infection. *(Chapter 5)*

Icosahedral symmetry The symmetry of the icosahedron, the solid with 20 faces and 12 vertices related by axes of two-, three-, and fivefold rotational symmetry. *(Chapter 4)*

Indirectly anchored proteins Proteins that are indirectly bound to the plasma membrane by interacting with either integral membrane proteins or the charged sugars of membrane glycolipids. *(Chapter 2)*

Infectious DNA clone A double-stranded DNA copy of the viral genome carried on a bacterial plasmid or other vector. *(Chapter 3)*

Initiation site The site at which transcription of a gene begins. *(Chapter 8)*

Initiator A short DNA sequence that is sufficient to specify the site at which RNA polymerase II initiates transcription. *(Chapter 8)*

Insertion mutation Addition of a nucleic acid sequence. *(Chapter 3)*

Integral membrane proteins Proteins that are embedded in a lipid bilayer, with external and internal domains connected by one or more membrane-spanning domains. *(Chapters 2 and 4)*

Introns Noncoding sequences that separate coding sequences (exons) in many cellular and viral pre-mRNAs. *(Chapter 10)*

Inverted terminal repetitions Sequences that are present in the opposite orientation at the ends of certain linear viral DNA genomes. *(Chapter 9)*

Koch's postulates Criteria developed by the German physician Robert Koch in the late 1800s to determine if a given agent is the cause of a specific disease. *(Chapter 1)*

Lagging strand The daughter DNA strand made by discontinuous synthesis during DNA replication. *(Chapter 9)*

Lariat An intermediate in pre-mRNA splicing containing the intron and 3′ exon, with the branch point A residue of the intron linked via a 2′-5′ phosphodiester bond to the nucleotide at the 5′ end of the intron. *(Chapter 10)*

Latency-associated transcript RNA produced specifically during a latent infection by herpes simplex virus. *(Chapter 8)*

Latent period The phase of viral infection during which no extracellular virus can be detected. *(Chapter 2)*

L domain sequences Short amino acid sequences required for membrane fusion during budding of enveloped viruses. *(Chapter 13)*

Leading strand The daughter DNA strand made by continuous synthesis during DNA replication; its synthesis begins before that of the lagging strand. *(Chapter 9)*

Lipid raft A microdomain of the plasma membrane that is enriched in cholesterol and saturated fatty acids and is more densely packed and less fluid than other regions of the membrane. *(Chapters 2 and 12)*

Long terminal repeat A direct repeat of genetic information that is present in the proviral DNA of retroviruses; it is formed by reverse transcription of the RNA template and includes *cis*-acting elements required for viral DNA integration and its subsequent transcription. *(Chapter 7)*

Lysogenic Pertaining to a bacterium that carries the genetic information of a quiescent bacteriophage, which can be induced to reproduce, and subsequently lyse, the bacterium. *(Chapter 1)*

Lysogeny The phenomenon by which the lysogenic state is established and maintained in bacteria. *(Chapter 1)*

Lysosome A vesicle in the cell that contains enzymes that degrade sugars, proteins, nucleic acids, and lipids. *(Chapter 5)*

Marker rescue Replacement of all local nucleic acids that include a mutation with wild-type nucleic acid. *(Chapter 3)*

Marker transfer Introduction of a mutation by replacement of a segment of viral nucleic acid with one containing the mutation. *(Chapter 3)*

Membrane-spanning domain A segment of an integral membrane protein that spans the lipid bilayer; often α-helical. *(Chapters 2 and 4)*

Metastable structure A structure that has not attained the lowest free energy state. *(Chapter 4)*

Microdomains Regions of the plasma membrane with distinct lipid and protein composition. *(Chapter 2)*

Minichromosome maintenance element A sequence of papillomavirus genomes required for maintenance of episomal viral genomes in a host cell population. *(Chapter 9)*

Missense mutation A change in a single nucleotide or codon that results in the production of a protein with a single amino acid substitution. *(Chapter 3)*

Molecular chaperone *See* Chaperone.

Monocistronic Encoding one polypeptide; refers to mRNA. *(Chapter 11)*

Monoclonal antibody An antibody of a single specificity made by a clone of antibody-producing cells. *(Chapter 2)*

Monolayer A layer of cultured cells growing in a cell culture dish. *(Chapter 2)*

Multiplicity of infection The number of infectious viruses added per cell. *(Chapter 2)*

Mutagen An agent that causes base changes in nucleic acids. *(Chapter 3)*

Negative [(–)] strand The strand of DNA or RNA that is complementary in sequence to the (+) strand. *(Chapter 1)*

Neutralize To block (by antibodies) the infectivity of viruses. *(Chapter 2)*

Nonsense mutation A substitution mutation that produces a translation termination codon. *(Chapter 3)*

Nuclear localization signal Amino acid sequence that is necessary and sufficient for import of a protein into the nucleus. *(Chapter 5)*

Nucleocapsid A nucleic acid-protein assembly packaged within the virion; the term is used when this complex is a discrete substructure of a complex particle. *(Chapter 4)*

Obligate parasites Organisms that are dependent on another living organism for reproduction. *(Chapter 1)*

Oligomerization Association of protein subunits, which may be the same or different, to form a protein with multiple subunits. *(Chapter 8)*

Oligosaccharide A short linear or branched chain of sugar residues (monosaccharides). *(Chapter 4)*

One-hit kinetics A linear relationship between plaque count and virus concentration that indicates that one infectious particle is sufficient to initiate infection. *(Chapter 2)*

One-step growth curve A single replication cycle that occurs synchronously in every infected cell. *(Chapter 2)*

Origin for plasmid maintenance The origin of Epstein-Barr virus DNA active in latently infected cells but not in productively infected cells. *(Chapter 9)*

Origins (of replication) Specific sites at which replication of DNA begins. *(Chapter 9)*

Packaging Incorporation of the viral genome during assembly of virus particles. *(Chapter 13)*

Packaging signal Nucleic acid sequence or structural feature directing incorporation of a viral genome into a virus particle. *(Chapter 13)*

Particle–to–plaque-forming-unit (PFU) ratio The inverse value of the absolute efficiency of plating. *(Chapter 2)*

Pathogen Disease-causing virus or other microorganism. *(Chapter 1)*

Permissive Able to support virus replication when viral nucleic acid is introduced; refers to cells. *(Chapter 2)*

Plaque A circular zone of infected cells that can be distinguished from the surrounding monolayer. *(Chapter 2)*

Plaque-forming units per milliliter A measure of virus infectivity. *(Chapter 2)*

Plaque purified Prepared from a single plaque (refers to virus stock); when one infectious virus particle initiates a plaque, the viral progeny within the plaque are clones. *(Chapter 2)*

Polarized cells Differentiated cells with surfaces divided into functionally specialized regions. *(Chapter 12)*

Polyadenylation The addition of ~200 A residues to the 3′ ends of cellular and viral transcripts made in eukaryotic cells. *(Chapter 10)*

Polycistronic Encoding several polypeptides; refers to mRNA. *(Chapter 11)*

Polyclonal antibodies The antibody repertoire against the many epitopes of an antigen produced in an animal. *(Chapter 2)*

Portal A specialized structure for entry of a viral genome into a preassembled protein shell. *(Chapter 4)*

Positive [(+)] strand The strand of DNA or RNA that corresponds in sequence to that of the messenger RNA. Also known as the sense strand. *(Chapter 1)*

Pregenomic mRNA The hepadnaviral mRNA that is reverse transcribed to produce the DNA genome. *(Chapter 7)*

Preinitiation complex A promoter-bound complex of an RNA polymerase and initiator proteins competent to initiate transcription. *(Chapter 8)*

Primary cell cultures Cell cultures prepared from animal tissues; these cultures include several cell types and have a limited life span, usually no more than 5 to 20 cell divisions. *(Chapter 2)*

Primary cells Cells that have been freshly derived from an organ or tissue. *(Chapter 1)*

Primase An enzyme that synthesizes RNA primers for DNA synthesis. *(Chapter 9)*

Primer A free 3′-OH group required for initiation of synthesis of DNA from DNA or RNA templates and initiation of synthesis of some viral RNA genomes. *(Chapters 6 and 9)*

Prions Infectious agents comprising an abnormal isoform of a normal cellular protein but no nucleic acid; implicated as the causative agents of transmissible spongiform encephalopathies. *(Chapter 1)*

Procapsid A closed, protein-only structure into which viral genomes are inserted; precursor to a capsid or nucleocapsid. *(Chapter 13)*

Processivity The ability of an enzyme to copy a nucleic acid template over long distances from a single site of initiation. *(Chapters 7, 8, and 9)*

Promoter A set of DNA sequences necessary for initiation of transcription by a DNA-dependent RNA polymerase. *(Chapter 8)*

Promoter occlusion The mechanism by which access to a promoter is blocked by passage of a transcribing RNA polymerase. *(Chapter 8)*

Proofreading Correction of mistakes made during chain elongation by exonuclease activities of DNA-dependent DNA polymerases. *(Chapter 6)*

Prophage The genome of the quiescent bacteriophage in a lysogenic bacterium. *(Chapter 1)*

Proteasome A complex containing multiple proteases that is responsible for degradation of proteins tagged with polyubiquitin. *(Chapter 8)*

Proteoglycans Proteins linked to glycosaminoglycans, which are unbranched polysaccharides made of repeating disaccharides. *(Chapter 2)*

Protomer *See* Asymmetric unit.

Proviral DNA *See* Provirus.

Provirion A noninfectious precursor of a mature virion. *(Chapter 13)*

Provirus Retroviral DNA that is integrated into its host cell genome and is the template for formation of retroviral mRNAs and genomic RNA. Also called proviral DNA. *(Chapter 7)*

Pseudodiploid Having two RNA genomes per virion that give rise to only one DNA copy, as is the case for retroviruses. *(Chapter 7)*

Pseudoreversion Phenotypic reversion caused by second-site mutation; also known as suppression. *(Chapter 3)*

Quasiequivalence The arrangement of structural units in a virus particle such that similar interactions among them are allowed. *(Chapter 4)*

Quasispecies Virus populations that exist as dynamic distributions of nonidentical but related replicons. *(Chapter 6)*

Reactivation A switch from a latent to a productive infection; usually applied to herpesviruses. *(Chapter 8)*

Reassortants Viral genomes that have exchanged segments after coinfection of cells with viruses with segmented genomes. *(Chapter 3)*

Reassortment The exchange of entire RNA molecules between genetically related viruses with segmented genomes. *(Chapters 3 and 6)*

Receptor The cellular molecule to which a virus attaches to initiate replication. *(Chapter 5)*

Receptor-mediated endocytosis The uptake of molecules into the cell from the extracellular fluid; in this process, the molecule binds a cell surface receptor, and the complex is taken into the cell by invagination of the membrane and formation of a vesicle. *(Chapter 5)*

Relative efficiency of plating A ratio of viral titers obtained on two different cell types: this number may be more or less than 1, depending on how well the virus replicates in the different host cells. *(Chapter 2)*

Replication centers Specialized nuclear structures in which viral DNA genomes are replicated. Also called replication compartments. *(Chapter 9)*

Replication forks The sites of synthesis of nascent DNA chains that move away from origin as replication proceeds. *(Chapter 9)*

Replication intermediate An incompletely replicated DNA molecule containing newly synthesized DNA. *(Chapter 9)*

Replication licensing Mechanisms which ensure that replication of cellular DNA is initiated at each origin once, and only once, per cell cycle. *(Chapter 9)*

Replicon A unit of replication in large genomes, defined by discrete origin and termini. *(Chapter 9)*

Resolution The minimal size of an object that can be distinguished by microscopy or other methods of structural analysis. *(Chapter 4)*

Resting state *See* G_0 state.

Retroid viruses Viruses that replicate their genomes via reverse transcription. *(Chapter 7)*

Revert To change to the parental, or wild-type, genotype or phenotype. *(Chapter 3)*

Ribosome A complex of RNAs and proteins that is the site of translation. *(Chapter 11)*

Ribozyme An RNA molecule with catalytic activity. *(Chapter 10)*

RNA-dependent RNA polymerase The protein assembly required to carry out RNA synthesis. *(Chapter 6)*

RNA editing The introduction into an RNA molecule of nucleotides that are not specified by a cellular or viral gene. *(Chapter 10)*

RNA interference A mechanism of posttranscriptional regulation of gene expression by small RNA molecules that induce mRNA degradation or inhibition of translation. *(Chapters 3 and 10)*

RNA processing The series of co- or posttranscriptional covalent modifications that produce mature mRNAs from primary transcripts. *(Chapter 10)*

RNA pseudoknot An RNA secondary structure formed when a single-stranded loop region base pairs with a complementary sequence outside the loop. *(Chapter 6)*

Rule of six The requirement that the (−) strand RNA genome of a paramyxovirus be copied efficiently only when its length in nucleotides is a multiple of 6. *(Chapter 6)*

Satellites Small, single-stranded RNA molecules that lack genes required for their replication but do replicate in the presence of another virus (the **helper virus**). *(Chapter 1)*

Satellite virus A satellite with a genome that encodes one or two proteins. *(Chapter 1)*

Scaffolding protein A viral protein that is required for assembly of an icosahedral protein shell but is absent from mature virions. *(Chapter 13)*

Secretory pathway The series of membrane-demarcated compartments (e.g., the endoplasmic reticulum and Golgi apparatus), tubules, and vesicles through which secreted and membrane proteins travel to the cell surface. *(Chapter 12)*

Self-priming A mechanism by which some viral DNA genomes serves as primers, as well as templates, for DNA synthesis. *(Chapter 9)*

Semiconservative replication Production of two daughter DNA molecules, each containing one strand of the parental template and a newly synthesized complementary strand. *(Chapter 9)*

Serotype A virus type as defined on the basis of neutralizing antibodies. *(Chapter 2)*

Signal peptide A short sequence (generally hydrophobic) that directs nascent proteins to the endoplasmic reticulum. The signal may be removed, or retained as a transmembrane domain. *(Chapter 12)*

Single-exon mRNAs mRNAs produced without splicing, because their precursors lack introns and splice sites. *(Chapter 10)*

siRNAs *See* Short interfering RNAs.

Site-specific recombination Exchange of DNA sequences at short DNA sequences that are specifically recognized by proteins that catalyze recombination. *(Chapter 9)*

Small interfering RNAs Small RNA molecules that induce mRNA cleavage or inhibition of translation. Abbreviated siRNAs. *(Chapter 3)*

Small nuclear ribonucleoproteins Structures that contain small nuclear RNAs and several proteins; several participate in pre-mRNA splicing. *(Chapter 10)*

S phase The phase of the cell cycle in which the DNA genome is replicated. *(Chapter 9)*

Spliceosome The large complex that assembles on an intron-containing pre-mRNA before splicing; in mammalian cells, it comprises the small nuclear ribonucleoproteins containing U1, U2, U4, U5, and U6 small nuclear RNAs and ~150 proteins. *(Chapter 10)*

Splice sites Sites at which pre-mRNA sequences are cleaved and ligated during splicing; defined by short consensus sequences. *(Chapter 10)*

Splicing The precise ligation of blocks of noncontiguous coding sequences (exons) in cellular or viral pre-mRNAs with excision of the intervening noncoding sequences (introns). *(Chapter 10)*

Stop transfer signal A hydrophobic sequence that halts translocation of a nascent protein across the endoplasmic reticulum membrane; serves as a transmembrane domain. *(Chapter 12)*

Structural unit *See* Asymmetric unit.

Substitution mutation Replacement of one or more nucleotides in a nucleic acid. *(Chapter 3)*

Subunit A single folded protein of a multimeric protein. *(Chapter 4)*

Supercoiling The winding of one duplex DNA strand around another. *(Chapter 9)*

Suppression *See* Pseudoreversion.

Susceptible Producing the receptor(s) required for virus entry; refers to cells. *(Chapter 2)*

Suspension cultures Cells propagated in suspension, in which a spinning magnet continuously stirs the cells. *(Chapter 2)*

Tegument The layer interposed between the nucleocapsid and the envelope of herpesvirus particles. *(Chapter 4)*

Termini Sites at which DNA replication stops. *(Chapter 9)*

Tight junctions The areas of contact between adjacent epithelial cells, circumscribing the cells at the apical edges of their lateral membranes. *(Chapter 2)*

Topology The geometric arrangement of, and connections among, secondary-structure units in a protein. *(Chapter 4)*

Transcriptional control region Local and distant DNA sequences necessary for initiation and regulation of transcription. *(Chapter 8)*

Transcytosis A mechanism of transport in which material in the intestinal lumen is endocytosed by M cells, transported to the basolateral surface, and released to the underlying tissues. *(Chapter 2)*

Transfection Introduction of viral nucleic acid into cells by transformation, resulting in the infection of cells. *(Chapter 3)*

Transfer RNAs Adapter molecules that align each amino acid with its corresponding codon on the mRNA. Abbreviated tRNAs. *(Chapter 11)*

Transport vesicles Membrane-bound structures with external protein coats that bud from compartments of the secretory pathway and carry cargo in anterograde or retrograde directions. *(Chapter 12)*

Triangulation Division of the triangular face of a large icosahedral structure into smaller triangles. *(Chapter 4)*

tRNAs *See* Transfer RNAs.

Tropism The predilection of a virus to invade, and replicate, in a particular cell type. *(Chapter 5)*

Tumor suppressor gene A cellular gene encoding a protein that negatively regulates cell proliferation; mutational inactivation of both copies of the genes is associated with tumor development. *(Chapter 9)*

Two-hit kinetics A parabolic relationship between plaque count and virus concentration which indicates that two different types of virus particle must infect a cell to ensure replication. *(Chapter 2)*

Type-specific antigens Epitopes, defined by neutralizing antibodies, which define viral serotypes (e.g., poliovirus types 1, 2, and 3). *(Chapter 2)*

Uncoating The release of viral nucleic acid from its protective protein coat or lipid envelope; in some cases, the liberated nucleic acid is still associated with viral proteins. *(Chapter 5)*

Vaccination Inoculation of healthy individuals with attenuated or related microorganisms, or their antigenic products, to elicit an immune response that will protect against later infection by the corresponding pathogen. *(Chapter 1)*

Variolation Inoculation of healthy individuals with material from a smallpox pustule, or in modern times from a related or attenuated cowpox (vaccinia) virus preparation, through a scratch on the skin (called scarification). *(Chapter 1)*

Viral pathogenesis The processes by which viral infections cause disease. *(Chapter 2)*

Virion An infectious virus particle. *(Chapters 1 and 4)*

Viroids Unencapsidated, small, circular, single-stranded RNAs that replicate autonomously when inoculated into plant cells. *(Chapter 1)*

Viruses Submicroscopic, obligate parasitic pathogens comprising genetic material (DNA or RNA) surrounded by a protective protein coat. *(Chapter 1)*

Virus replication The sum total of all events that occur during the infectious cycle. *(Chapter 2)*

Virus titer The concentration of a virus in a sample. *(Chapter 2)*

Wild type The original (often laboratory-adapted) virus from which mutants are selected and which is used as the basis for comparison. *(Chapter 3)*

Zoonotic Transmitted among humans and other vertebrates; refers to infections and diseases. *(Chapter 1)*

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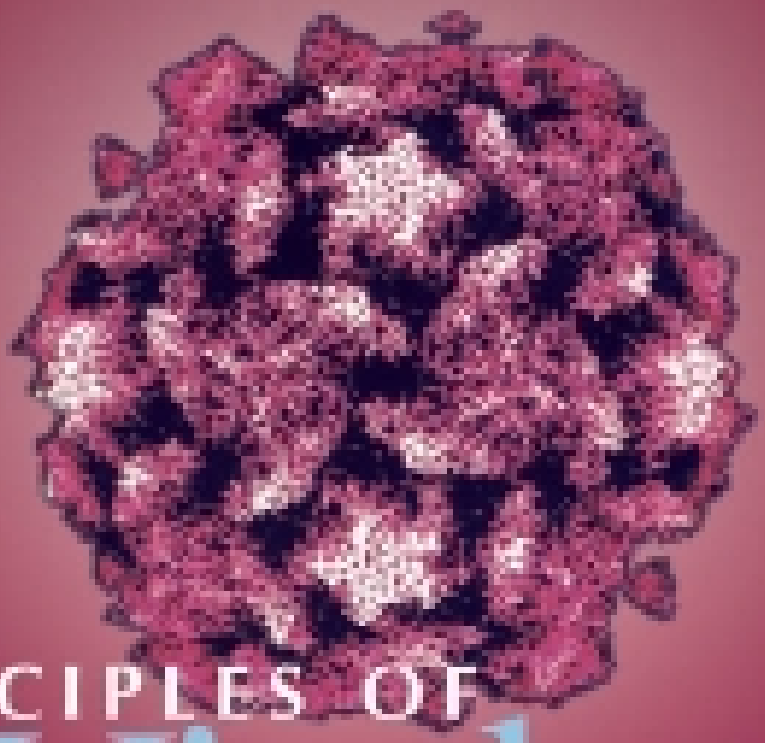
AMERICAN
SOCIETY FOR
MICROBIOLOGY

VOLUME II *Pathogenesis and Control*

S. J. FLINT • L. W. ENQUIST
V. R. RACANIELLO • A. M. SKALKA



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VOLUME II *Pathogenesis and Control*

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Front cover illustration: A model of the atomic structure of the poliovirus type 1 Mahoney strain. The model has been highlighted by radial depth cuing so that the portions of the model that are farthest from the center are bright. Prominent surface features include a star-shaped mesa at each of the fivefold axes and a propeller-shaped feature at each of the threefold axes. A deep cleft or canyon surrounds the star-shaped feature. This canyon is the receptor-binding site. Courtesy of Robert Grant, Stéphane Crainic, and James Hogle (Harvard Medical School).

Back cover illustration: Progress in the global eradication of poliomyelitis has been striking, as illustrated by maps showing areas of known or probable circulation of wild-type poliovirus in 1988, 1998, and 2008. Dark red indicates the presence of virus. In 1988, the virus was present on all continents except Australia. By 1998, the Americas were free of wild-type poliovirus, and transmission was interrupted in the western Pacific region (including the People's Republic of China) and in the European region (with the exception of southeastern Turkey). By 2008, the number of countries reporting endemic circulation of poliovirus had been reduced to four: Afghanistan, Pakistan, India, and Nigeria.

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We dedicate this book to the students, current and future scientists and physicians, for whom it was written. We kept them ever in mind.

We also dedicate it to our families:

Jonn, Gethyn, and Amy Leedham

Kathy and Brian

Doris, Aidan, Devin, and Nadia

Rudy, Jeanne, and Chris

Oh, be wiser thou!
Instructed that true knowledge leads to love.

WILLIAM WORDSWORTH
Lines left upon a Seat in a Yew-tree
1888

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Contents

Preface

The enduring goal of scientific endeavor, as of all human enterprise, I imagine, is to achieve an intelligible view of the universe. One of the great discoveries of modern science is that its goal cannot be achieved piecemeal, certainly not by the accumulation of facts. To understand a phenomenon is to understand a category of phenomena or it is nothing. Understanding is reached through creative acts.

A. D. HERSHEY
Carnegie Institution Yearbook 65

The major goal of all three editions of this book has been to define and illustrate the basic principles of animal virus biology. In this information-rich age, the quantity of data describing any given virus can be overwhelming, if not indigestible, for student and expert alike. Furthermore, the urge to write more and more about less and less is the curse of reductionist science and the bane of those who write textbooks meant to be used by students. Consequently, in the third edition, we have continued to distill information with the intent of extracting essential principles, while retaining some descriptions of how the work is done. Our goal is to illuminate process and strategy as opposed to listing facts and figures. We continue to be selective in our choice of topics, viruses, and examples in an effort to make the book readable, rather than comprehensive. Detailed encyclopedic works like *Fields Virology* (2007) have made the best attempt to be all-inclusive, and *Fields* is recommended as a resource for detailed reviews of specific virus families.

What's New

The major change in the third edition is the separation of material into two volumes, each with its unique appendix(es) and general glossary. Volume I covers molecular aspects of the biology of viruses, and Volume II focuses on viral pathogenesis, control of virus infections, and virus evolution. The organization into two volumes follows a natural break in pedagogy and provides considerable flexibility and utility for students and teachers alike. The smaller size and soft covers of the two volumes make them easier for students to carry

and work with than the single hardcover volume of earlier editions. The volumes can be used for two courses, or as parts I and II of a one-semester course. While differing in content, the volumes are integrated in style and presentation. In addition to updating the material for both volumes, we have used the new format to organize the material more efficiently and to keep chapter size manageable.

As in our previous edition, we have tested ideas for inclusion in the text in our own classes. We have also received constructive comments and suggestions from other virology instructors and their students. Feedback from students was particularly useful in finding typographical errors, clarifying confusing or complicated illustrations, and pointing out inconsistencies in content.

For purposes of readability, references again are generally omitted from the text, but each chapter ends with an updated and expanded list of relevant books, review articles, and selected research papers for readers who wish to pursue specific topics. In general, if an experiment is featured in a chapter, one or more references are listed to provide more detailed information.

Principles Taught in Two Distinct, but Integrated Volumes

These two volumes outline and illustrate the strategies by which all viruses are propagated in cells, how these infections spread within a host, and how such infections are maintained in populations. The principles established in Volume I enable understanding of the topics of Volume II: viral disease, its control, and the evolution of viruses.

Volume I: the Science of Virology and the Molecular Biology of Viruses

This volume features the molecular processes that take place in an infected host cell. Chapters 1 and 2 discuss the foundations of virology. A general introduction with historical perspectives as well as definitions of the unique properties of viruses is provided first. The unifying principles that are the foundations of virology, including the concept of a common strategy for viral propagation, are then described. Chapter 2 establishes the principle of the infectious cycle with an introduction to cell biology. The basic techniques for cultivating and assaying viruses are outlined, and the concept of the single-step growth cycle is presented.

Chapter 3 introduces the fundamentals of viral genomes and genetics, and it provides an overview of the perhaps surprisingly limited repertoire of viral strategies for genome replication and mRNA synthesis. Chapter 4 describes the architecture of extracellular virus particles in the context of providing both protection and delivery of the viral genome in a single vehicle. In Chapters 5 through 13, we describe the broad spectrum of molecular processes that characterize the common steps of the reproductive cycle of viruses in a single cell, from decoding genetic information to genome replication and production of progeny virions. We describe how these common steps are accomplished in cells infected by diverse but representative viruses, while emphasizing principles applicable to all.

The appendix in Volume I provides concise illustrations of viral life cycles for the main virus families discussed in the text. It is intended to be a reference resource when one is reading individual chapters and a convenient visual means by which specific topics may be related to the overall infectious cycles of the selected viruses.

Volume II: Pathogenesis, Control, and Evolution

This volume addresses the interplay between viruses and their host organisms. Chapters 1 to 7 focus on principles of virus replication and pathogenesis. Chapter 1 provides a brief history of viral pathogenesis and addresses the basic concepts of how an infection is established in a host as opposed to infection of single cells in the laboratory. In Chapter 2, we focus on how viral infections spread in populations. Chapter 3 presents our growing understanding of crucial autonomous reactions of cells to infection and describes how these actions influence the eventual outcome for the host. Chapter 4 provides a virologist's view of immune defenses and their integration with events that occur when single cells are infected. Chapter 5 describes how a particular virus replication strategy and the ensuing host response influence the outcome of infection such that some are short and others are of long duration. Chapter 6 is devoted entirely to the AIDS virus, not only because it is the causative agent of the most serious current worldwide epidemic, but also because of its unique and informative interactions with the human immune defenses. In Chapter 7, we discuss virus infections that transform cells in culture and promote oncogenesis (the formation of tumors) in animals.

Chapters 8 and 9 outline the principles involved in treatment and control of infection. Chapter 8 focuses on vaccines, and chapter 9 discusses the approaches and challenges of antiviral drug discovery. In Chapter 10, the final chapter, we present a foray into the past and future, providing an introduction to viral evolution. We illustrate important principles taught by zoonotic infections, emerging infections, and humankind's experiences with epidemic and pandemic viral infections.

Appendix A summarizes the pathogenesis of common viruses that infect humans in three "slides" (viruses and diseases, epidemiology, and disease mechanisms) for each virus or virus group. This information is intended to provide a simple snapshot of pathogenesis and epidemiology. Appendix B provides a concise discussion of unusual infectious agents, such as viroids, satellites, and prions, that are not viruses but that (like viruses) are molecular parasites of the cells in which they replicate.

Reference

Knipe, D. M., and P. M. Howley (ed. in chief). 2007. *Fields Virology,* 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.

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Since the inception of this work, our belief has been that the illustrations must complement and enrich the text. Execution of this plan would not have been possible without the support of Jeff Holtmeier (Director, ASM Press) and the technical expertise and craft of our illustrator. The illustrations are an integral part of the exposition of the information and ideas discussed, and credit for their execution goes to the knowledge, insight, and artistic talent of Patrick Lane of ScEYEnce Studios. As noted in the figure legends, many of the figures could not have been completed without the help and generosity of our many colleagues who provided original images. Special thanks go to those who crafted figures tailored specifically to our needs or provided multiple pieces: Mark Andrake (Fox Chase Cancer Center), Edward Arnold (Rutgers University), Bruce Banfield (The University of Colorado), Christopher Basler and Peter Palese (Mount Sinai School of Medicine), Amy Brideau (Peregrine Pharmaceuticals), Roger Burnett (Wistar Institute), Rajiv Chopra and Stephen Harrison (Harvard University), Marie Chow (University of Arkansas Medical Center), Bob Craigie (NIDDK, National Institutes of Health), Richard Compans (Emory University), Friedrich Frischknecht (European Molecular Biology Laboratory), Wade Gibson (Johns Hopkins University School of Medicine), Ramón González (Universidad Autónoma del Estado de Morelos), David Knipe (Harvard Medical School), Thomas Leitner (Los Alamos National Laboratory), Maxine Linial (Fred Hutchinson Cancer Center), Pedro Lowenstein (University of California, Los Angeles), Paul Masters (New York State Department of Health), Rolf Menzel (National Institutes of Health), Thomas Mettenleiter (Federal Institute for Animal Diseases, Insel Reims, Germany), Heather Ongley and Michael Chapman (Oregon Health and Science University), B. V. Venkataram Prasad (Baylor College of Medicine), Botond Roska (Friedrich Miescher Institute, Basel, Switzerland), Michael Rossmann (Purdue University), Alasdair Steven (National Institutes of Health), Phoebe Stewart (Vanderbilt University), Wesley Sundquist (University of Utah), Jose Varghese (Commonwealth Scientific and Industrial Research Organization), Robert Webster (St. Jude's Children's Research Hospital), Thomas Wilk (European Molecular Biology Laboratory), Alexander Wlodawer (National Cancer Institute), and Li Wu (Medical College of Wisconsin).

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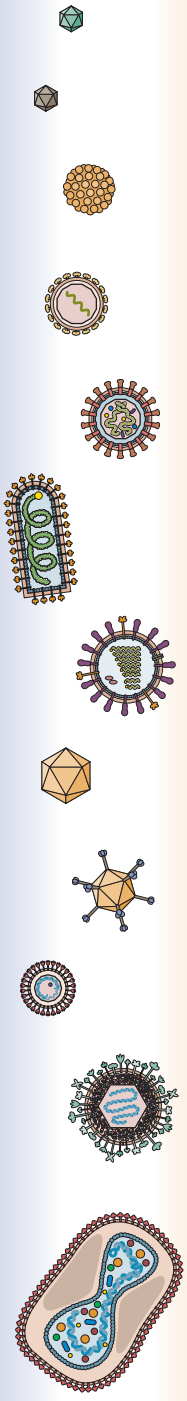

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Infection of a Susceptible Host

Before I came here I was confused about this subject. Having listened to your lecture, I am still confused—but at a higher level.

ENRICO FERMI

Introduction

While the field of viral pathogenesis is almost 100 years old, the viral and host genes that control this process are only now being enumerated and analyzed. Even though many such genes have been identified, this information rarely provides more than a glimpse of the molecular mechanisms responsible. For example, knowing that a gene controls the spread of infection via the blood or the nervous system does not answer the question of how the gene product determines which route will be taken. Although it is difficult to obtain such mechanistic knowledge, many investigators believe that viral pathogenesis is the most exciting field in virology today, simply because more fundamental questions remain to be answered than in any other area. The challenge is to build on our understanding of viral replication that was established in Volume I of this book to provide a comprehensive molecular description of how viruses cause disease. One obvious consequence of such knowledge will be new approaches to preventing, treating, and curing viral disease. In this chapter, we begin our analysis of viral pathogenesis and control, by outlining some history and the basic principles of how a viral infection is established in a single host.

A Brief History of Viral Pathogenesis

Microbes as Infectious Agents

From the earliest times, poisonous air (miasma) was generally invoked to account for **epidemics** of contagious diseases, and there was little recognition of the differences among their causative agents. The association of particular microorganisms, initially bacteria, with specific diseases can be attributed to the ideas of the German physician Robert Koch. He developed and applied a set of criteria for identification of the agent responsible for a specific disease (a **pathogen**). These criteria, **Koch's postulates**, are still applied in the identification of pathogens that can be propagated in the laboratory and tested in an appropriate animal model. The postulates are as follows.

- The organism must be associated regularly with the disease and its characteristic lesions.
- The organism must be isolated from the diseased host and grown in culture.
- The disease must be reproduced when a pure preparation of the organism is introduced into a healthy, susceptible host.
- The same organism must be reisolated from the experimentally infected host.

Guided by these postulates, and the methods for the sterile culture and isolation of pure preparations of bacteria developed by Pasteur, researchers identified and classified many pathogenic bacteria (as well as yeasts and fungi) during the last part of the 19th century. From these beginnings, investigation into the causes of infectious disease was placed on a secure scientific foundation, the first step toward rational treatment and ultimately control. During the last decade of the 19th century, failures of the paradigm that bacterial or fungal agents are responsible for all diseases led to the identification of a new class of infectious agents—submicroscopic pathogens that came to be called **viruses** (see Volume I, Chapter 1).

The First Human Viruses

The first human virus to be identified, in 1901, was that responsible for yellow fever. The high lethality of this disease, the lack of effective treatments, and the complicated transmission and life cycle of the virus made this achievement all the more remarkable. Yellow fever is known to have been widespread in tropical countries since the 15th century. It was responsible for devastating epidemics associated with such high rates of mortality (e.g., 28% in the New Orleans epidemic of 1853) that normal life became impossible (Volume I, Appendix; also see Box 10.15 in this volume). However, this disease is not directly contagious, and an infectious agent could not be demonstrated in yellow fever patients. These puzzling properties defeated efforts to establish the origin of yellow fever until 1880, when the Cuban physician Carlos Juan Finlay proposed that a bloodsucking insect, most likely a mosquito, played a part in the transmission of the disease. A commission to study the etiology of yellow fever was established in 1899 by the U.S. Army under Colonel Walter Reed, in part because of the high incidence of the disease among soldiers who were occupying Cuba. Jesse Lazear, a member of Reed's commission, provided leadership and ultimately gave his life to demonstrate that mosquitoes transmitted yellow fever. Lazear was the first experimentally infected person who died from the disease. The members of this courageous team are depicted in a dramatic 1939 painting by Dean Cornwell (Fig. 1.1). The results of the Reed Commission's study proved conclusively that mosquitoes are the vectors for this disease. In 1901, Reed and James Carroll injected diluted, filtered serum from an experimentally infected yellow fever patient into three nonimmune individuals. Two subsequently developed yellow fever. Reed and Carroll concluded that a filterable virus was the cause of the disease. In the same year, Juan Guiteras, a professor of pathology and tropical medicine at the University of Havana, attempted to produce immunity to yellow fever by exposing volunteers to mosquitoes carrying yellow fever virus. Of 19 volunteers, 8 contracted yellow fever and 3 died. One of the dead was Clara Louise Maass, a U.S. Army nurse from New Jersey. Yellow fever had been a constant scourge of Havana for 150 years, but the conclusions of Reed and his colleagues were a revelation. Rapid introduction of mosquito control by the mayor of Havana, William Gorgas, dramatically reduced the incidence of disease within a year. To this day, mosquito control remains an important method for reducing the incidence of yellow fever.

Figure 1.1 ***Conquerors of Yellow Fever.*** This painting by Dean Cornwell (1939) depicts the experimental infection of a soldier volunteer with yellow fever virus, via transmission through mosquitoes. Standing (left to right) are Carlos Finlay, in a dark suit; Aristedes Agramonte, holding a hat; Jesse Lazear, applying a cage with mosquitos to the arm of the soldier; and Walter Reed, in a white uniform. Reproduced with the generous permission of Wyeth Laboratories.

Other human viruses were identified during the early decades of the 20th century (Fig. 1.2). However, the pace of discovery was slow, not least because of the dangers and difficulties associated with experimental manipulation of human viruses so amply illustrated by the experience with yellow fever virus. Consequently, agents of some

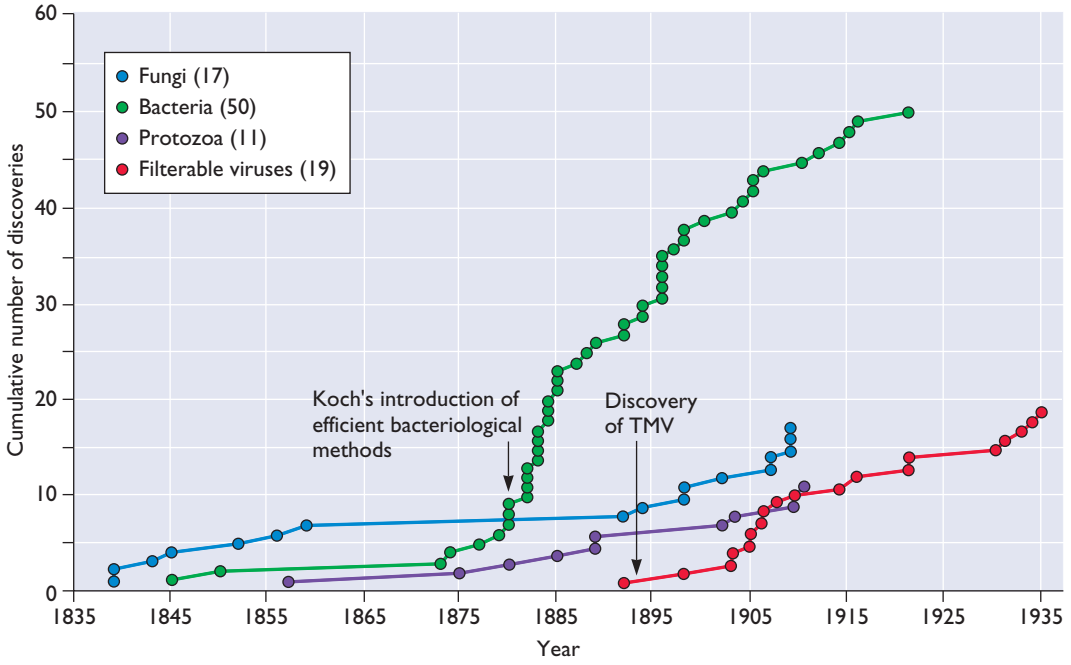


Figure 1.2 The pace of early discovery of new infectious agents. Koch's introduction of efficient bacteriological techniques spawned an explosion of new discoveries of bacterial agents in the early 1880s. Similarly, the discovery of filterable agents launched the field of virology in the early 1900s. Despite an early surge of virus discovery, only 19 distinct human viruses had been reported by 1935. TMV, tobacco mosaic virus. Adapted from K. L. Burdon, *Medical Microbiology* (MacMillan Co., New York, NY, 1939), with permission.

important human diseases were not identified for many years, and then only with some good luck. A classic case in point is the virus responsible for influenza, a name derived in the mid-1700s from Italian because of the belief that the disease resulted from the "influence" of miasma (bad air) and adverse astrological signs. The human disease is now thought to have arisen as a result of the transfer of virus among humans and livestock following domestication of animals about 10,000 years ago. Worldwide epidemics, called **pandemics**, of influenza have been documented in humans for well over 100 years. These pandemics were typically associated with mortality among the very young and the very old, but the 1918 to 1919 pandemic following the end of World War I was especially devastating. Over 40 million people died, more than were killed in the preceding war. Despite many efforts, a human influenza virus was not isolated until 1933. This virus was first identified by Wilson Smith, Christopher Andrewes, and Patrick Laidlaw only because they found a host suitable for its propagation. They infected ferrets with human throat washings and isolated the virus now known as influenza A virus. Ferrets may seem to be an exotic animal host, and, in fact, the success achieved with these animals was serendipitous: Laidlaw was using ferrets in studies of canine distemper virus and therefore they were available in his laboratory. Subsequently, influenza A virus was shown to infect adult mice and chicken embryos. The latter proved to be an especially valuable host system, for vast quantities of the virus are produced in the allantoic sac. Indeed, chicken eggs are still used today to produce influenza vaccines.

The Golden Age of Viral Pathogenesis

While the first 50 years of virology saw ground-breaking work in describing the viral etiology of diseases, the study of viral pathogenesis was placed on a firm foundation during the next 25 years. In the first half of the 20th century, methods were developed to isolate and identify viruses and use embryonated chicken eggs in quantitative studies of viral infection. However, in the 1950s, several new technologies were developed to usher in what some now call the golden age of **viral pathogenesis**. Indeed, the basic principles of this field were established in the 25 years after 1950. The development of seemingly simple techniques was critical. For example, the plaque assay for lytic viruses and the focus-forming assay for viral transformation brought methods and concepts developed by phage biologists to analysis of animal virus infections. Synchronous infection and analysis of a single reproductive

cycle, as pioneered by Max Delbrück and colleagues for bacteriophage, were now applicable to animal viruses. Tissue culture techniques and methods for centrifugation enabled early forays into biochemistry. The revolutionary use of fluorescent antibodies to identify viral proteins enabled scientists to observe viral infection and spread in cells and tissues. Electron microscopy became routine, and the amazing subcellular changes in virus-infected cells could be catalogued and analyzed. The development of methods to measure the immune response was probably the most significant accelerant of the golden age. In particular, the ability to identify antibodies and their activities transformed the study of viral pathogenesis. Complement fixation, neutralization, and hemagglutination inhibition are but three of the many innovative assays developed in the 1950s. With these techniques in hand, scientists performed the classic studies of the pathogenesis of poliovirus, mousepox virus, rabies virus, and lymphocytic choriomeningitis virus, which stand to this day as models of elegance and careful analyses.

The New Millennium and Viral Pathogenesis

The time from the mid-1970s to the end of the 20th century saw a true revolution in biology. Recombinant DNA technology enabled the cloning, sequencing, and manipulation of host and viral genomes. The polymerase chain reaction (PCR) was first among the many new offshoots of recombinant DNA technology that transformed biology. One can point to the Nobel Prizes of the 1980s and 1990s to see the transformative power of recombinant DNA technology: transgenic animals, gene targeting, and RNA interference are prime examples.

The concepts and methods of molecular and cell biology marked a transition from the descriptive phase of virology to the reductionist phase. Genomes were isolated, proteins were identified, functions were deduced by genetic and biochemical methods, and new models of disease were established. This reductionist approach was remarkably fruitful, not only for mechanistic understanding, but also for practical applications including the development of diagnostic reagents, antiviral drugs, and vaccines. As the 20th century came to a close, many scientists moved from reductionism to a more holistic philosophy of analysis. They embraced the concept of "systems biology," the idea that by using appropriate technologies, one could know all the molecules or reactions of a biological system, monitor them during an infection, and discover new mechanisms of host and viral biology missed by the classical "one gene at a time" methods. These ideas were first developed using DNA microarray technology, with which it is possible to measure the global transcriptional response of both host and viruses after infection of single cells and tissues (Box 1.1). The systematic compilation of data has revealed common and cell-specific responses to infection, as well as host-specific responses to a given infectious agent. Other massive data-gathering technologies enable similar profiling of proteins and even small-molecule metabolites of cells and tissues (Box 1.2). Computer programs capable of handling and integrating these massive databases are available to most scientists. Not surprisingly, this veritable gold mine of information can be overwhelming. It is likely that reductionist approaches will be required to test the many ideas that are emerging from systems biology. Furthermore, despite the new approaches, some fundamental tenets of viral pathogenesis remain unchallenged. These principles are considered in the next sections.

Infection Basics

A Series of Unfortunate Events

Infection of a susceptible host can be viewed as a sequence of individual events (Fig. 1.3A). The pathogenesis of mousepox is a classic example of such a sequence (Fig. 1.4). After local viral multiplication in the foot, the virus spreads via the bloodstream to the spleen, liver, and skin. The steps involved in infection have been defined for many viral pathogens by using animal models of infection. A contrary view is that infection comprises a series of random (stochastic) events that permit infection to bypass bottlenecks imposed by the host (Fig. 1.3B). The architecture of tissues and the immune system are examples of such bottlenecks. Selection of viruses that can bypass the bottleneck occurs because of the the diversity of viral populations (Chapter 10). These two views of infection are not as difficult to reconcile as they appear; the stochastic view does not exclude the idea that infection is a series of defined steps, but rather adds random elements to the outcome of each step.

Initiating an Infection

Three requirements must be satisfied to ensure successful infection in an individual host: sufficient **virions** must be available to initiate infection; the cells at the site of infection must be physically accessible to virions, **susceptible** (bear receptors for entry), and **permissive** (contain intracellular gene products needed for viral replication); and the local host antiviral defense systems must be absent or at least initially ineffective.

The first requirement erects a substantial barrier to any infection, and represents a significant weak link in the transmission of infection from host to host. Free virus particles face both a harsh environment and rapid dilution that can reduce their concentration. Viruses that are spread in contaminated water and sewage must be stable in the presence of osmotic shock, pH changes, and sunlight, and must not adsorb irreversibly to debris. Aerosol-dispersed virus particles must remain hydrated and highly concentrated to infect

BOX 1.1 EXPERIMENTS

Determining the host response to infection by transcriptional profiling

Since the first paper in 1998 demonstrating the use of DNA microarrays to monitor the host transcriptional response to human cytomegalovirus infection, hundreds of similar and more advanced studies have been published covering most of the well-known viral infections.

The results of these studies are always complex. For example, it is difficult to determine if an RNA changes in abundance because of new synthesis or changes in stability or even if the transcript levels produce corresponding changes in protein abundance. However, it is possible to define some common host transcriptional responses to infection.

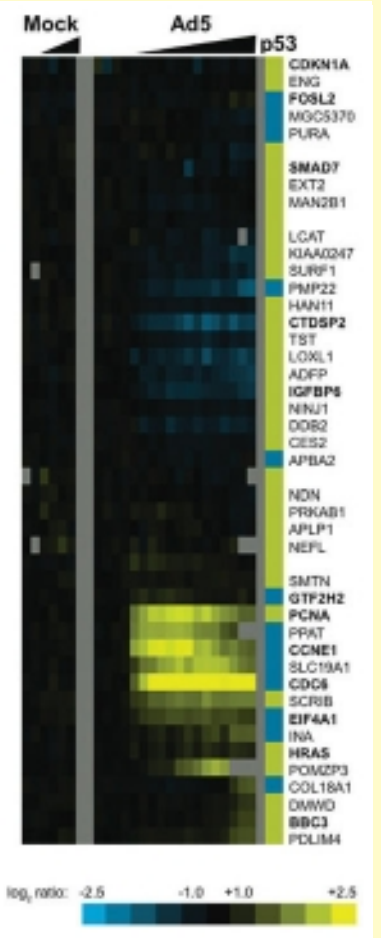
One such study provided a systematic cluster analysis of 32 studies that compared 785 experiments and 77 different host-pathogen interactions including a variety of viruses and bacteria. The authors found a cluster of 511 coregulated RNAs that they designated the "common host response." The authors postulated that some of these gene products constituted a general alarm signal for infection as well as the common intrinsic, cell-autonomous defenses. They also suggest that pathogens can modulate these responses to enhance virulence.

The products of these 511 genes fall into five general groups:

1. Inflammation mediators (proinflammatory cytokines, chemokines, prostaglandin synthesis)
2. Interferon stimulated
3. Activators of intrinsic and immune defenses (transcription proteins such as Nf-κB, activators of cytoplasmic nucleic acid detectors)
4. Negative regulators of cellular and immune defenses (Iκbα, Iκbε, various kinases, antiapoptosis genes)
5. Host response (lymphocyte activation, cell adhesion, tissue invasion)

However, despite being clustered by the computer, these responses are cell type and pathogen specific, and vary in degree and temporal appearance. The challenge is to understand the relationship of transcriptional changes in cultured cells to those found in infected tissues and hosts.

Jenner, R., and R. Young. 2005. Insights into host responses against pathogens from transcriptional profiling. *Nat. Rev. Microbiol.* **3:**281–294.



Changes in expression of cellular genes during adenovirus type 5 infection. Human foreskin fibroblasts were infected or mock infected, and RNA was prepared at different times after infection and analyzed by microarray. Shown are the results for ~50 genes that are targeted by p53. Blue indicates a reduction in RNA; yellow indicates an increase. The column labeled p53 summarizes genes that are transcriptionally activated (yellow) or repressed (blue) by the p53 protein. Ramps above columns indicate time after infection. Reprinted from D. L. Miller et al., *Genome Biol.* **8:**R58, 2007, with permission.

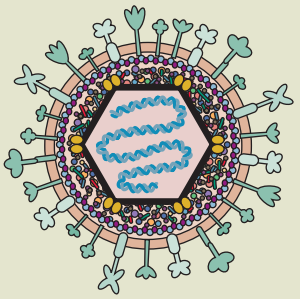
the next host. Viruses that are spread in this way do best in populations in which individuals are in close contact. In contrast, viruses that are spread via biting insects, contact with mucosal surfaces, or other means of direct contact, including contaminated needles, have little environmental exposure.

Even if one virus particle survives the passage from one host to another, infection may fail simply because the concentration is not sufficient. In principle, a single virion should be able to initiate an infection, but host physical and immune defenses, coupled with the complexity

BOX 1.2 DISCUSSION

Virus infection markedly affects cellular metabolic pathways: a genomics and metabolomics approach

The biochemical infrastructure of host cells is essential for virus propagation. An underlying, but poorly understood, fact is that this infrastructure includes the cellular metabolic machinery that provides the energy and building blocks necessary for their replication. Liquid chromatography-mass spectrometry has been used to quantitate directly the concentrations of a large number of metabolic compounds (energy molecules and biochemical building blocks) during human cytomegalovirus infection of cultured human cells. In addition, changes in cellular RNA were measured by microarray analysis in parallel. After infection, the concentration of many metabolites increased dramatically, far more than is seen when cells switch from resting to growing states. Often the change in metabolite levels coincided with an apparent increase in the level of RNA associated with the production of that metabolite. One striking conclusion was that virus-infected cells produced a characteristic metabolic program.



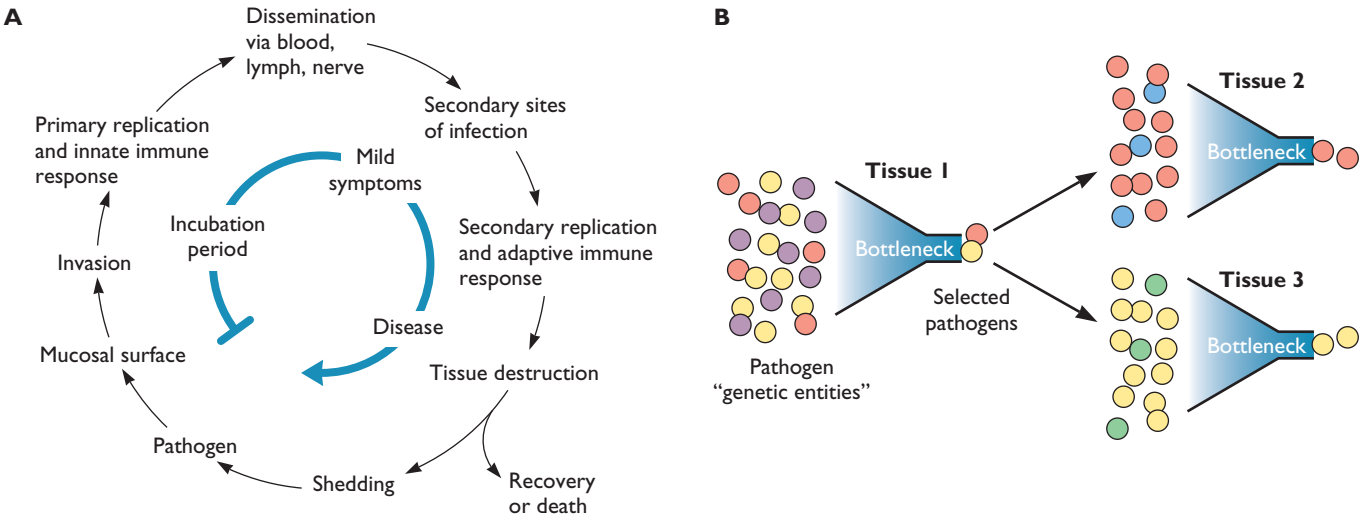
We can expect more studies like this one to provide a comprehensive characterization of the metabolic environment of virus-infected cells. It is likely that these substantial metabolic changes will affect the global host response to infection. Moreover, it may be that these changes are required for virus reproduction and spread and may therefore be targets for antiviral therapies.

Munger J., S. Bajad, H. Coller, T. Shenk, and J. Rabinowitz. 2006. Dynamics of the cellular metabolome during human cytomegalovirus infection. *PLoS Pathog.* **2:**1165–1175.

of the infection process itself, demand the presence of many particles. The number of particles required to initiate and maintain an infection depends on the particular virus, the site of infection, and the physiology and age of the host. However, some basic facts help to guide us.

Statistical analysis of infections in cultured cells demonstrates that on average a single virus particle can initiate an infection, but that many perfectly competent virions fail to do so. Such failure can be explained in part by the complexity of the infectious cycle: there are many distinct reactions, and the probability of a single virus particle

Figure 1.3 Views of viral pathogenesis. (A) Infection viewed as a series of steps with a predictable outcome. **(B)** Infection seen as a series of stochastic events. Viruses are selected that can pass through bottlenecks in cells, organs, or hosts. Adapted from H. W. Virgin, *Nat. Immunol.* **8:**1143–1147, 2007, with permission.



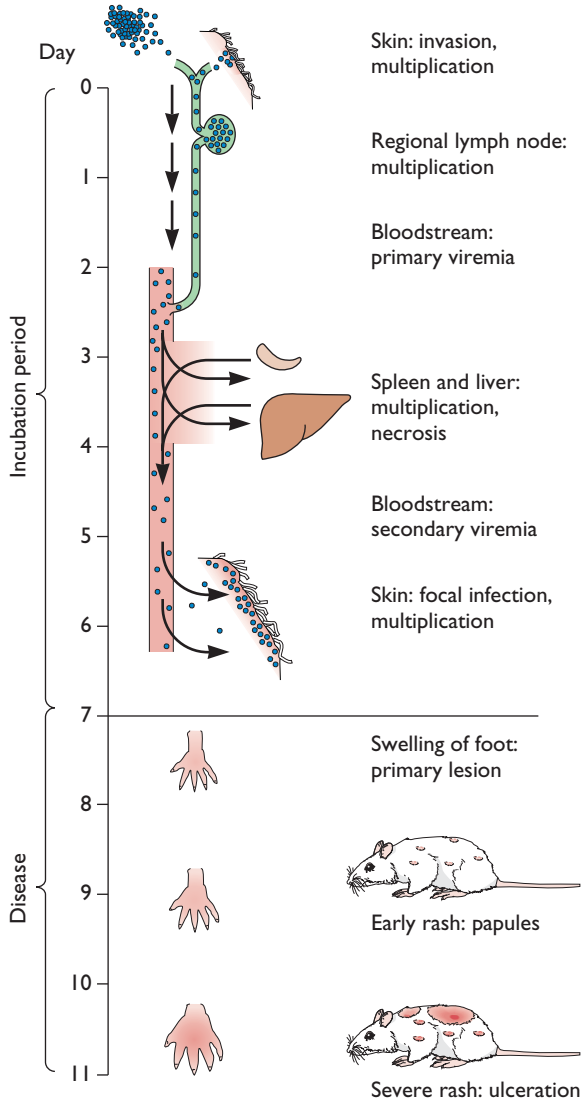


Figure 1.4 Pathogenesis of mousepox. Sequence of events in the pathogenesis of mousepox after inoculation of virus into the footpad. The figure is based on the classic studies of Frank Fenner, which were the first to demonstrate how disseminated viral infections develop from local multiplication to primary and secondary viremia. In the case of mousepox, after local multiplication in the foot, the host response leads to swelling at the site of inoculation; after viremia, the host response to replication in the skin results in a rash. Adapted from F. Fenner et al., *The Biology of Animal Viruses* (Academic Press, New York, NY, 1974), with permission.

completing any one is not 100%. For example, there are many potentially nonproductive interactions of virus particles with debris and extracellular material during their initial encounter with the cell surface. Even if a virus attaches successfully to a permissive cell, it may be delivered to a digestive lysosome upon entry. Many of these false starts or inappropriate interactions are irreversible, aborting infection by the virion.

In addition, populations of viruses often contain particles that are not capable of completing an infectious cycle. For example, defective particles can be produced by mistakes during virus replication or from interaction with inhibitory compounds in the environment. In the laboratory, a quantitative measure of the proportion of infectious viruses is the particle–to–**plaque-forming-unit** (PFU) ratio. As described in Volume I, Chapter 2, the number of physical particles in a given preparation are counted, usually with an electron microscope, and compared with the number of infectious units, or PFU, per unit volume. This ratio is a useful indicator of the quality of a virus preparation, as it should be constant for a given virus prepared by identical or comparable procedures.

Viral Entry

In general, virions must first enter cells at a body surface. Common sites of entry include the mucosal linings of the respiratory, alimentary, and urogenital tracts, the outer surface of the eyes (conjunctival membranes or cornea), and the skin (Fig. 1.5).

Respiratory Tract

Probably the most common route of viral entry is through the respiratory tract. In a human lung there are about 300 million terminal sacs, called alveoli, which function in gaseous exchange between inspired air and the blood. To accomplish this function, each sac is in close contact with capillary and lymphatic vessels. The combined absorptive area of the human lung is almost 140 m^2. Humans have a resting ventilation rate of 6 liters of air per min. Consequently, large numbers of foreign particles and aerosolized droplets are introduced into the lungs with every breath. Many of these particles and droplets contain virions. Fortunately, there are numerous host defense mechanisms to block respiratory tract infection. Mechanical barriers play a significant role in antiviral defense. For example, the tract is lined with a mucociliary blanket consisting of ciliated cells, mucus-secreting goblet cells, and subepithelial mucus-secreting glands (Fig. 1.6). Foreign particles deposited in the nasal cavity or upper respiratory tract are trapped in mucus, carried to the back of the throat, and swallowed. In the lower respiratory tract, particles trapped in mucus are brought up from the lungs to the throat by ciliary action. The lowest portions of the tract, the alveoli, lack cilia or mucus, but macrophages lining the alveoli are responsible for ingesting and destroying particles. Other cellular and humoral immune responses also intervene.

Many viruses enter the respiratory tract in the form of aerosolized droplets expelled by an infected individual by

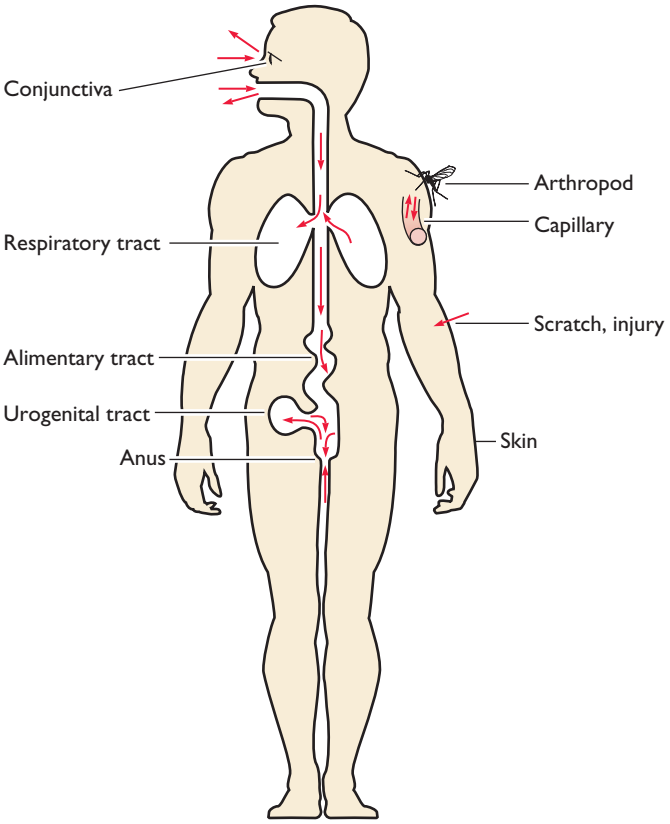


Figure 1.5 Sites of viral entry into the host. A representation of the human host is shown, with sites of virus entry and shedding indicated. The body is covered with skin, which has a relatively impermeable (dead) outer layer. However, there are accessible layers of living cells to absorb food, exchange gases, and release urine and other fluids. These layers offer easier pathways for the entry of viruses than the skin. Virions can be introduced through the skin by a scratch or injury, a vector bite, or inoculation with a needle.

coughing or sneezing (Table 1.1). Infection can also spread through contact with saliva from an infected individual. Larger virus-containing droplets are deposited in the nose, while smaller droplets find their way into the airways or the alveoli. To infect the respiratory tract successfully, viruses must not be swept away by mucus, neutralized by antibody, or destroyed by alveolar macrophages.

Alimentary Tract

The alimentary tract is a common route of infection and dispersal (Table 1.1). This tube, which connects the oral cavity to the anus, is always in motion. Eating, drinking, and some social activities routinely place viruses in the alimentary tract. It mixes, digests, and absorbs food, providing a good opportunity for viruses to encounter a susceptible cell and to interact with cells of the circulatory, lymphatic, and immune systems. However, it is an extremely hostile environment for virions. The stomach is acidic, the intestine is alkaline, digestive enzymes and bile detergents abound, mucus lines the epithelium, and the lumenal surfaces of intestines carry antibodies and phagocytic cells.

Virions that infect by the intestinal route must, at a minimum, be resistant to extremes of pH, proteases, and bile detergents. Indeed, virions that lack these features are destroyed when exposed to the alimentary tract, and must infect at other sites. The family *Picornaviridae* comprises both acid-labile viruses (e.g., rhinoviruses) and acid-resistant viruses (e.g., poliovirus) (Box 1.3). Rhinoviruses are respiratory pathogens and cannot infect the upper intestine. They spread in a population as inhaled aerosols. In contrast, poliovirus can survive ingestion and establish an infection of the upper intestine, and spreads by the fecal-oral route. The hostile environment of the alimentary tract actually facilitates infection by some viruses. For example, reovirus particles are converted by host proteases in the intestinal lumen into infectious subviral particles, the forms that subsequently infect intestinal cells. As might be expected, most enveloped viruses do not initiate infection in the alimentary tract, because viral envelopes are susceptible to dissociation by detergents such as bile salts. Enteric coronaviruses are notable exceptions, but it is not known why these enveloped viruses can withstand the harsh conditions in the alimentary tract.

Nearly the entire intestinal surface is covered with columnar villous epithelial cells with apical surfaces that are densely packed with microvilli (Fig. 1.7). This brush border, together with a surface coat of glycoproteins and glycolipids, and the overlying mucus layer, is permeable to electrolytes and nutrients but presents a formidable barrier to microorganisms. Nevertheless, viruses such as enteric adenoviruses and Norwalk virus, a calicivirus, replicate extensively in intestinal epithelial cells. The mechanisms by which they bypass physical barriers and enter susceptible cells are beginning to be understood (Volume I, Chapter 5). Scattered throughout the intestinal mucosa are lymphoid follicles that are covered on the lumenal side with a specialized follicle-associated epithelium consisting mainly of columnar absorptive cells and M (membranous epithelial) cells. The M cell cytoplasm is very thin, resulting in a membrane-like bridge that separates the lumen from the subepithelial space. As discussed in Chapter 4, M cells ingest and deliver antigens to the underlying lymphoid tissue by **transcytosis**. In this process, material taken up on the lumenal side of the M cell traverses the cytoplasm virtually intact, and is delivered to the underlying basal membranes and extracellular space (Fig. 1.7). It is thought that M cell transcytosis provides the mechanism by which some enteric viruses gain entry

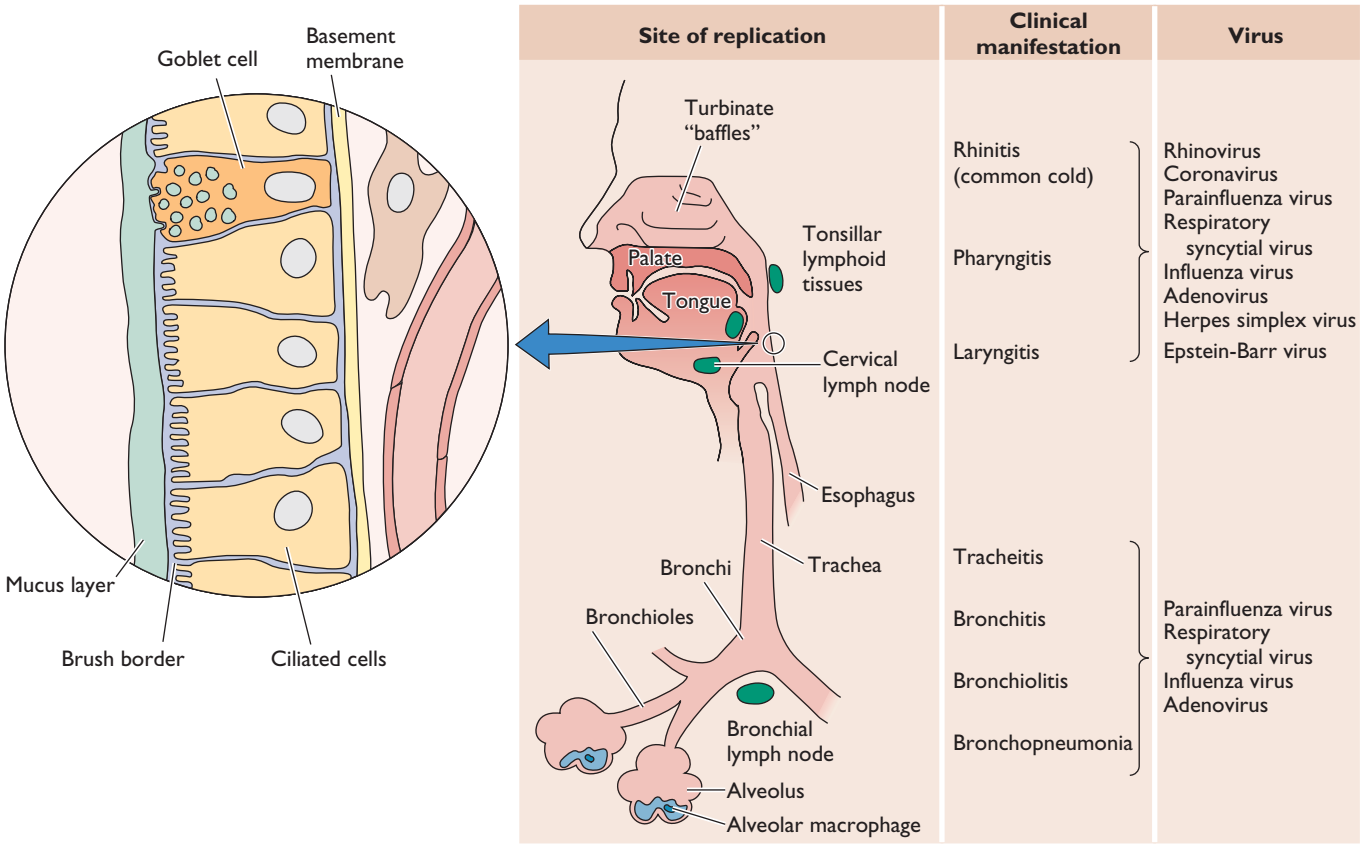


Figure 1.6 Sites of viral entry in the respiratory tract. (Left) A detailed view of the respiratory epithelium. A layer of mucus, produced by goblet cells, is a formidable barrier to virion attachment. Virions that pass through this layer may multiply in the ciliated cells or pass between them, reaching another physical barrier, the basement membrane. Beyond this extracellular matrix are tissue fluids from which particles may be taken into lymphatic capillaries and reach the blood. Local macrophages patrol the tissue fluids in search of foreign particles. Adapted from C. A. Mims et al., *Mims' Pathogenesis of Infectious Disease* (Academic Press, Orlando, FL, 1995), with permission. **(Right)** Viruses that replicate at different levels of the respiratory tract, with the associated clinical syndromes.

to deeper tissues of the host from the intestinal lumen. After crossing the mucosal epithelium in this manner, a virus particle could enter lymphatic vessels and capillaries of the circulatory system, facilitating spread within the host. A particularly well studied example is transcytosis of reovirus. After attaching to the M cell surface, reovirus subviral particles are transported to cells underlying the lymphoid follicle, where they replicate and spread to other tissues. Rather than spread by transcytosis across the M cell, some viruses actively replicate in them and do not spread to underlying tissues. For example, infection by human rotavirus and the coronavirus transmissible gastroenteritis virus destroys M cells, resulting in mucosal inflammation and diarrhea.

It is possible for virions to enter the body through the lower gastrointestinal tract without passing through the upper tract and its defensive barriers. For example, human immunodeficiency virus can be introduced into the body by anal intercourse. As M cells abound in the lower colon, these cells are likely to provide a portal of entry for this virus into susceptible lymphocytes in the underlying lymphoid follicles. Once in the follicle, the virus can infect migratory lymphoid cells and spread throughout the body.

Urogenital Tract

Some viruses enter the urogenital tract as a result of sexual activities (Table 1.1). The urogenital tract is well protected by physical barriers, including mucus and low pH (in the case of the vagina). Normal sexual activity can result in minute tears or abrasions in the vaginal epithelium or the urethra, allowing virions to enter. Some viruses infect the epithelium and produce local lesions (certain human papillomaviruses, which cause genital warts). Other viruses gain access to cells in the underlying tissues and infect cells of the

Table 1.1 Different routes of viral entry into the host

Location	Virus(es)
Respiratory tract	
Localized upper tract	Rhinovirus; coxsackievirus; coronavirus; arenaviruses; hantavirus; parainfluenza virus types 1–4; respiratory syncytial virus; influenza A and B viruses; human adenovirus types 1–7, 14, 21
Localized lower tract	Respiratory syncytial virus; parainfluenza virus types 1–3; influenza A and B viruses; human adenovirus types 1–7, 14, 21
Entry via respiratory tract followed by systemic spread	Rubella virus, arenaviruses, hantavirus, mumps virus, measles virus, varicella-zoster virus, poxviruses
Alimentary tract	
Systemic	Enterovirus, reovirus, adenovirus
Localized	Coronavirus, rotavirus
Urogenital tract	
Systemic	Human immunodeficiency virus type 1, hepatitis B virus, herpes simplex virus
Localized	Papillomavirus
Eyes	
Systemic	Enterovirus 70, herpes simplex virus
Localized	Adenovirus types 8, 22
Skin	
Arthropod bite	Bunyavirus, flavivirus, poxvirus, reovirus, togavirus
Needle puncture, sexual contact	Hepatitis C and D viruses, cytomegalovirus, Epstein-Barr virus, hepatitis B virus, human immunodeficiency virus, papillomavirus (localized)
Animal bite	Rhabdovirus

immune system (human immunodeficiency virus type 1), or sensory and autonomic neurons (herpes simplex virus). Infection by these two viruses invariably spreads from the urogenital tract to establish lifelong persistent or latent infections, respectively.

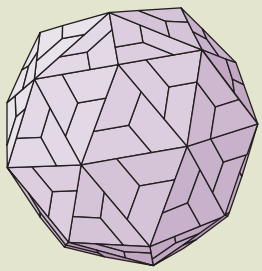
Eyes

The epithelium covering the exposed part of the sclera (the outer fibrocollagenous coat of the globe of the eye) and the inner surfaces of the eyelids (conjunctivae) is the route of entry for several viruses. Every few seconds the eyelid

BOX 1.3 DISCUSSION

Why is rhinovirus but not poliovirus sensitive to low pH?

Low pH induces irreversible disassembly of the rhinovirus capsid, but the changes in the poliovirus capsid that occur under acidic conditions are fully reversible. Although high-resolution crystallographic structures of rhinovirus and poliovirus virions have been determined, they do not provide information about the basis for the difference in pH sensitivity. Acid-resistant mutants of rhinovirus contain amino acid changes near the fivefold axes of symmetry. Such mutants behave similarly to poliovirus, in that low pH induces reversible conformational changes in the capsid. The



mutations responsible for acid resistance probably result in stabilization of the particle, so that it does not dissociate at low pH. Such mutations are not selected during natural infections, because they are not necessary for the virus to replicate in the respiratory tract.

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Skern, T., H. Torgersen, H. Auer, E. Kuechler, and D. Blaas. 1991. Human rhinovirus mutants resistant to low pH. *Virology* **183:**757–763.

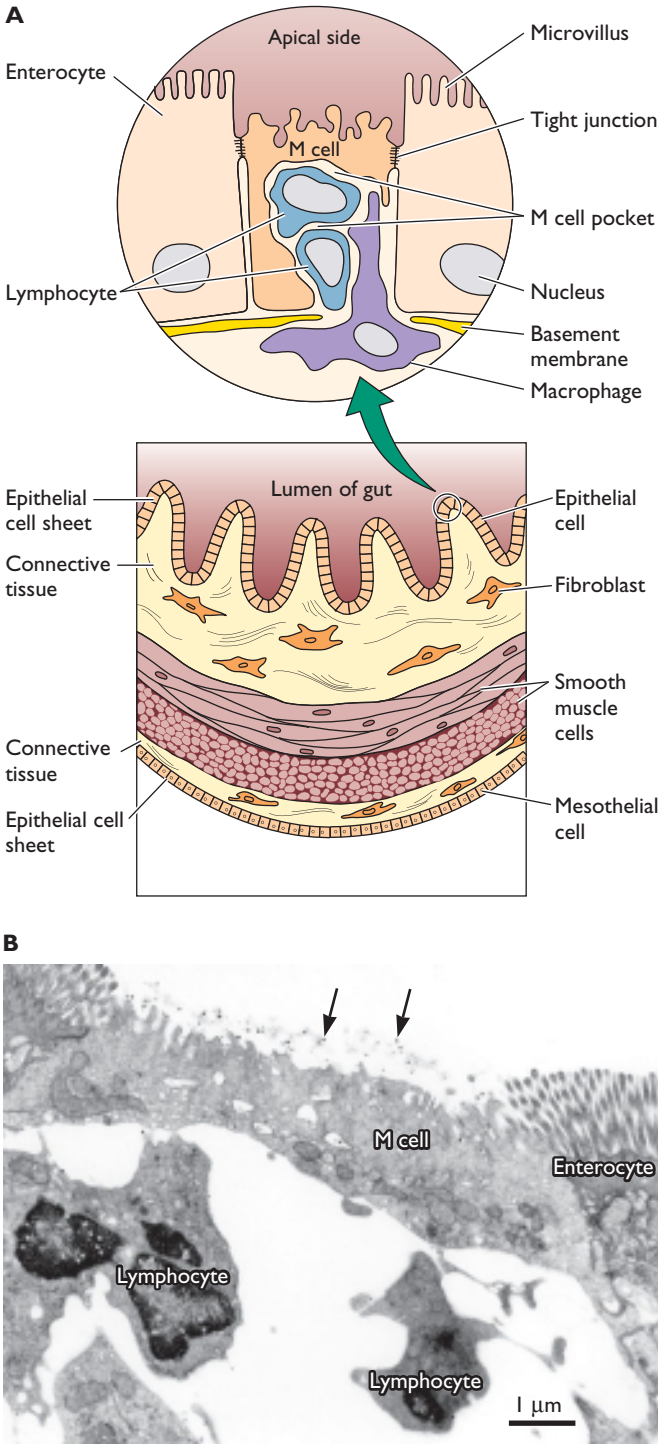


Figure 1.7 Viral entry through M cells in the intestine. (A) Schematic drawing of the intestinal wall. This organ is made up of epithelial, connective, and muscle tissues. Each is formed by different cell types that are organized by cell-cell adhesion within an extracellular matrix. A section of the epithelium has been enlarged, and a typical M cell is shown, surrounded by two enterocytes. Lymphocytes and macrophages move in and out of invaginations on the basolateral side of the M cell. Adapted from A. Siebers and B. B. Finlay, *Trends Microbiol.* **4:**22–28, 1996, and B. Alberts et al., *Molecular Biology of the Cell* (Garland Publishing, New York, NY, 1994), with permission. **(B)** Reovirus attached to, and within vesicles of, an M cell. An electron micrograph of the gut epithelium shows reovirus (small black arrows) attached to the surface of an M cell and also within intracellular vesicles. Reprinted from J. L. Wolf and W. A. Bye, *Annu. Rev. Med.* **35:**95–112, 1984, with permission. Photo courtesy of R. Finberg, Harvard Medical School.

passes over the sclera, bathing it in secretions that wash away foreign particles. There is usually little opportunity for viral infection of the eye, unless it is injured by abrasion. Direct inoculation into the eye may occur during ophthalmologic procedures or from environmental contamination, such as improperly sanitized swimming pools and hot tubs. In most cases, replication is localized and results in inflammation of the conjunctiva, a condition called conjunctivitis. Systemic spread of the virus from the eye is rare, although it does occur; paralytic illness after enterovirus 70 conjunctivitis is one example. Herpesviruses, in particular herpes simplex virus type 1, can also infect the cornea, mainly at the site of a scratch or other injury. Such an infection may lead to immune destruction of the cornea and blindness. Inevitably, herpes simplex virus infection of the cornea is followed by spread of the virus to sensory neurons, and then to neuronal cell bodies in the sensory ganglia, where a latent infection is established.

Skin

The skin protects the body yet provides sensory contact with the environment. The external surface of the skin, or **epidermis**, is composed of several layers, including a basal germinal layer of proliferating cells, a granular layer of dying cells, and an outer layer of dead, keratinized cells (Fig. 1.8). The skin of most animals is an effective barrier against viral infections, as the dead outer layer cannot support viral replication. Replication is usually limited to the site of entry, because the epidermis is devoid of blood or lymphatic vessels that could provide pathways for further spread. Entry through this organ occurs primarily when its integrity is breached by breaks or punctures. Examples of viruses that can gain entry in this manner are some human papillomaviruses and certain poxviruses (e.g., myxoma virus) that are transmitted mechanically by insect vectors such as arthropods (Fig. 1.5; Table 1.1). However, the epidermis is supported by the highly vascularized **dermis**. Other viruses can gain entry to the dermis through the bites of arthropod vectors such as mosquitoes, mites, ticks, and sand flies. Even deeper inoculation, into the tissue and muscle below the dermis, can occur by hypodermic

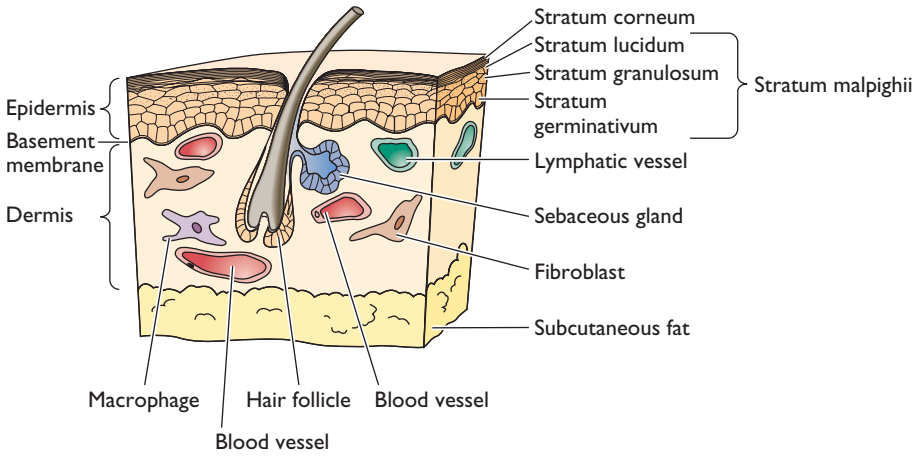


Figure 1.8 Schematic diagram of the skin. The epidermis consists of a layer of dead, keratinized cells (stratum corneum) over the stratum malpighii. The latter may have two layers of cells with increasing numbers of keratin granules (stratum granulosum and stratum lucidum) and a basal layer of dividing epidermal cells (stratum germinativum). Below this is the basement membrane. The dermis contains blood vessels, lymphatic vessels, fibroblasts, and macrophages. A hair follicle and a sebaceous gland are shown. Adapted from F. Fenner et al., *The Biology of Animal Viruses* (Academic Press, New York, NY, 1974), with permission.

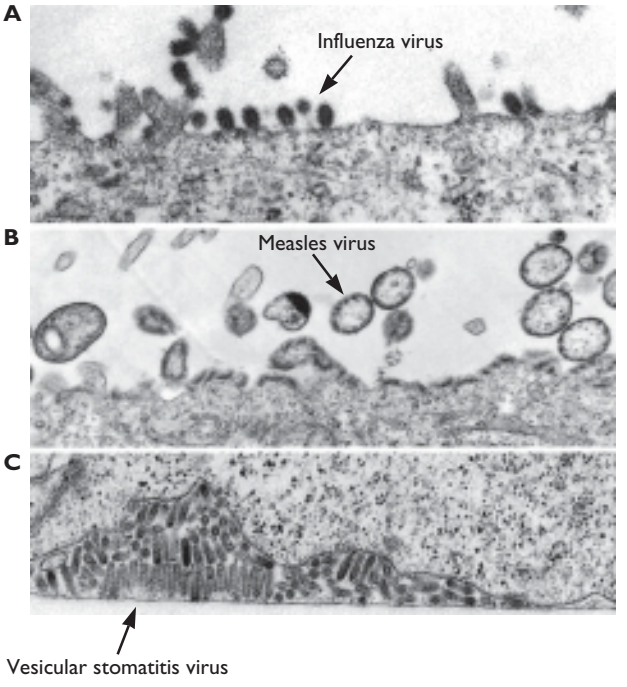
needle punctures, body piercing or tattooing, or sexual contact when body fluids are mingled through skin abrasions or ulcerations (Table 1.1). Animal bites can introduce rabies virus into tissue and muscle rich with nerve endings, through which virions can invade motor neurons. In contrast to the strictly localized replication of viruses in the epidermis, viruses that initiate infection in dermal or subdermal tissues can reach nearby blood vessels, lymphatic tissues, and cells of the nervous system. As a consequence, they may spread to other sites in the body.

Successful Infections Must Modulate or Bypass Host Defenses

To initiate any infection, there must be viral mechanisms for countering the host defenses: these mechanisms may be active or passive, or a combination of the two. The actual pattern of infection that ensues is determined by the kinetics of virus replication in the face of host defenses. The interplay between virus offense and host defense is dynamic: there will be different consequences of a fast-replicating or a slow-replicating virus, in combination with strong or weak host defenses. We discuss many such mechanisms in Chapters 3 and 4.

Some host defenses may be overcome passively by an overwhelming inoculum of virus particles. Single droplets found in the aerosol produced by sneezing can contain as many as 100 million rhinovirus particles; a similarly large number of hepatitis B virus particles can be found in 1 ml of blood from a patient with hepatitis. At these concentrations, it may be impossible for physical and intrinsic

Figure 1.9 Polarized release of viruses from cultured epithelial cells visualized by electron microscopy. (A) Influenza virus released by budding from the apical surface of canine kidney cells. **(B)** Budding of measles virus on the apical surface of human colon carcinoma cells. **(C)** Release of vesicular stomatitis virus at the basal surface of canine kidney cells. Arrows indicate virus particles. Magnification, ×324,000. Reprinted from D. M. Blau and R. W. Compans, *Semin. Virol.* **7:**245–253, 1996, with permission. Courtesy of D. M. Blau and R. W. Compans, Emory University School of Medicine.



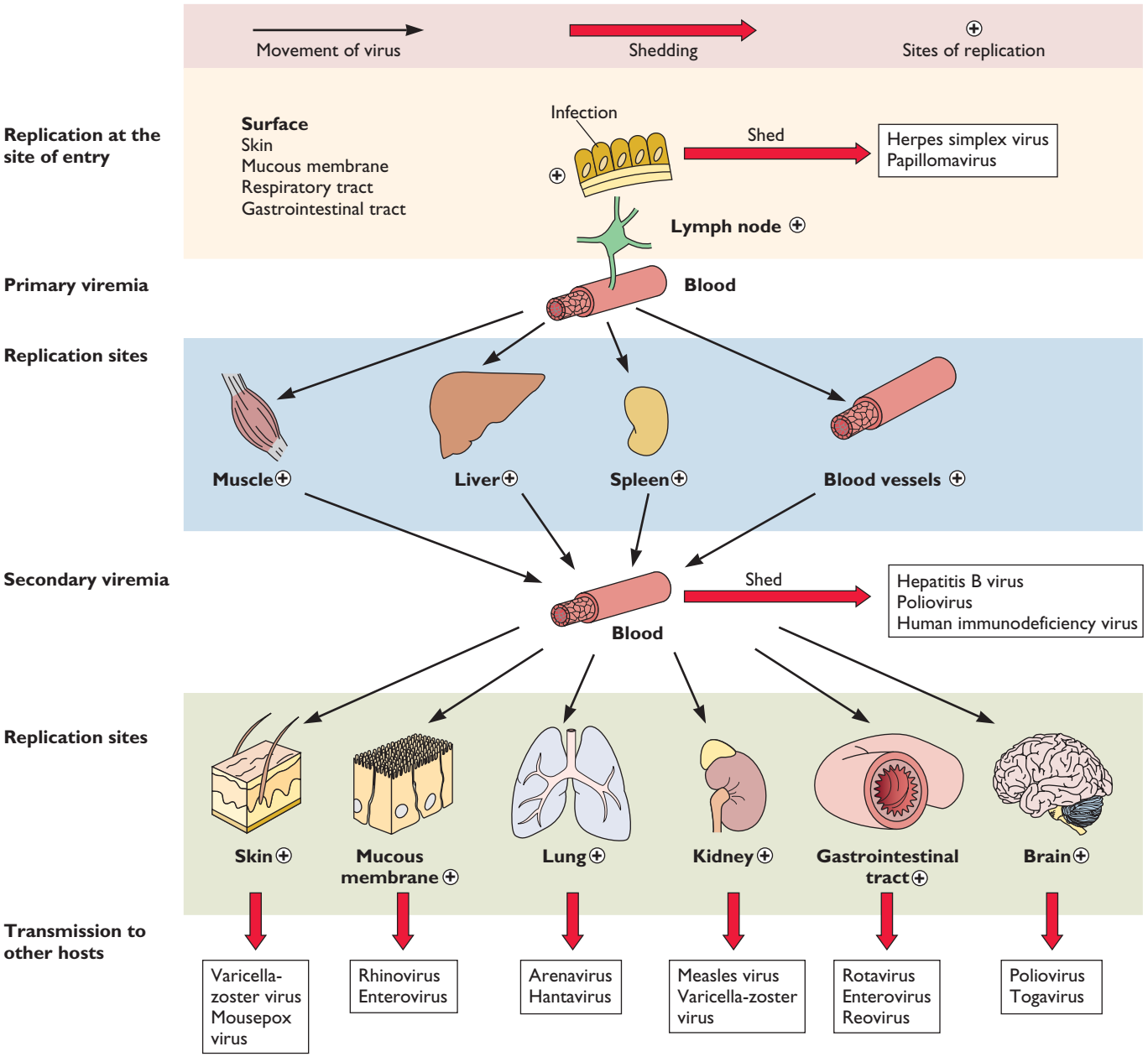


Figure 1.10 Entry, dissemination, and shedding of blood-borne viruses. Shown are the target organs for some viruses that enter at epithelial surfaces and spread via the blood. The sites of virus shedding (red arrows), which may lead to transmission to other hosts, are shown. Adapted from N. Nathanson (ed.), *Viral Pathogenesis* (Lippincott-Raven Publishers, Philadelphia, PA, 1997), with permission.

defenses to block every infecting virus particle. Free passage of virus through the primary physical barriers of skin and mucus layers, made possible by a cut, abrasion, or needle stick, may also allow passive evasion of defenses. Some viruses, including herpesviruses, papillomaviruses, and rabies virus, establish unique infections because they infect organs or cells not exposed to antibodies or cytotoxic lymphocytes. A more egregious breach of both primary and secondary defenses may occur during organ transplantation, which places potentially infected tissues in direct contact with potentially susceptible cells in immunosuppressed patients.

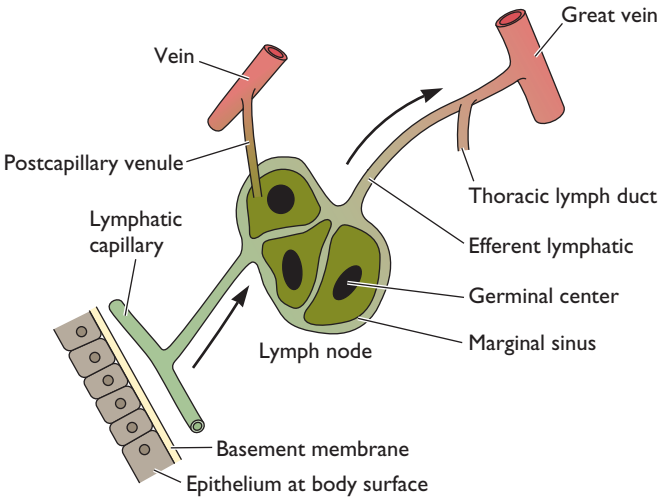


Figure 1.11 The lymphatic system. Lymphocytes flow from the blood into the lymph node through postcapillary venules. Adapted from C. A. Mims et al., *Mims' Pathogenesis of Infectious Disease* (Academic Press, Orlando, FL, 1995), with permission.

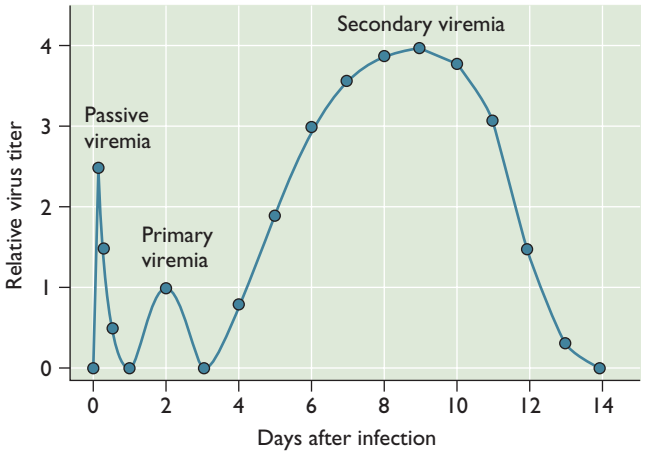


Figure 1.12 Characteristics of viremia. The graph was produced using data from different viral infections. For passive viremia, La Crosse virus, a bunyavirus, was injected into weanling mice, and virus titers in plasma, brain, and muscle were determined at different times afterward. No virus can be detected in the blood after 1 day. For primary viremia, ectromelia virus was inoculated into the footpad of mice; after local multiplication, the virus enters the blood. For secondary viremia, viral progeny produced by multiplication of the ectromelia virus in the target organs were counted. Virus reaches these organs during primary viremia Adapted from N. Nathanson (ed.), *Viral Pathogenesis and Immunity* (Academic Press, London, United Kingdom, 2007), with permission.

Viral Spread

Following replication at the site of entry, virus particles can remain localized or can spread to other tissues (Table 1.1). Local infections in the epithelium are usually contained by the physical constraints of the tissue and brought under control by the intrinsic and immune defenses discussed in Chapters 3 and 4. In general, an infection that spreads beyond the primary site of infection is said to be **disseminated**. If many organs become infected, the infection is described as **systemic**. Spread of an infection beyond the primary site requires that physical and immune barriers be breached. For example, after crossing an epithelium, virus particles reach the basement membrane (Fig. 1.7). The integrity of that structure may be compromised by epithelial cell destruction and inflammation. Below the basement membrane are subepithelial tissues, where virions encounter tissue fluids, the lymphatic system, and phagocytes. All three play significant roles in clearing foreign particles, but also may disseminate infectious virus from the primary site of infection.

One important mechanism for avoiding local host defenses and facilitating spread within the body is the directional release of virions from polarized cells at a mucosal surface (Volume I, Chapter 12). Virions can be released from the apical surface, from the basolateral surface, or from both (Fig. 1.9). After replication, particles released from the apical surface are back where they started, "outside" the host. Such directional release facilitates the dispersal of many newly replicated enteric viruses in the feces (e.g., poliovirus). In general, virions released at apical membranes establish a localized or limited infection. In this case, local lateral spread from cell to cell occurs in the infected epithelium, but the underlying lymphatic and circulatory vessels are rarely invaded. In contrast, virus particles released from the basolateral surfaces of polarized epithelial cells have been moved away from the defenses of the lumenal surface. Release of particles at the basal membrane provides access to the underlying tissues and may facilitate systemic spread. Directional release is therefore a major determinant of the infection pattern.

The consequences of directional release are striking. Sendai virus, which is normally released from the apical surfaces of polarized epithelial cells, causes only a localized infection of the respiratory tract. In stark contrast, a mutant virus, which is released from both apical and basal surfaces, is disseminated.

Hematogenous Spread

Virions that escape from local defenses to produce a disseminated infection often do so by entering the bloodstream (**hematogenous spread**). Virus particles may enter the blood directly through capillaries, by replicating in endothelial cells, or through inoculation by a vector bite. Once in the blood, virions have access to almost every tissue in the host (Fig. 1.10). Hematogenous spread begins when newly replicated particles produced at the entry site

BOX 1.4 **TERMINOLOGY**

Infection of the nervous system: definitions and distinctions

A **neurotropic virus** can infect neurons; infection may occur by neural or hematogenous spread initiating from a peripheral site.

A **neuroinvasive virus** can enter the central nervous system (spinal cord and brain) after infection of a peripheral site.

A **neurovirulent** virus can cause disease of nervous tissue, manifested by neurological symptoms and often death.

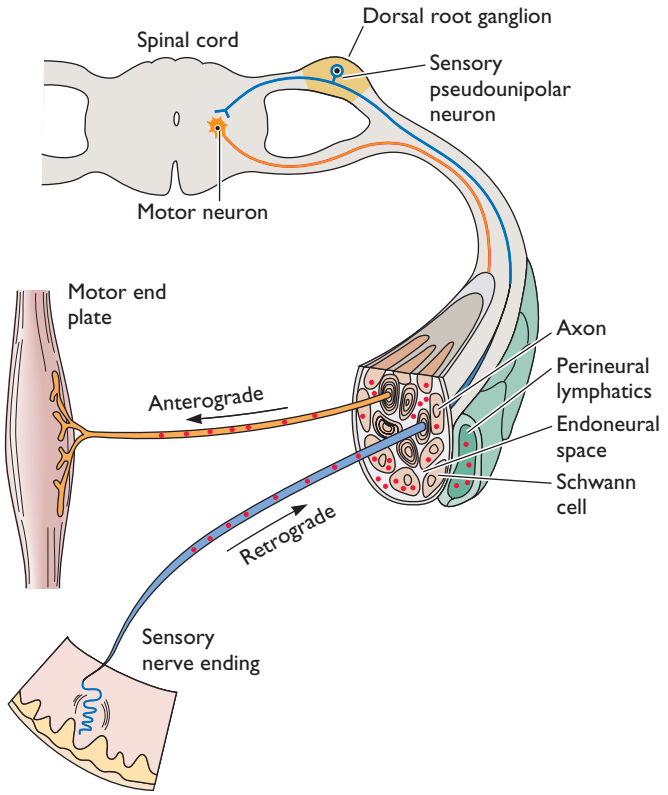
Examples:

Herpes simplex virus has low neuroinvasiveness of the central nervous system, but high neurovirulence. It always enters the peripheral nervous system but rarely enters the central nervous system. When it does, the consequences are almost always severe, often fatal.

Mumps virus has high neuroinvasiveness but low neurovirulence. Most infections lead to invasion of the central nervous system, but neurological disease is mild.

Rabies virus has high neuroinvasiveness and high neurovirulence. It readily infects the peripheral nervous system and spreads to the central nervous system with 100% lethality unless antiviral therapy is administered shortly after infection.

Figure 1.13 Possible pathways for the spread of infection in nerves. Virus particles may enter the sensory or motor neuron endings. They may be transported within axons, in which case viruses taken up at sensory endings reach dorsal root ganglion cells. Those taken up at motor endings reach motor neurons. Viruses may also travel in the endoneural space, in perineural lymphatics, or in infected Schwann cells. Directional transport of virus particles inside the sensory neuron is defined as anterograde [movement from the (−) to the (+) ends of microtubules] or retrograde [movement from the (+) to the (−) ends of microtubules]. Adapted from R. T. Johnson, *Viral Infections of the Nervous System* (Raven Press, New York, NY, 1982), with permission.



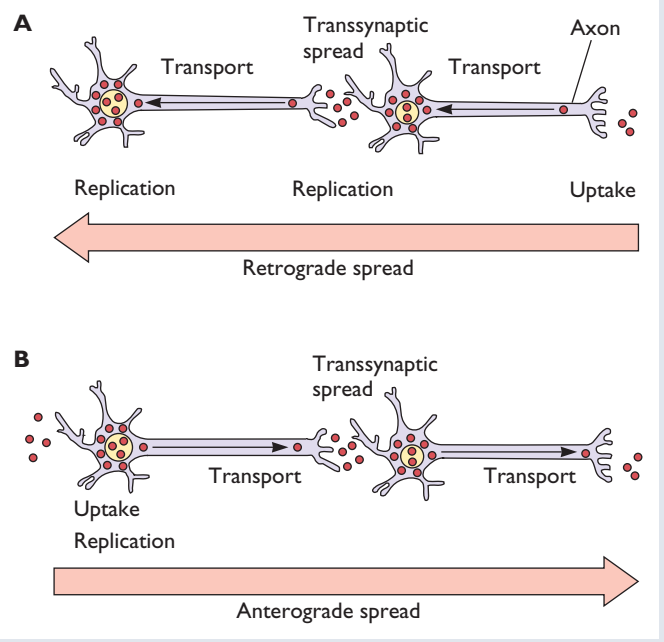
are released into the extracellular fluids, which can be taken up by the local lymphatic vascular system (Fig. 1.11). Lymphatic capillaries are considerably more permeable than circulatory system capillaries, facilitating virus entry. As lymphatic vessels ultimately drain into the circulatory system, virus particles in lymph have free access to the bloodstream. In the lymphatic system, virions pass through lymph nodes, where they encounter migratory cells of the immune system. Viral pathogenesis resulting from the direct infection of immune system cells is initiated in this fashion (e.g., human immunodeficiency virus and measles virus). Some viruses replicate in the infected lymphoid cells, and progeny are released into the blood plasma. The infected lymphoid cell may also migrate away from the local lymph node to distant parts of the circulatory system. There may be little viral replication while the cell is in the bloodstream. However, when the infected cell receives chemotactic signals that direct it to enter other tissues, new virus particles may be produced in the activated cell.

The term **viremia** describes the presence of infectious virus particles in the blood. These virions may be free in the blood or contained within infected cells such as lymphocytes. **Active viremia** is produced by replication, while **passive viremia** results when particles are introduced into the blood without viral replication at the site of entry (injection of a virion suspension into a vein) (Fig. 1.12). Progeny virions released into the blood after initial replication at the site of entry constitute **primary viremia**. The concentration of particles during primary viremia is usually low. However, the subsequent disseminated infections that result are often extensive, releasing considerably more virions. Such delayed appearance of a high concentration of infectious virus in the blood is termed **secondary viremia**. The two phases of viremia were first described in classic studies of mousepox (Fig. 1.4).

BOX 1.5 TERMINOLOGY

Which direction, anterograde or retrograde?

Those who study virus spread in the nervous system often use the words **retrograde** and **anterograde** to describe direction. Unfortunately, confusion arises because the terms can be used to describe directional movement of virus particles inside a cell as well as spread between synaptically connected neurons. Spread from the primary neuron to the second-order neuron in the direction of the nerve impulse is said to be anterograde spread (see figure). Spread in the opposite direction is said to be retrograde. Spread inside a neuron is defined by microtubule polarity. Transport on microtubules from (–) to (+) ends is said to be anterograde, while transport on microtubules from (+) to (–) ends is said to be retrograde.



Retrograde and anterograde spread of virus in nerves. **(A)** Retrograde spread of infection. Virus invades at axon terminals and spreads to the cell body, where replication ensues. Newly replicated virus particles spread to a neuron at sites of synaptic contact. Particles enter the axon terminal of the second neuron to initiate a second cycle of replication and spread. **(B)** Anterograde spread of infection. Virus invades at dendrites or cell bodies and replicates. Virus particles then spread to axon terminals, where virions cross synaptic contacts to invade dendrites or cell bodies of the second neuron.

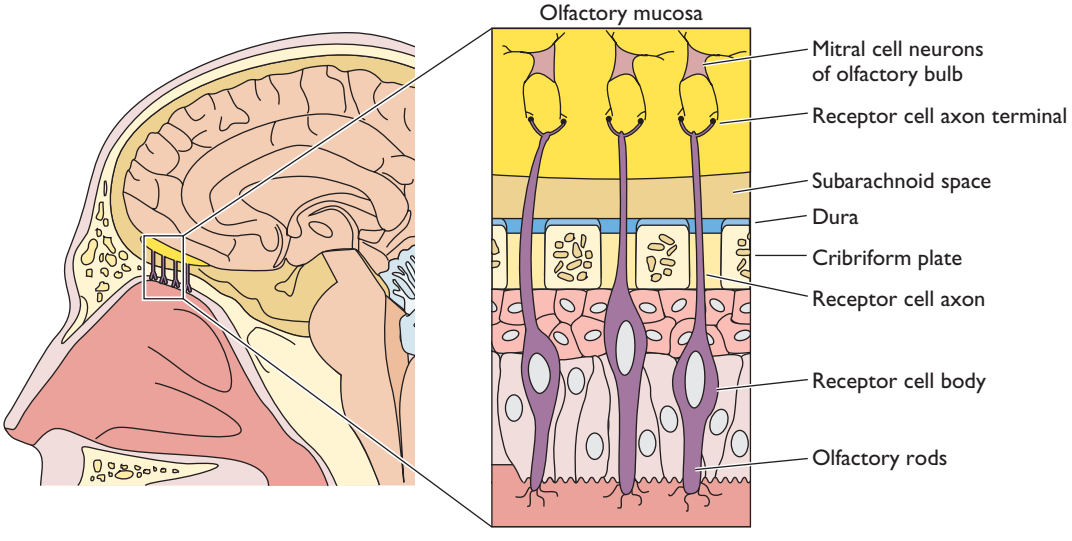
The concentration of virions in blood is determined by the rates of their synthesis in permissive tissues, and by how quickly they are released into, and removed from, the blood. Circulating particles are removed by phagocytic cells of the reticuloendothelial system in the liver, lungs, spleen, and lymph nodes. When serum antibodies appear, virions in the blood may bind these antibodies and be neutralized, as described in Chapter 4. Formation of a complex of antibodies and virus particles facilitates uptake by Fc receptors carried by macrophages lining the circulatory vessels. Virion-antibody complexes can be sequestered in significant quantities in the kidneys, spleen, and liver. The time a virion is present in the blood usually varies from 1 to 60 min, depending on such parameters as the physiology of the host (e.g., age and health) and the size of the virus (large particles are cleared more rapidly than small particles). Some viral infections are noteworthy for the long-lasting presence of infectious particles in the blood. Hosts infected with hepatitis B and C viruses or lymphocytic choriomeningitis virus may have viremia that lasts for years.

Viremia obviously is of diagnostic value and can be used to monitor the course of infection, but it also presents practical problems. Infections can be spread inadvertently in the population, when pooled blood from thousands of individuals is used directly for therapeutic purposes (transfusions) or as a source of therapeutic proteins (e.g., gamma globulin or blood-clotting factors). We have learned from unfortunate experience that hepatitis and acquired immunodeficiency syndrome can be spread by contaminated blood and blood products. Obviously, sensitive detection methods and stringent purification protocols are required to protect those who use and dispense these products. Frequently, it may be difficult, or technically impossible, to quantify infectious particles in the blood, as is currently the case for hepatitis B virus. Accordingly, the presence

BOX 1.6 BACKGROUND

The path rarely taken: direct entry into the central nervous system by olfactory routes

Olfactory neurons are unusual in that their cell bodies are present in the olfactory epithelia and their axon termini are in synaptic contact with olfactory bulb neurons. Literally, these direct conduits to the brain project from cells that are in direct contact with the environment. The olfactory nerve fiber passes through the skull via an opening called the arachnoid. Remarkably, few viral infections enter the brain by the olfactory route, despite significant replication of many in the nasopharyngeal cavity.



Adapted from R. T. Johnson, *Viral Infections of the Nervous System* (Raven Press, New York, NY, 1982), with permission.

of characteristic viral proteins provides surrogate markers for viremia.

Neural Spread

Many viruses spread from the primary site of infection by entering local nerve endings. In some cases, neuronal spread is the definitive characteristic of their pathogenesis (e.g., rabies virus and alphaherpesviruses). For others, invasion of the nervous system is a rare but important diversion from their normal site of replication (e.g., poliovirus and reovirus). Mumps virus, human immunodeficiency virus, and measles virus replicate in the brain, but spread by the hematogenous route. The molecular mechanisms that dictate spread by neural or hematogenous pathways are generally not well understood. While viruses that infect the nervous system are often said to be **neurotropic** (Box 1.4), they are generally capable of infecting a variety of cell types. Viral replication usually occurs first in nonneuronal cells, with virions subsequently spreading into afferent (e.g., sensory) or efferent (e.g., motor) nerve fibers innervating the infected tissue (Fig. 1.13).

Neurons are polarized cells with functionally and structurally distinct processes (axons and dendrites) that can be separated by enormous distances. For example, in adult humans the axon terminals of motor neurons that control stomach muscles can be 50 cm away from the cell bodies and dendrites in the brain stem. We currently have a limited understanding of how viral particles move in and among cells of the nervous system. It is likely that virions enter neurons by the same mechanisms used to enter other cells. Virus particles must be transported over relatively long distances to the site of viral replication in the neuronal cell body. All evidence indicates that virions are carried in the infected neuron by cellular systems, but viral proteins may facilitate the direction of spread.

Directionality of movement within a neuron is most likely to be mediated by microtubules and their attendant motor proteins, including kinesin and dynein (Box 1.5). Drugs, such as colchicines, that disrupt microtubules efficiently block the spread of many neurotropic viruses from the site of peripheral inoculation to the central nervous system. The precise intracellular form of any virus

BOX 1.7 DISCUSSION

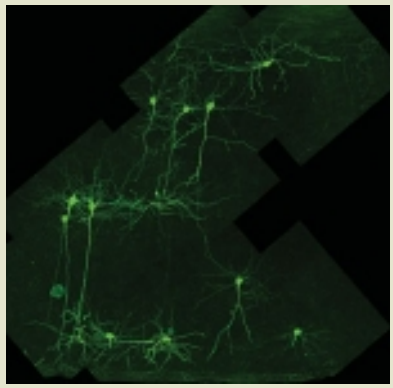
Tracing neuronal connections in the nervous system with viruses

The identification and characterization of synaptically linked multineuronal pathways in the brain are important in understanding the functional organization of neuronal circuits. Conventional tracing methodologies have relied on the use of markers such as wheat germ agglutinin-horseradish peroxidase or fluorochrome dyes. The main limitations of these tracers are their low specificity and sensitivity. During experimental manipulation, it is difficult to restrict the diffusion of tracers to a particular cell group or nucleus, and so uptake occurs in neighboring neurons or adjacent unconnected axon fibers, producing false-positive labeling of a circuit. Neurons located one or more synapses away from the injection site receive progressively less label, because the tracer is diluted at each stage of transneuronal transfer.

Some alphaherpesviruses and rhabdoviruses have considerable promise as self-amplifying tracers of synaptically connected neurons. Under proper conditions, second- and third-order neurons show the same labeling intensity as those infected initially. Moreover, the specific pattern of infected neurons observed in tracing studies is consistent with transsynaptic passage of virus rather than lytic spread through the extracellular space.

The detection of viruses typically involves immunohistochemical localization of viral antigens by light microscopy. More recently, reporter genes such as that encoding green fluorescent protein gene from *Aequorea victoria* have been introduced into the genomes of neurotropic viruses for simpler visualization of viral infection.

Ekstrand, M., L. Pomeranz, and L. W. Enquist. 2008. The alpha-herpesviruses: molecular pathfinders in nervous system circuits. *Trends Mol. Med.* **14:**134–140.



Identification of a possible microcircuit in the rodent visual cortex (V2) after injection of a GFP-expressing strain of pseudorabies virus into the synaptically connected, but distant V1 region. Infection spread via V1 axons (V1 cell bodies are located far out of the field of view) in a retrograde manner to a subset of V2 cell bodies seen here. Confocal microscopy and image reconstruction by Botond Roska, Friedrich Miescher Institute, Basel, Switzerland.

particle that spreads in the nervous system has not been established. Both mature virions and nucleocapsids have been observed in axons and dendrites of animals infected by rabies virus and alphaherpesviruses.

In general, viral replication in neurons is preceded by replication in epithelial cells. Virions must enter the neuron at axon terminals and virions or subviral particles (e.g., the nucleocapsid) then must be transported to the cell body of the neuron where replication may occur. After replication, virions or subviral particles are assembled, and may be released directly from the neuronal cell body, may spread directly to glia or support cells in contact with the neuron, or may be transported within the axon, to axon terminals for release. Under some circumstances, virions can enter neurons directly with no prior replication in nonneuronal cells. However, this event is probably infrequent, as nerve endings are rarely exposed to an infecting virus.

With rare exceptions (Box 1.6), cells of the peripheral nervous system are the first to be infected. These neurons represent the first cells in circuits connecting the innervated peripheral tissue with the spinal cord and brain. Once in the peripheral nervous system, alphaherpesviruses, some rhabdoviruses (e.g., rabies virus), and some flaviviruses (e.g., West Nile virus) can spread among neurons connected by synapses (Box 1.7). Nonneuronal support cells and satellite cells in ganglia may also be infected. Virus spread by this mode can continue through chains of connected neurons of the peripheral nervous system to the spinal cord and brain, with devastating results (Fig. 1.14).

An important component of neuronal infection is movement and release of infectious particles in polarized neurons. As with polarized epithelial cells discussed earlier, directional release of infectious virus from neurons affects the outcome of infection. For example, alphaherpesviruses become latent in peripheral neurons that innervate the site of infection (Fig. 1.14). Reactivation from the latent state results in viral replication in the primary neuron and subsequent transport of progeny virus particles from the neuron cell body back to the innervated peripheral tissue. Virions can then spread from the peripheral to the central nervous system, or it can spread back to the peripheral site serviced by that particular group of neurons in the ganglion (Fig. 1.14). The direction taken is the difference

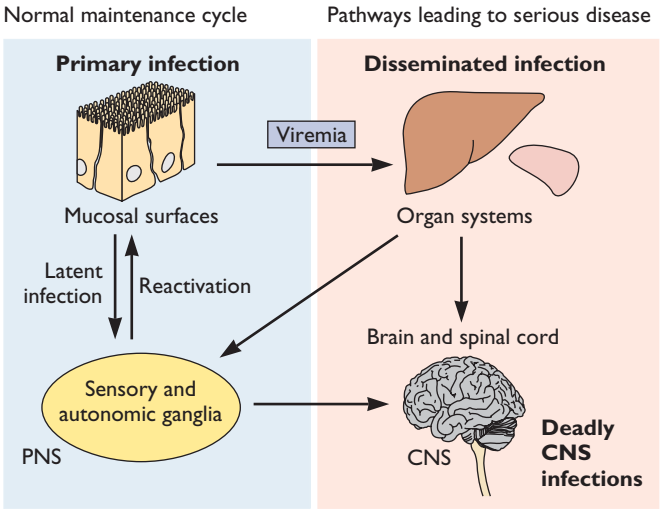


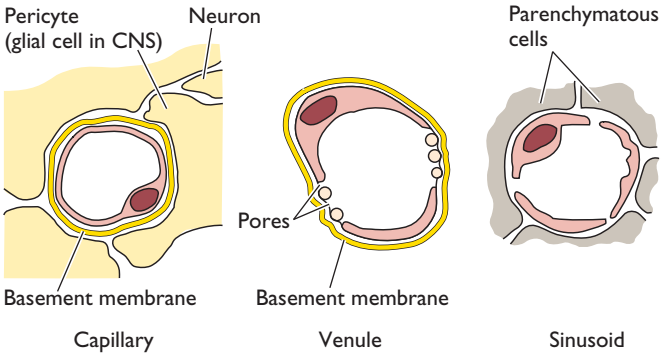
Figure 1.14 Outline of the spread of alphaherpesviruses and relationship to disease. Abbreviations: PNS, peripheral nervous system; CNS, central nervous system.

between a minor local infection (a cold sore) or a lethal viral encephalitis. Luckily, spread back to the peripheral site is by far more common.

Organ Invasion

Once virions enter the blood and are dispersed from the primary site, any subsequent replication requires invasion of new cells and tissues. There are three main types of blood vessel-tissue junctions that serve as routes for tissue invasion (Fig. 1.15). In some tissues the endothelial cells are continuous with a dense basement membrane. At other sites, the endothelium contains gaps, and at still others there may be **sinusoids**, in which macrophages form part of the blood-tissue junction.

Figure 1.15 Blood-tissue junction in a capillary, venule, and sinusoid. (Left) Continuous endothelium and basement membrane found in the central nervous system, connective tissue, skeletal and cardiac muscle, skin, and lungs. **(Center)** Fenestrated endothelium found in the choroid plexus, villi of the intestine, renal glomerulus, pancreas, and endocrine glands. **(Right)** Sinusoid, lined with macrophages of the reticuloendothelial system, as found in the adrenal glands, liver, spleen, and bone marrow. Adapted from C. A. Mims et al., *Mims' Pathogenesis of Infectious Disease* (Academic Press, Orlando, FL, 1995), with permission.



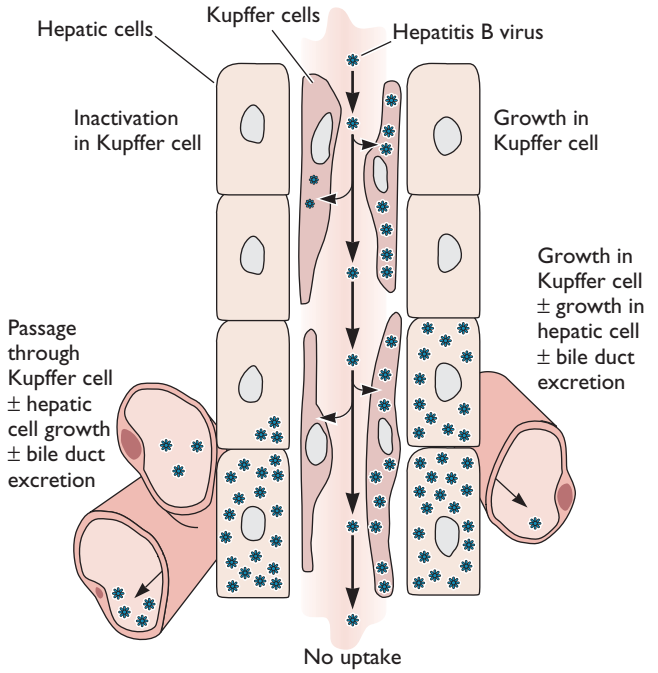


Figure 1.16 Routes of viral entry into the liver. Two layers of hepatocytes are shown, with the sinusoid at the center lined with Kupffer cells. Endothelial cells are not shown. Adapted from C. A. Mims et al., *Mims' Pathogenesis of Infectious Disease* (Academic Press, Orlando, FL, 1995), with permission.

The Liver, Spleen, Bone Marrow, and Adrenal Glands

The liver, spleen, bone marrow, and adrenal glands are characterized by the presence of sinusoids lined with macrophages. Such macrophages, known as the reticuloendothelial system, function to filter the blood and remove foreign particles. They often provide a portal of entry into tissues. For example, viruses that infect the liver, the major filtering and detoxifying organ of the body, usually enter from the blood. The presence of virus particles in the blood invariably leads to the infection of **Kupffer cells**, the macrophages that line liver sinusoids (Fig. 1.16). Virions may be transcytosed across Kupffer and endothelial cells without replication to reach the underlying hepatic cells. Alternatively, viruses may multiply in these cells and then infect underlying hepatocytes. Either mechanism may induce inflammation and necrosis of liver tissue, a condition termed **hepatitis**.

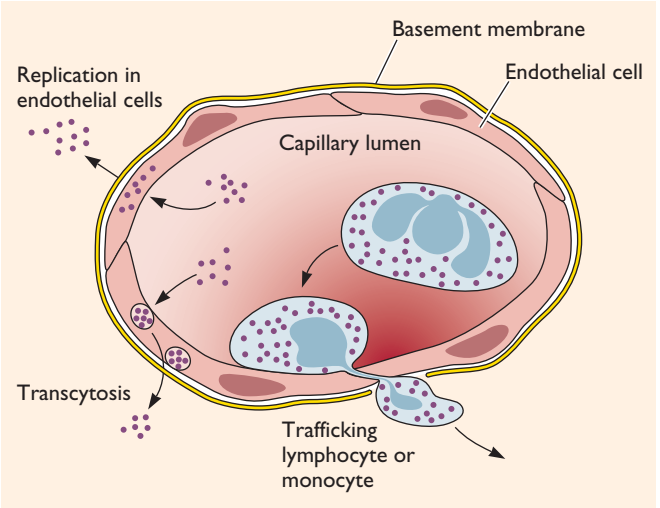


Figure 1.17 How viruses travel from blood to tissues. Schematic of a capillary illustrating different pathways by which viruses may leave the blood and enter underlying tissues. Adapted from N. Nathanson (ed.), *Viral Pathogenesis and Immunity* (Academic Press, London, United Kingdom, 2007), with permission.

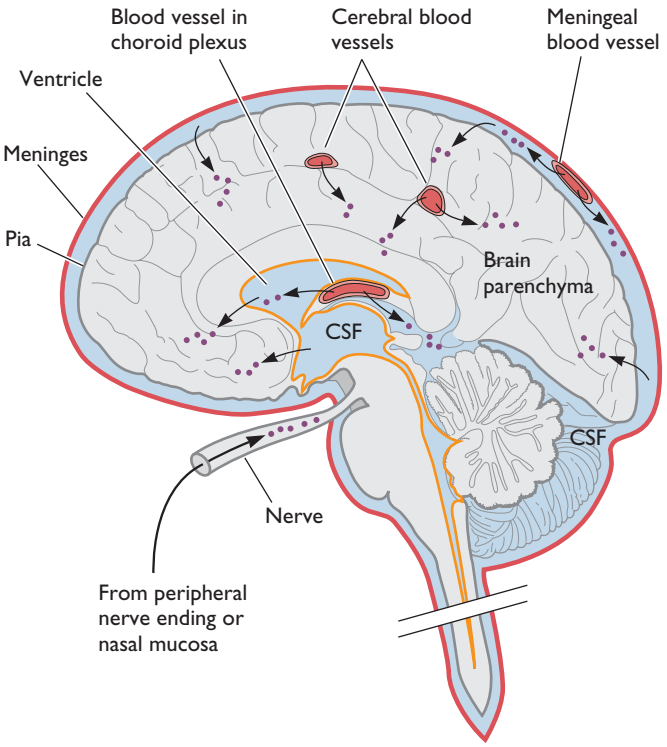


Figure 1.18 How viruses gain access to the central nervous system. A summary of the mechanisms by which viruses can enter the brain is shown. CSF, cerebrospinal fluid. Adapted from C. A. Mims et al., *Mims' Pathogenesis of Infectious Disease* (Academic Press, Orlando, FL, 1995), with permission.

Central Nervous System, Connective Tissue, and Skeletal and Cardiac Muscle

In the central nervous system, connective tissue, and skeletal and cardiac muscle, capillary endothelial cells are backed by a dense basement membrane (Fig. 1.15 and 1.17). In the central nervous system the basement membrane is the foundation of the blood-brain barrier. However, it is not so much a barrier as a selective permeability system. Much study has been devoted to determining how infection spreads to the brain from the blood. Routes of invasion into the central nervous system are summarized in Fig. 1.18.

In several well-defined parts of the brain, the capillary epithelium is fenestrated (with "windows" between cells; loosely joined together), and the basement membrane is sparse. These highly vascularized sites include the choroid plexus, a tissue that produces more than 70% of the cerebrospinal fluid that bathes the spinal cord and ventricles of the brain. Some viruses (mumps virus and certain togaviruses) pass through the capillary endothelium and enter the stroma of the choroid plexus, where they may cross the epithelium into the cerebrospinal fluid either by transcytosis or by replication and directed release. Once in the cerebrospinal fluid, infection spreads to the ependymal cells lining the ventricles and the underlying brain tissue (Fig. 1.18). Other viruses (picornaviruses and togaviruses) may infect directly, or be transported across, the capillary endothelium. Some viruses (human immunodeficiency virus and measles virus) cross the endothelium within infected monocytes or lymphocytes. Increased local permeability of the capillary endothelium, caused, for example, by certain hormones, may also permit virus entry into the brain and spinal cord.

Coxsackieviruses, members of the *Picornaviridae*, multiply in skeletal and cardiac muscle. The mechanisms by which these viruses enter muscles from the blood have not been adequately studied. It is known that certain coxsackieviruses can replicate in cultured endothelial cells. Virions may be released into muscle cells after multiplying within cells of the capillary walls. Some coxsackieviruses infect B lymphocytes, which might carry the infection from the blood into muscle.

The Renal Glomerulus, Pancreas, Ileum, and Colon

To enter tissues that lack sinusoids (Fig. 1.15), virions must first adhere to the endothelial cells lining capillaries or venules, where the blood flow is slowest and the walls are thinnest. Endothelial cells are not highly phagocytic, and therefore adhesion of virus particles to these cells requires the presence of cellular receptors. To increase

the chances of adhesion, virions must be present in a high concentration and circulate for a sufficient period. Clearly there is a "race" between adhesion and removal of virions by the reticuloendothelial system. Once bloodborne virus particles have adhered to the vessel wall, they can readily invade the renal glomerulus, pancreas, ileum, or colon, because the endothelial cells that make up the capillaries are fenestrated, permitting virions or virus-infected cells to cross into the underlying tissues. Some viruses cross the endothelium while being carried by infected monocytes or lymphocytes, in a process called **diapedesis**.

Skin

In a number of systemic viral infections, rashes are produced when virions leave blood vessels (Table 1.2). Different types of skin lesions are recognized. **Macules** and **papules** develop when inflammation occurs in the dermis, with the infection confined in or near the vascular bed. **Vesicles** and **pustules** occur when viruses spread from the capillaries to the superficial layers of the skin. Destruction of cells by virus replication is the primary cause of lesions.

During some viral infections, lesions may also occur in mucosal tissues such as those in the mouth and throat. Because these surfaces are wet, vesicles break down more rapidly than on the skin. During measles infection, vesicles in the mouth become ulcers before the appearance of skin lesions. Such Koplik spots are diagnostic for measles virus infection. In the respiratory tract, after virions leave the subepithelial capillaries, only a single layer of cells must be traversed before particles reach the exterior. Hence, during infections with measles virus and varicella-zoster virus, virions appear in respiratory tract secretions before the skin rash appears. By the time that the infection is recognized from the skin rash, viral transmission to other persons may already have occurred.

Table 1.2 Viruses that cause skin rashes in humans

Virus	Disease	Features
Coxsackievirus A16	Hand-foot-and-mouth disease	Maculopapular rash
Measles virus	Measles	Maculopapular rash
Parvovirus	Erythema infectiosum	Maculopapular rash
Rubella virus	German measles	Maculopapular rash
Varicella-zoster virus	Chickenpox, zoster	Vesicular rash

The Fetus

The basement membrane is less well developed in the fetus, and infection can occur by invasion of the placental tissues and subsequent invasion of fetal tissue. Infected circulating cells such as monocytes may enter the fetal bloodstream directly. In a pregnant female, viremia may result in infection of the developing fetus. The risk of fetal infection in infants whose mothers have been infected with rubella virus during the first trimester is approximately 80%. Similarly, intrauterine transmission of human cytomegalovirus occurs in approximately 40% of pregnant women with primary infection. Human immunodeficiency virus can be transmitted from mother to infant.

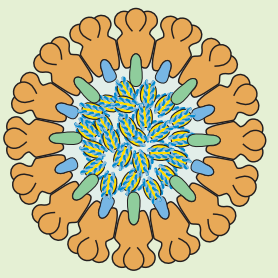
Tropism

Most viruses do not infect all the cells of a host, but are restricted to specific cell types of certain organs. **Tropism** is the predilection of viruses to infect certain tissues and

BOX 1.8 BACKGROUND

JC virus, a ubiquitous human polyomavirus

JC virus is widespread in the human population. Most humans experience inapparent childhood infections, but the virus then persists for life in the kidneys or brain with little consequence. If the immune system is compromised by pregnancy or chemotherapy, virus often reactivates from kidney tissues, and infectious virus particles can be found in the urine. On rare occasions, JC virus reactivates in the brain, causing more serious problems. The ensuing disease is called progressive multifocal leukoencephalopathy, a demyelinating disease affecting oligodendrocytes. This disease is often seen in patients with acquired immunodeficiency syndrome and after immunosuppressive therapy for organ transplant procedures. Given the rarity of the disease and the lack of suitable animal models, it has been difficult to determine how the genome is maintained and virus replication is reactivated.



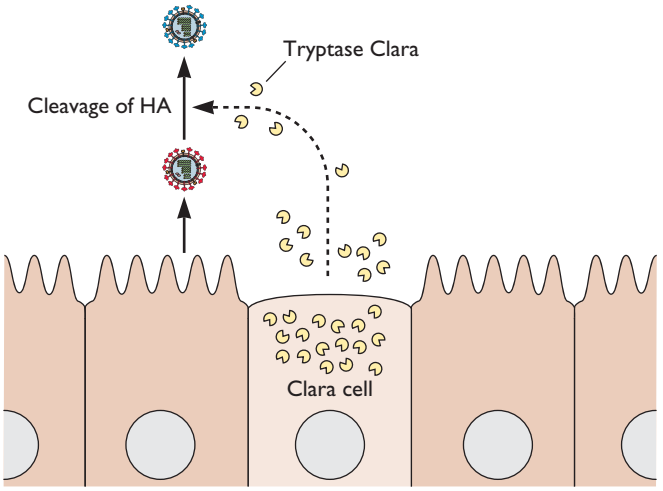


Figure 1.19 Cleavage of influenza virus HA0 by tryptase Clara. Influenza viruses replicate in respiratory epithelial cells in humans. These virus particles contain the uncleaved form of HA (HA0) and are noninfectious. Clara cells secrete the protease tryptase Clara, which cleaves the HA0 of extracellular particles, thereby rendering them infectious. Adapted from M. Tashiro and R. Rott, *Semin. Virol.* **7:**237–243, 1996, with permission.

not others. For example, an **enterotropic** virus replicates in the gut, whereas a **neurotropic virus** replicates in cells of the nervous system. Some viruses are **pantropic**, infecting and replicating in many cell types and tissues. Tropism is governed by at least four parameters. It can be determined by the distribution of receptors for entry (**susceptibility**), or by a requirement for differentially expressed intracellular gene products to complete the infection (**permissivity**). However, even if the cell is susceptible and permissive, infection may not occur because virus particles are physically prevented from interacting with the tissue (**accessibility**). Finally, an infection may not occur even when the tissue is accessible and the cells are susceptible and permissive, because of local intrinsic and innate immune defenses. In most cases, tropism is determined by a combination of two or more of these parameters.

Tropism influences the pattern of infection, pathogenesis, and long-term virus survival. Human herpes simplex virus is often said to be neurotropic because of its noteworthy ability to infect, and be reactivated from, the nervous system. But in fact, herpes simplex virus is pantropic and replicates in many cells and tissues in the host. By infecting neurons, it may establish a stable latent infection, but, because it is pantropic, infection may spread to other tissues and host cells. One serious consequence is that, if an infection is not contained by host defenses at the site of infection, it spreads widely, causing disseminated disease, as can occur when herpes simplex virus infects infants and immunocompromised adults (Fig. 1.14). Herpes simplex virus neurotropism leads to yet another serious result of infection. On rare occasions, this virus can enter the central nervous system and cause encephalitis that is often fatal. This lethal excursion is not due to a breach in immune defenses because immunodeficient individuals experience the same low rate of brain infections as do immunocompetent individuals.

Cell Receptors for Viruses

A cell may be susceptible to infection if the viral receptor(s) is present and functional. If the proper form of viral receptor is not made, the tissue cannot become infected. The location of the receptor might also be a determinant of cellular susceptibility. If the cellular receptor is present only on the basal cell membrane of polarized epithelial cells, a virus cannot replicate unless it first reaches that location by some means. In some cases, virus particles bound to antibodies can be taken up by Fc receptors on nonsusceptible cells (see "Immunopathological lesions caused by B cells" in Chapter 4).

As cell receptors for viruses have been identified and studied, it has become evident that they can be determinants of tropism. Despite the simplicity of this mechanism, the distribution of cellular receptors in host tissues generally is more widespread than the observed tropism of the virus (Table 1.3). Clearly, receptors are necessary for infection but are not sufficient to explain viral tropism.

Host Cell Proteins That Regulate Viral Transcription

Sequences in viral genomes that control transcription of viral genes such as enhancer regions may be determinants of viral tropism. In the brain, JC polyomavirus replicates only in oligodendrocytes (Box 1.8). The results of *in vitro* and *in vivo* experiments indicate that the JC virus enhancer is active only in this cell type. Other examples include the liver-specific enhancer of hepatitis B virus, the keratinocyte-specific enhancer of human papillomavirus type 11, and the enhancers in the long terminal repeat of human immunodeficiency virus type 1 that are specific for T cells.

Table 1.3 Viral receptors and viral tropism

Virus	Tropism	Receptor (distribution)
Major-group rhinovirus	Respiratory tract	Icam-1 (ubiquitous)
Influenza virus	Respiratory tract	Sialic acid (ubiquitous)
Poliovirus	Oropharyngeal and intestinal mucosa, motor neurons	CD155 (most organs)
Herpes simplex virus	Mucoepithelial cells, neurons	Glycosaminoglycans (ubiquitous)

BOX 1.9 DISCUSSION

A mechanism for expanding the tropism of influenza virus is revealed by analyzing infections that occurred in 1940

Until the isolation of the H5N1 virus from 16 persons in Hong Kong, viruses with the HA0 cleavage site change that permits cleavage by intracellular furin proteases had not been found in humans. However, the WSN/33 strain of influenza virus, produced in 1940 by passage of a human isolate in mouse brain, is pantropic in mice. Unlike most human influenza virus strains, WSN/33 can replicate in cell culture in the absence of added trypsin, because its HA can be cleaved by serum plasmin. Surprisingly, it was found that the NA of WSN/33 is necessary for HA cleavage by serum plasmin. This altered NA protein can bind plasminogen, sequestering it on the cell surface, where it can be converted to the active form, plasmin (see figure, panel A). Plasmin then cleaves HA into HA1 and HA2. Therefore, a change in NA, not in HA, allowed cleavage of HA by a ubiquitous cellular protease. This property may, in part, explain the pantropic nature of WSN/33.

WSN/33 can replicate in the mouse brain, causing encephalitis. Interestingly, recombinant viruses containing only NA from WSN/33 did not cause encephalitis; replication in the brain requires the presence of the M and NS genes in addition to the NA from WSN/33. The virulence of WSN/33 is polygenic and cannot be explained solely by the expanded tropism of this strain.

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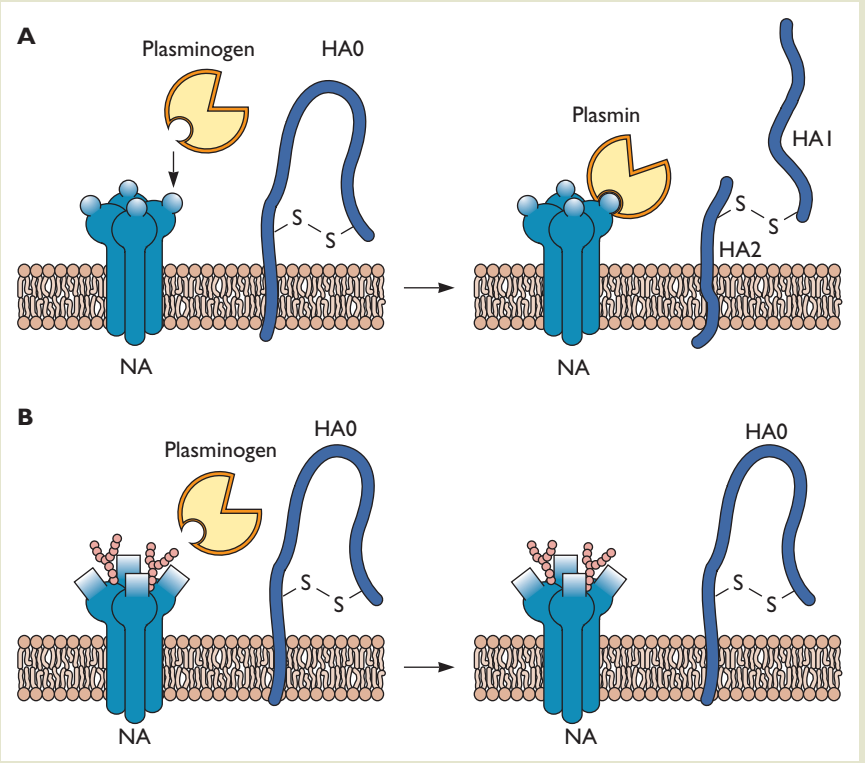

Proposed mechanism for activation of plasminogen and cleavage of HA. **(A)** Plasminogen binds to NA, which has a lysine at the carboxyl terminus. A cellular protein converts plasminogen to the active form, plasmin. Plasmin then cleaves HA0 into HA1 and HA2. **(B)** When NA does not contain a lysine at the carboxyl terminus, plasminogen cannot interact with NA and is not activated to plasmin. Therefore, HA is not cleaved. Adapted from H. Goto and Y. Kawaoka, *Proc. Natl. Acad. Sci. USA* **95:**10224–10228, 1998, with permission.

Cellular Proteases

Cellular proteases are often required to cleave viral proteins to form the mature infectious virus particle. A cellular protease cleaves the influenza virus HA0 precursor into two subunits so that fusion of the viral envelope and cell membrane can proceed. In mammals, the replication of influenza virus is restricted to epithelial cells of the upper and lower respiratory tract. The tropism of this virus is thought to be influenced by the limited production of the protease that processes HA0. This serine protease, called tryptase Clara, is secreted by nonciliated Clara cells of the bronchial and bronchiolar epithelia (Fig. 1.19). The purified enzyme can cleave HA0 and activate HA0 in virions *in vitro*. Alteration of the HA cleavage site so that it can be recognized by other cellular proteases dramatically influences the tropism of the virus and its pathogenicity: some highly virulent avian influenza virus strains contain an insertion of multiple basic amino acids at the cleavage site of HA0. This new sequence

permits processing by ubiquitous intracellular proteases such as furins. As a result, these variant viruses are released in an active form and are able to infect many organs of the bird, including the spleen, liver, lungs, kidneys, and brain. Naturally occurring mutants of this type cause high mortality in poultry farms. Avian influenza viruses isolated from 16 people in Hong Kong contained similar amino acid substitutions at the HA cleavage site. Indeed, many of these individuals had gastrointestinal, hepatic, and renal symptoms as well as respiratory symptoms. A virus with such an HA site alteration had not been previously identified in humans, and its isolation led to fears that an influenza pandemic was imminent. To prevent the virus from spreading, all chickens in Hong Kong were slaughtered. The concern was based on the knowledge that the HA of a pantropic influenza virus strain isolated over 50 years ago is processed by ubiquitous proteases (Box 1.9).

The Site of Entry Often Establishes the Pathway of Spread

For viruses that spread by neural pathways, the innervation at the primary site of inoculation determines the neuronal circuits that become infected. The only areas in the brain or spinal cord that become infected by herpes simplex virus are those that contain neurons with axon terminals or dendrites connected to the site of inoculation. After peripheral infection, poliovirus never reaches certain areas of the spinal cord and brain. However, replication occurs if the virus is placed directly into these sites. The conclusion is that accessibility of susceptible cells can determine the tropism of infection.

As we have discussed, the brain can be infected by either hematogenous or neural spread. When the neurotropic NWS strain of influenza virus is inoculated intraperitoneally into mice, it is disseminated to the brain by hematogenous spread and replicates in the meninges, choroid plexus, and ependymal cells lining the brain and spinal cord. However, when the virus inoculum is placed in the nose, there is no viremia. Instead, virus enters the brain by neural spread. It first replicates in olfactory epithelia, and subsequently enters the sensory neurons that richly endow the nasopharyngeal cavity (Box 1.6). Virions then can be found in the trigeminal ganglia, neurons in the brain stem that are in contact with trigeminal neurons, and the olfactory bulb in the brain. The experiment with the NWS influenza virus strain makes a significant point: the site of inoculation can determine the pathway of spread of a particular virus.

Perspectives

In this chapter, we begin discussion of the transition from the well-controlled environment of single cells in the tissue culture dish to the constantly changing "real world." Infection of a single susceptible host is the first step in the complicated process by which a viral infection is established and maintained in a host population. Every viral pathogen has a distinctive route of infection, which leads ultimately to shedding of infectious particles. Primary sites of replication are often the mucosal membranes of the nasopharyngeal tract, the respiratory system, the gastrointestinal tract, and the genital tract. Injection of virions or infected cells into the bloodstream by insect vectors, needles, or wounds provides another common route of infection. Remarkably, some viral infections remain localized and do not spread throughout the infected host. Others show characteristic spread through the blood or nervous system. Subsequent rounds of viral replication during systemic spreading lead to characteristic features of viral pathogenesis (e.g., the rash of measles, herpes simplex virus encephalitis, or viral hepatitis). After infection and replication, virions are released from the infected individual in a form that can be passed on to other susceptible hosts. Events in a single infected host set the stage for an essential, and even more complicated, process, serial transmission of infection in a susceptible host population, which is the topic of the next chapter.

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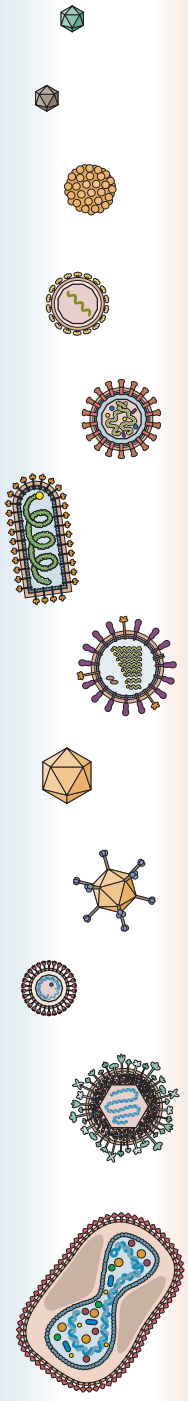


2

Infection of Populations

Good tests kill flawed theories; we remain alive to guess again.
KARL POPPER

Unless you have measured it, you do not know what you are talking about.
LORD KELVIN

Introduction

Uncountable numbers of viral particles are released constantly into the environment from all living things. The number of virions that impinge on any one individual is unknowable, yet most encounters are of no consequence for many reasons (discussed in Chapter 10). However, in some instances, not only do we become infected, we become ill and may even die. Furthermore, the disease may spread rampantly to many other individuals for a time. Perhaps surprisingly, there is no one answer to the seemingly straightforward questions: how does a viral infection cause disease in its hosts and how is disease transmitted and maintained in populations? In this chapter, we discuss the complicated problem of viral disease and its transmission in populations.

Principles of Viral Pathogenesis

Viral pathogenesis refers to the series of events that occur during viral infection of a host. It is the sum of the effects on the host of virus replication and of the immune response (Table 2.1). The simple fact is that interaction between virus-infected cells and host defense systems determines the severity of disease. However, these interactions are complex, multifactorial processes that are difficult to control and study (Table 2.2). Conclusions derived from reductionist approaches to studying pathogenesis, such as focusing on the function of a viral receptor protein in cultured cells, frequently are called into question when tested in animals (Box 2.1). Studying pathogenesis in living animals is thorny because so many variables come into play that it is often impossible to prove mechanisms. Consequently, viral pathogenesis has often been called a phenomenological discipline. With the recent development of new experimental tools, pathogenesis is becoming a mechanistic science.

Some human viruses have a broad host range and can infect different animals such as monkeys, ferrets, and guinea pigs. These various animal models have proven invaluable for understanding viral diseases. The mouse has become a particularly fruitful host for studying viral pathogenesis. Because the

Table 2.1 Determinants of viral pathogenesis

Interaction with target tissue
Access to target tissue
Presence of receptors
Stability of virus particles in body
Temperature
Acid and bile of gastrointestinal tract
Capacity to establish viremia
Capacity to spread through the reticuloendothelial system
Ability to kill cells (cause cytopathology)
Efficiency of viral replication in the cell
Best temperature for replication
Cell permissivity
Cytotoxic viral proteins
Inhibition of macromolecular synthesis
Production of viral proteins and structures (inclusion bodies)
Altered cell metabolism
Host response to infection
Intrinsic cell response
Innate immune response
Acquired immune response
Viral immune escape mechanisms
Immunopathology
Interferon: systemic symptoms
T-cell responses: delayed-type hypersensitivity
Antibody: complement, antibody-dependent cellular cytotoxicity, immune complexes

Table 2.2 Determinants of viral disease

Nature of the disease
Target tissue
Site of entry
Ability of virus to gain access to target tissue
Viral tropism
Permissivity of cells
Strain of virus
Severity of disease
Ability to kill cells (cytopathic effect)
Immunity to virus
Intact immune response
Immunopathology
Quantity of virions inoculated
Duration of infection
General health of the host
Host nutritional status
Other infections which might affect immune response
Host genotype
Age of the host

mouse genome can be manipulated readily, it is possible to engineer this host to allow susceptibility to some human viruses (Box 2.2). The ability to disrupt specific genes in mice enables assessment of the role of individual proteins in pathogenesis. In some cases, insights into human disease are gleaned by studying close relatives of human viruses. An example is simian immunodeficiency virus, which has proven invaluable as a model for human immunodeficiency virus infection. While the knowledge obtained from animal models is essential for understanding how viruses cause disease in humans, the results of such studies must be interpreted with caution. No human disease is completely reproduced in an animal model: what is true for a mouse is not always true for a human. For example, simple differences in size, metabolism, and development can have substantial effects on pathogenesis. Nevertheless, principles and mechanisms obtained from the study of animal models of virus infections often apply to human infections.

Interest in viral pathogenesis stems in large part from the desire to treat or eliminate viral diseases that affect humans. However, treatment of disease requires not only an understanding of pathogenesis mechanisms but also information about transmission and propagation. Progress in understanding the basis for these processes has been complicated, because the molecular correlates of pathogenesis are often defined in animal models, while the understanding of disease spread has come from analyzing human populations.

Statistics

When studying viral infections *in vivo*, scientists rarely obtain results that are so clear and obvious that everyone agrees with the conclusions. Often the effects are subtle, or the data are noisy with wide variation from sample to sample or study to study. Indeed, authors sometimes are stunned to realize that their colleagues do not accept their conclusions. Statistical methods (Table 2.3), properly employed, provide the common language of critical analysis to determine whether the differences between groups are significant. Unfortunately, surveys of articles published in virology journals indicate that errors in statistical analyses abound, which makes it even more difficult for the reader to interpret results. In fact, the term "significant difference" may be one of the most misused phrases in scientific papers, because the actual statistical support for the statement is often absent. While a detailed presentation of basic statistical considerations for virology experiments is beyond the scope of this textbook, critical principles are provided.

It is essential to consider experimental design carefully **before** going to the bench or to the field (Fig. 2.1). The

BOX 2.1 EXPERIMENTS

Of mice and humans

The conclusion that human influenza virus strains are preferentially bound by sialic acids attached to galactose via an α(2,6) linkage was derived by studying the binding of virus particles to cultured cells and to purified sugars. This is the major sialic acid present on human respiratory epithelium, suggesting that it is the receptor bound by virus during infection of animals. This hypothesis was tested using mice that lack the gene encoding ST6Gal I sialyltransferase, the main enzyme used for linking of α(2,6) sialic acid to glycoproteins. Such mice have no detectable α(2,6) sialic acid in the respiratory tract. Nevertheless, human influenza viruses efficiently replicate in the lung and trachea of these mice, indicating that α(2,6) sialic acid is not essential for influenza virus infection of mice.

The lesson to be learned from this experiment is clear: the findings of reductionist experiments must always be validated by experiments with animals.

Glaser, L., G. Conenello, J. Paulson, and P. Palese. 2007. Effective replication of human influenza viruses in mice lacking a major α(2,6) sialyltransferase. *Virus Res.* **126:**9–18.

fundamental problem in study design is to understand the number of observations required to detect a significant difference. The significance level is defined as the probability of mistakenly saying that a difference is meaningful; typically this probability is set at 0.05. An important concept is **power**, the probability of detecting a difference that truly is significant. In the simplest case, power can be increased by having a larger sample size. As an example, consider a study of vaccine efficacy in which laboratory animals are used. Animals are injected with a placebo or with a vaccine, and then challenged with a pathogenic virus. How many animals do you need before you can be confident that the vaccine is effective? Suppose that three animals are in each of the control groups and three animals are in each of the vaccine groups. After the challenge, all control animals die and all vaccinated animals survive. The result seems to be clear cut; the vaccine works—or does it? In fact, with this number of animals, the result will never be significant ($P < 0.05$) (Table 2.4). There is simply insufficient power in a study with so few animals to make a statistically meaningful conclusion.

It is critical to have a detailed description of how statistical analyses were performed (preferably in the Methods section of the paper). These facts are just as important as a description of laboratory methods. More complex data, study design issues, and analyses may require consultation with a statistician. The modern world of virology is incorporating more quantitative and data-rich components into

Table 2.3 Statistical terms[a]

Term	Definition
Alternative hypothesis	Hypothesis that contradicts the null hypothesis
Binary data	Data that consist of only two values (e.g., positive, negative)
Cardinal data	Data that are on a scale in which common arithmetic is meaningful
Confidence interval	Likely range of the true value of a parameter of interest
Hypothesis testing	Use of statistical testing to objectively assess whether results seen in experiments are real or due to random chance
Nonparametric test	Statistical test that requires no assumptions regarding the underlying distribution of the data
Normally distributed data	Data which, when plotted in a histogram, look approximately like a bell-shaped curve
Null hypothesis	Hypothesis which presumes that there are no differences between treated and untreated groups; if hypothesis testing results in a statistically significant difference, the null hypothesis is rejected
P value	Probability of getting a result as extreme as or more extreme than the value obtained in one's sample, given that the null hypothesis is true
Parametric test	Statistical test that assumes the data follow a particular distribution (e.g., normal)
Power	Probability of detecting a statistically significant difference that truly exists
Sample size	Number of experimental units in a study
Significance level	Probability of falsely finding a statistically significant difference

[a]Reprinted from B. A. Richardson and J. Overbaugh, *J. Virol.* **79:**669–676, 2005, with permission.

BOX 2.2 BACKGROUND

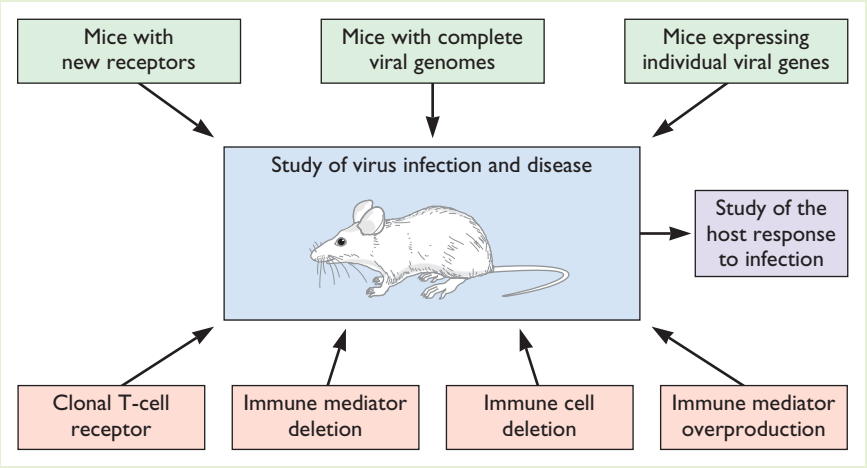
Transgenic and knockout mice for studying viral pathogenesis

Mice have always played an important role in the study of viral pathogenesis (see figure). Because it is possible to manipulate this animal genetically, a wealth of new information about how viruses cause disease is emerging. Introducing a gene into the mouse germ line to produce a transgenic mouse and ablating specific genes (gene knockouts) both have wide use in virology.

New mouse models have been established for poliomyelitis and measles by producing transgenic mice that synthesize the human viral receptors. When viral receptors have not been identified, or are not sufficient for infection, an alternative approach is to express either the entire viral genome or a selected viral gene in mice. For example, transgenic mice expressing the hepatitis B virus genome have been used to study interactions between the virus and the host immune response. Transgenic mice that express T-cell-receptor transgenes or genes encoding soluble immune mediators have also been produced. Such mice have been used to study the effect of immune cells on virus clearance, and the protective and deleterious effects of cytokines.

Mice lacking specific components of the immune response have proven invaluable for studying immunity and immunopathogenesis. For example, mice lacking the gene encoding perforin, a molecule essential for the ability of cytotoxic T lymphocytes to lyse target cells, cannot clear infection by lymphocytic choriomeningitis virus, despite the presence of an otherwise intact immune response. Studies of mice with disruptions in genes encoding components of the immune response have led to the identification of cells that are important for mediating recovery from a variety of viral infections, including measles, influenza, and lymphocytic choriomeningitis.

Rall, G. F., D. M. P. Lawrence, and C. E. Patterson. 2000. The application of transgenic and knockout mouse technology for the study of viral pathogenesis. *Virology* **271:**220–226.



analyses. Consequently, statistical methods will be even more important for interpreting results and drawing conclusions. The objective is to be precise and consistent in determining whether observed biological differences are real, or due to random chance.

Epidemiology

While all viral infections begin with events in single cells, the expansion of infection within an individual must lead to the subsequent spread of infection in many individuals, if the virus population is to survive. **Epidemiology** is the study of the events and actions that affect the health and illness of populations. It is the cornerstone of public health research, providing the rationale for intervention and control.

A viral epidemiologist is an expert in communicable disease who investigates disease outbreaks by undertaking careful data collection and statistical analysis. An epidemiologist specializing in viral diseases must be knowledgeable about not only viral biology and pathogenesis, but also social science disciplines. Social actions and group dynamics are an integral part of the consideration of mechanisms of viral transmission, risk factors for infection, size of the population needed for virus transmission, geography, season, and means of control (Box 2.3; Table 2.5).

Incidence and Prevalence

The quantitation of disease occurrence is the primary result of epidemiology studies. **Incidence** (attack rate)

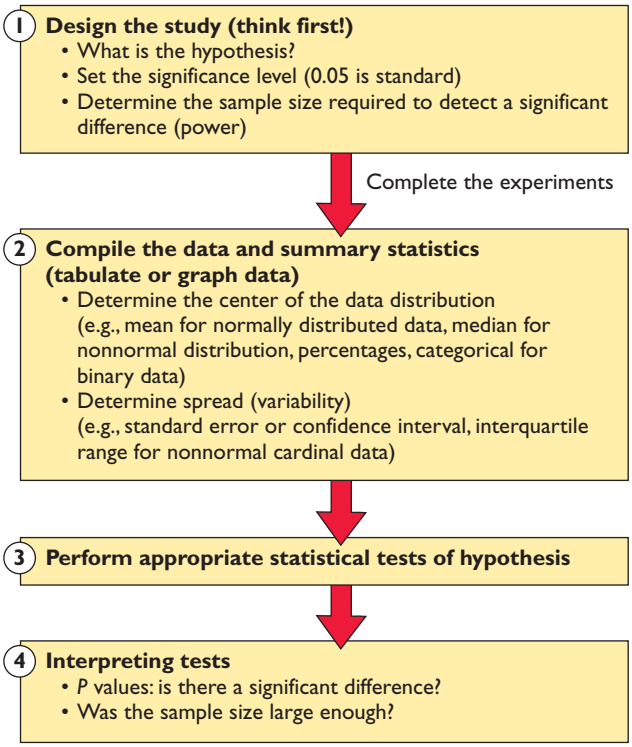


Figure 2.1 Experimental design, execution, and interpretation.

is computed as a ratio of the number of cases of disease divided by some measure of population size and time frame. Incidence is of use mainly for acute infections. For example, the incidence of influenza in New York City is expressed as the number of reported cases per year. Disease **prevalence**, an alternative way to express attack rate, is often used for persistent infections where disease onset is not easily determined. In this case, a particular date is selected and the number of cases of a particular disease on that day is divided by an appropriate measure of population. Prevalence is often expressed as cases per million at a particular time.

Prospective and Retrospective Studies

Infections of natural populations obviously differ from those under controlled conditions in the laboratory. Nevertheless, it is possible to determine if one or more variables affect disease incidence and spread in nature. Two general experimental protocols are used: **prospective** (cohort or longitudinal) and **retrospective** (case-control). In prospective studies, the population is divided into two groups, such that one variable is present in one group but not the other. The incidence of disease or side effect is determined. Protocols of this type are often used for drug or vaccine trials where one group is treated and the other is not. Experimental design is critical in these studies. Placebo controls as well as single- or double-blind analyses are essential to remove investigator bias and patient expectations. Prospective studies require a large number of subjects who often are studied for months or years. The number of subjects and time required depend on the incidence of the disease or side effect under study, and the statistical significance required for decision-making.

In contrast, retrospective studies are not burdened by the need for large numbers of subjects and long study times. The protocol simply is to choose a number of subjects with the disease or side effect and an equal number who do not have the malady and classify them as to the variable to be analyzed. For example, in one retrospective study of measles vaccine, a group of 100 children with an adverse side effect and 100 age-matched controls were chosen randomly and classified as vaccinated or not vaccinated. The variable was a particular adverse side effect that may or may not be associated with vaccination. The incidence of the side effect in each group can be computed,

Table 2.4 *P* values for the differences in infection rates between experimental and control groups[a]

No. of animals per group (*n*)	*P* value for indicated group[b]		
	All control animals infected and no experimental animals infected	All control animals and one experimental animal infected or one control animal infected and no experimental animal infected	One control animal infected and one experimental animal infected
3	0.1	0.4	1.0
4	0.03	0.1	0.5
5	0.008	0.05	0.2
6	0.002	0.02	0.08
7	<0.001	0.005	0.03
8	<0.001	0.001	0.01

[a]Reprinted from B. A. Richardson and J. Overbaugh, *J. Virol.* **79:**669–676, 2005, with permission.

[b]Determined by Fisher's exact test, using a two-sided hypothesis test with the significance level fixed at 0.05. Fisher's exact test is used because it is appropriate for experiments with small numbers of observations.

BOX 2.3 DISCUSSION

Video games model infectious-disease epidemics

The hugely popular online video game *World of Warcraft* recently became a model for the transmission of virus infections. In this game, players adventure in a fantasy world populated by humans, elves, and orcs and other exotic beasts. In late 2005, a dungeon was added in which players could confront and kill a powerful creature called Hakkar. In his death throes, Hakkar hits foes with "corrupted blood" that contains a virus and causes a fatal infection. The infection was meant to affect only those in the immediate vicinity of Hakkar's corpse, but the virus spread as players and their virtual pets traveled to other cities in the game. Within hours after the software update that installed the new dungeon, a full-blown epidemic ensued as millions of characters became infected.

Although such games are meant only for entertainment, they do model disease spread in a realistic manner. For example, the spread of the virus depended on the ease of travel within the game, interspecies transmission by pets, and transmission via asymptomatic carriers. These aspects of the game world mirrored real-world epidemiology, except for how the disease was halted: the game developers removed Hakkar's dungeon and rebooted their computers.

Epidemiologists are limited to observational and retrospective studies when studying human infectious diseases. Computer models of epidemics have been developed, but they lack the variability and unexpected outcomes found in real-world epidemics. Massively multiplayer online role-playing games have large numbers of participants (10 million for *World of Warcraft*) and therefore are excellent pools for experimental study of infectious diseases. While enjoyment and entertainment are the central focus of such games, the players are serious and devoted, and their responses to situations of danger approximate real-world reactions. For example, during the "corrupted-blood" epidemic, players with healing ability were the first to attempt to help the infected players. This action probably affected the dynamics of the epidemic since infected players survived longer and were able to travel and spread the infection. Multiplayer video games provide an excellent opportunity to examine the consequences of human actions within a statistically significant and controlled computer simulation.

The computer game disease model differs from a real-word virus infection in one significant way: the death of a character in *World of Warcraft* is not permanent. Resurrection is as simple as the click of a mouse.

Lofgren, E. T., and N. H. Fefferman. 2007. The untapped potential of virtual game worlds to shed light on real world epidemics. *Lancet Infect. Dis.* **7:**625–629.

and the ratio of these values yields the relative risk associated with vaccination.

Shedding of Virions

The release of infectious virus particles from an infected host is called **shedding** (see also Fig. 1.10). Shedding is usually an absolute requirement for viral propagation in the host population. The exceptions are direct transmission of viral genomes in the germ lines of their hosts and infections transmitted in the blood supply or by organ transplantation such as acquired immunodeficiency syndrome or hepatitis. During localized infections, shedding takes place from the primary site of replication at one of the body openings. In contrast, release of virions that cause disseminated infections can occur at many sites. Effective transmission of virions from one host to another depends directly on the concentration of released particles, and the mechanisms by which the virions are introduced into the next host. The shedding of small quantities of virus particles may be irrelevant to transmission, while the shedding of high concentrations may facilitate transmission with minute quantities of tissue or fluid. For example, the concentration of hepatitis B virus in blood can be so high that as little as a few microliters can be sufficient to initiate an infection. How well virions survive in the environment also influences the efficiency of transmission.

Respiratory Secretions

Respiratory transmission depends on the production of airborne particles, or aerosols, that contain viruses. Aerosols are produced during speaking, singing, and normal breathing, while coughing produces even more forceful expulsion. Shedding from the nasal cavity requires sneezing and is much more effective if infection induces the production of nasal secretions. A sneeze produces up to 20,000 droplets (in contrast to several hundred expelled by coughing), and all may contain rhinovirus if the individual has a common cold. The largest droplets fall to the ground within a few meters. Many virus particles are inactivated by drying (e.g., measles virus, influenza virus, and rhinovirus), and therefore close proximity is required for transmission. The remaining droplets travel a distance determined by their size. Droplet nuclei, which are 1 to 4 μm in diameter, may remain suspended indefinitely, because air is continually in motion. Such particles may reach the lower respiratory tract. Nasal secretions also frequently contaminate hands or tissues. The infection may be transmitted when these objects contact another person's fingers and

Table 2.5 The many components of epidemiology

Mechanisms of transmission
Aerosol
Food and water
Fomites
Body secretions
Sexual activity
Birth
Transfusion or transplantation
Zoonoses (animals, insects)
Factors that promote transmission
Virion stability
Presence in aerosols and secretions
Asymptomatic shedding
Ineffective immune response
Geography and season
Vector ecology (habitat and season)
School year
Home-heating season
Risk factors
Age
Health
Immunity
Occupation
Travel
Lifestyle
Children (school, day care centers)
Sexual activity
Critical population size
Numbers of seronegative susceptible individuals
Means of control
Quarantine
Vector elimination
Immunization
Antivirals

that person in turn touches his or her nose or conjunctiva. In today's crowded society, the physical proximity of people may select for viruses that spread efficiently by this route.

Saliva

Some viruses that replicate in the lungs, nasal mucosa, or salivary glands are shed into the oral cavity. Transmission may occur through aerosols, as discussed above, via contaminated fingers, or by kissing or spitting. Animals that lick, nibble, and groom may also transmit infections in saliva. Human cytomegalovirus, mumps virus, and some retroviruses are known to be transmitted by this route.

Feces

Enteric and hepatic virus particles are shed in the feces and are generally more resistant to inactivation by environmental conditions than those released from other sites. An important exception is hepatitis B virus, which is shed in bile into the intestine, but is inactivated as a consequence and not transmitted in feces. Instead, hepatitis B virus is transmitted through the blood. Viruses transmitted by fecal spread usually survive dilution in water, as well as drying. Inefficient or no sewage treatment, contaminated irrigation systems, and the use of animal manures are prime sources of fecal contamination of food, water supplies, and living areas. Any one of these conditions provides an efficient mode for continual reentry of these viruses into the alimentary canal of their hosts. Two hundred years ago, such contamination was inevitable in most of the world, as disposal of human feces in the streets was a common practice. With modern sanitation, the flow of feces into human mouths has been largely interrupted in developed countries, but is still common throughout the rest of the world. Even so, the closing of beaches because of high coliform counts and the contamination of clam and oyster beds by sewage outflows provide regular reminders of our continuous exposure to enteric and hepatic viruses.

Blood

Viremias are a common feature of many viral infections, and viremic blood is a prime vehicle of virus transmission. Arthropods acquire virions when they bite viremic hosts. Hepatitis and acquired immunodeficiency syndrome can be transmitted by virus-laden blood during transfusions and injections. Infections may be transmitted from viremic blood during coitus or childbirth, and eating raw meat may place viremic blood in contact with the alimentary and respiratory tracts. Health care and emergency rescue workers and dentists are exposed routinely to viremic blood. Indeed, for many of the fatal hemorrhagic fevers caused by viruses (such as members of the *Bunyaviridae* and *Filoviridae*), their only mode of transmission to humans is via blood and body fluids. Consequently, health care workers often are the first people to die in an outbreak of such viral diseases.

Urine, Semen, and Milk

Virus-containing urine is a common contaminant of food and water supplies. The presence of virus particles in the urine is called **viruria**. Hantaviruses and arenaviruses that infect rodents cause persistent viruria. Consequently,

humans may be infected by exposure to dust that contains dried urine of infected rodents. A few human viruses replicate in the kidneys and are shed in urine. However, viruria is not important for transmission of most human viruses.

Some retroviruses, including human immunodeficiency virus type 1, herpesviruses, and hepatitis B virus, are shed in semen and transmitted during coitus. Herpesviruses that infect the genital mucosa are also shed from lesions and transmitted by genital secretions, as are papillomaviruses.

Mouse mammary tumor virus is spread primarily by milk, as are some tick-borne encephalitis viruses. Mumps virus and cytomegalovirus are shed in human milk, but are probably not often transmitted by this route.

Skin Lesions

Many viruses replicate in the skin, and the lesions that form contain infectious virus particles that can be transmitted to other hosts. Spread of infection is usually by direct body contact. For example, herpes simplex virus causes a common rash in wrestlers, known as herpes gladiatorum. Warts caused by certain poxviruses and papillomavirus may also be transmitted by contact.

Transmission of Viral Infection

The chain of infection can be maintained only by spreading from one susceptible host to another (**transmission**). There are two general patterns of viral transmission: (i) the perpetuation of infection in one species and (ii) alternate infection of insect and vertebrate hosts. Some viruses such as rabies virus and influenza viruses spread across species, but transmission within each species is relatively self-contained. Most human viruses are transmitted from human to human, although there are some for which an extrahuman cycle is needed for maintenance. Measles and hepatitis A viruses are transmitted from human to human and are maintained solely in the human population. Humans are therefore the **reservoir** for these viruses. In contrast, rabies virus is transmitted from animal to human, but is maintained in animal-to-animal cycles. Some arboviruses are transmitted and maintained in vector-to-human cycles (dengue virus and urban yellow fever virus), while others are maintained in vector-to-vertebrate cycles (St. Louis encephalitis virus and western equine encephalitis virus). Viral diseases shared by humans and animals or insects are called **zoonoses** (see also Chapter 10).

Viral infections are transmitted among hosts in specific ways (Table 2.6). The site of virion excretion and the physical stability of the virion determine the route of transmission. The presence or absence of a lipid envelope is a major determinant of the mode of transmission. Enveloped virions are fragile and sensitive to low pH. Consequently, they are most often transmitted by aerosols or secretions, by injection, or by organ transplantation. In contrast, nonenveloped virions can withstand drying, detergents, low pH, and higher temperatures. These virions can be transmitted by the respiratory and fecal-oral routes, and are often acquired via contaminated objects (**fomites**). Various descriptions are used to characterize transmission (Box 2.4). Vertical transmission may occur during gestation, when infection crosses the placenta, during birth, or by close physical contact (Table 2.7). Most maternal infections have little effect on the fetus, but some viral infections that spread by viremia can infect the placenta and even reach the fetal circulation. As a result, the fetus may die and be aborted. The immature state of the fetal immune system may contribute to infections that cause congenital birth defects, including deafness, blindness, and abnormalities of the heart and nervous system. Other symptoms of fetal infection are obvious upon birth. For example, congenital rubella syndrome is recognized in the newborn by liver and spleen enlargement, jaundice, and skin discoloration. Human cytomegalovirus infection has been implicated in many fetal and neonatal birth defects.

Acute viral infections are transmitted efficiently to new hosts and are usually transmitted by the respiratory or fecal-oral route (e.g., common cold virus or poliovirus infections). Such infections often result in excretion of large numbers of virus particles, another property that ensures efficient transmission. Persistent infections present more complicated issues for transmission in populations. In some cases, large quantities of virions are found in the blood (e.g., hepatitis B virus infections), and transmission occurs

Table 2.6 Viral transmission

Route of transmission	Examples
Respiratory	Paramyxoviruses, influenza viruses, picornaviruses, varicella-zoster virus
Fecal-oral	Picornaviruses, rotavirus, adenovirus
Contact: lesions, saliva, fomites	Herpes simplex virus, rhinovirus, poxvirus, adenovirus
Zoonoses: insects, animals	Togaviruses (arthropod bite), flaviviruses (arthropod bite), bunyaviruses (urine, arthropod bite), arenaviruses (urine), rabies virus (animal bite)
Blood	Human immunodeficiency virus, human T-lymphotropic virus, hepatitis B virus, hepatitis C virus, cytomegalovirus
Sexual contact	Herpes simplex virus, human papillomavirus
Maternal-neonatal	Rubella virus, cytomegalovirus, echovirus, herpes simplex virus, varicella-zoster virus
Germ line	Retroviruses

BOX 2.4 **TERMINOLOGY**
Types of viral transmission

Iatrogenic transmission occurs when some activity of a health care worker leads to infection of the patient. Such transmission can occur when nonsterile instruments and needles are used or if a health care worker is infectious. **Nosocomial** transmission occurs when an individual is infected while in a hospital or health care facility. **Vertical transmission** refers to the transfer of infection between parent and offspring, while **horizontal transmission** includes all other forms. In **germ line transmission** the agent is transmitted as part of the host genome (e.g., integrated proviral DNA).

via shared needles or blood transfusions. In other cases, viremia is absent and no visible lesions can be detected (e.g., latent herpes simplex virus infections). Transmission is probable only for a short time after reactivation and only then by close contact.

Most arthropod-borne viruses can replicate in both an insect and a vertebrate host, and infections are maintained by cycling transmission between these diverse animals. Such viruses have a major or exclusive insect vector, and these insects in turn prefer to feed on particular vertebrate species. Humans are the major vertebrate host for dengue virus and urban yellow fever virus. In most other arthropod-mediated viral infections, humans are incidental hosts and are accidentally infected during vector bites (Chapter 10). In these so-called dead-end host infections, the insects have a preferred nonhuman target such as birds or woodland rodents. Examples of the dead-end host interaction are rabies, hantavirus pneumonia, and Korean hemorrhagic fever.

Geography and Season

Some viruses are found only in specific geographical locations. Such restrictions may reflect the requirement for a specific vector or animal reservoir and, in turn, the subsequent interactions of host and virus. For example, migratory animals may carry potential zoonotic infections and therefore the disease is confined to areas encompassing the highest concentrations of humans and other animals. Serial transmission of some acute viral infections occurs only if the host population is quite large and interactive (e.g., measles virus can be maintained only in populations that exceed 200,000). These infections are rarely found in isolated small groups that might populate islands or areas with extreme climates. Before global travel was possible, isolated host populations were the norm and the distribution of viruses was far more limited. Now, viral infections are transported routinely around the globe in planes, trains, and automobiles. A striking example of how the vector can affect localization of viral infection is the global spread of the hitherto exotic chikungunya virus caused by a viral mutation leading to a change in the mosquito vector (Box 2.5).

Despite the increasing propensity for global mixing of hosts and viruses, most acute viral infections have a striking seasonal variation in incidence (Fig. 2.2). Respiratory virus infections are more frequent in winter months, and enteric virus infections predominate in the summer. Seasonal differences in diseases caused by arthropod-borne viruses are clearly a consequence of the life cycle of the vector or the animal reservoir. However, the basis for the seasonal nature of non-arthropod-borne virus infections is less

Table 2.7 Some congenital viral infections

Syndrome	Virus	Fetus
Fetal death and abortion	Smallpox virus	Human
	Parvovirus	Human
	Various alphaherpesviruses	Swine, horses, cattle
Congenital defects	Cytomegalovirus	Human
	Rubella virus	Human
Immunodeficiency	Human immunodeficiency virus type 1	Human
Inapparent (lifelong carrier)	Lymphocytic choriomeningitis virus	Mouse
	Noncytopathic bovine viral diarrhea virus	Cattle
	Murine leukemia virus	Mouse
	Avian leukosis virus	Chicken

BOX 2.5 DISCUSSION

An exotic virus on the move

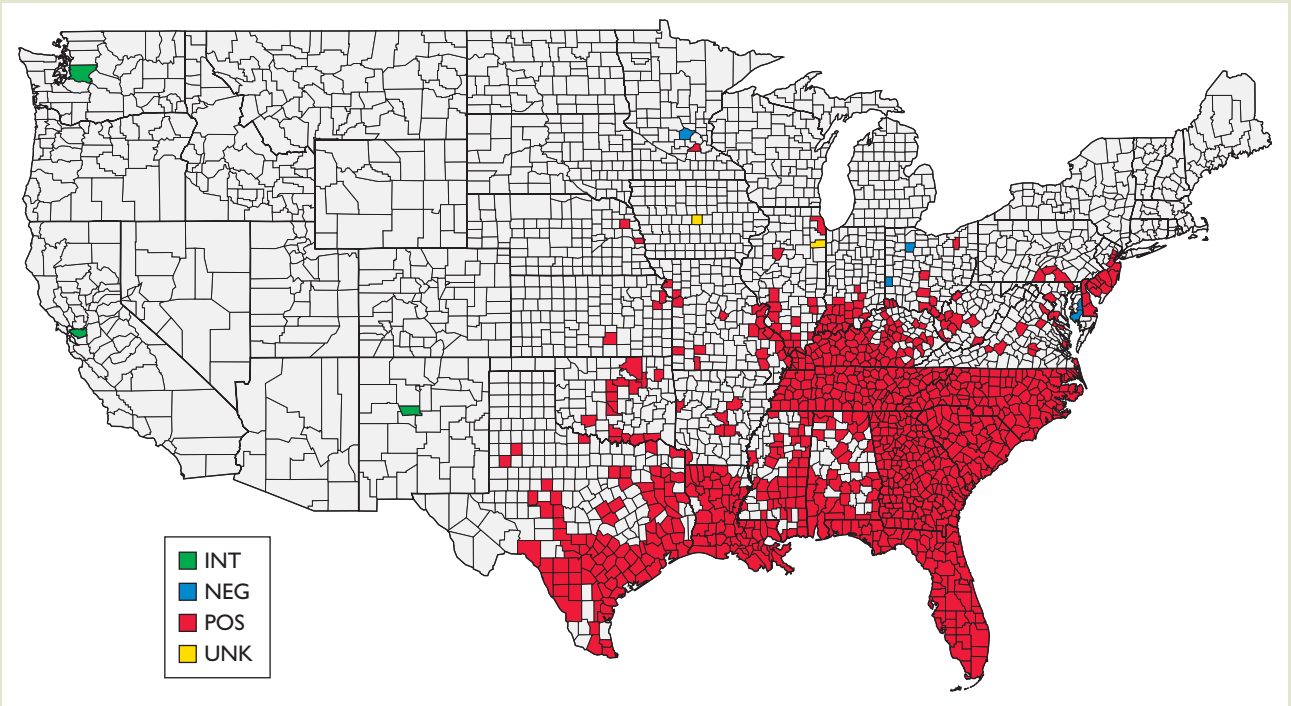
Chikungunya virus is a togavirus in the alphavirus genus. The infection is spread by mosquitoes (primarily the notorious *Aedes aegypti*). The viral disease has been known for more than 50 years in the tropics and savannahs of developing countries of Asia and Africa, but had never been a problem of the developed countries in Europe. The disease is uncomfortable (rashes and joint pains), but not fatal and certainly nothing out of the ordinary for an alphavirus disease. In the last 5 years, something changed dramatically and brought this once third-world viral disease into the forefront of public concern.

In 2004, outbreaks of chikungunya disease spread rapidly from Kenya to islands in the Indian Ocean and then to India (where it had not been reported in over 30 years). In 2007, there was an outbreak in Italy, the first ever in Europe. In some of the Indian Ocean islands, more than 40% of the population fell ill (e.g., the island of Réunion, population 785,000). What had happened to change the pattern of infection?

An alarming finding for the developed world was that the Asian tiger mosquito (*A. albopictus*) apparently is an efficient new vector for the virus. A point mutation in the viral genome appears to be the cause of this vector switch and, perhaps, for the epidemic spread of the disease where it had been unknown. *A. albopictus* is spreading across the globe from eastern Asia and is now found in mainland Europe and the United States. *A. albopictus* is a maintenance (occasionally epidemic) vector of dengue viruses in parts of Asia, and is a competent vector of several other viral diseases. Since its discovery in the United States, five arboviruses (eastern equine encephalomyelitis, Keystone, Tensaw, Cache Valley, and Potosi viruses) have been isolated from this mosquito.

Enserink, M. 2007. Chikungunya: no longer a third world disease. *Science* **318:**1860–1861.

Distribution of *Aedes albopictus* mosquitoes in the United States, 2000. *A. albopictus*, an Asian mosquito, is thought to have been introduced into Hawaii in the late 19th century. The mosquito was not found in the New World until 1985, when it was isolated in Houston, TX. As of 2000, it had been detected in 26 states in the continental United States. INT, intermediate; NEG, negative; POS, positive; UNK, unknown. From http://www.cdc.gov/ncidod/dvbid/arbor/albopic_new.htm.



obvious. Variations in disease incidence correlate with changes in climate. For example, poliomyelitis was seasonal in New England but not in Hawaii. It has been suggested that seasonality of infections is due to differences in sensitivity to humidity or to stability of virions to temperature. According to this hypothesis, during winter months when humidity is low, poliovirus is inactivated but influenza virus remains infectious. The results of recent experiments demonstrate that transmission of influenza A virions is more efficient at low temperature and humidity (Box 2.6).

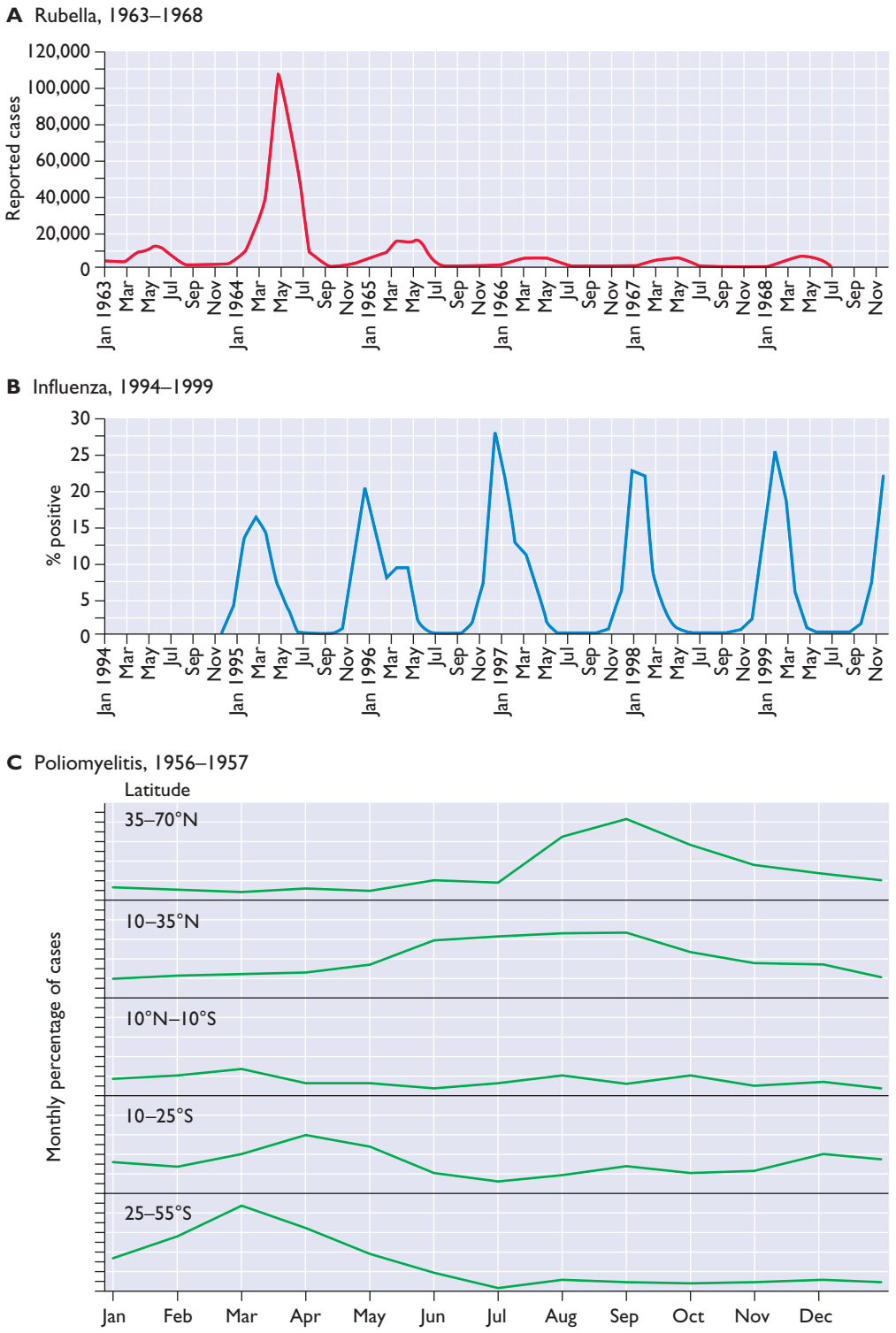


Figure 2.2 Seasonal variation in disease caused by three human pathogens in the United States. (A) Annual cycles of rubella between larger epidemics, which occurred every 6 to 9 years. **(B)** Percentage of specimens testing positive for influenza viruses. **(C)** Monthly incidence of poliomyelitis at different latitudes. Adapted from S. F. Dowell, *Emerg. Infect. Dis.* **7:**369–374, 2001, with permission.

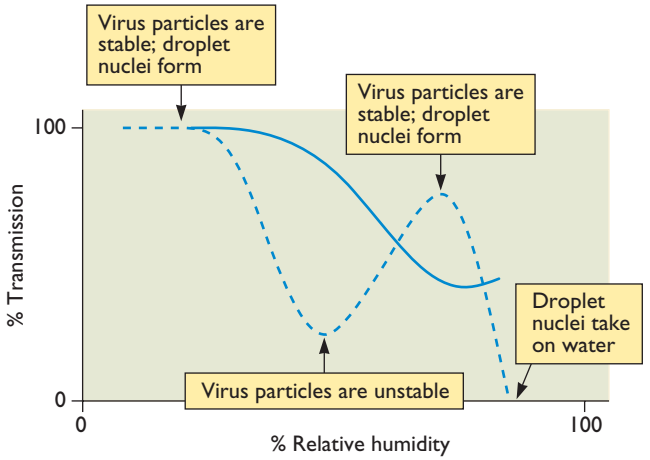
BOX 2.6 EXPERIMENTS

Seasonal factors that affect transmission of influenza virus

Seasonality is a familiar feature of influenza: in temperate climates the infection occurs largely from November to March in the northern hemisphere and from May to September in the southern hemisphere. There have been many hypotheses to explain this seasonality, but none have been supported by experimental data. Recently a guinea pig model was used to show that spread of the virus in aerosols is dependent upon both temperature and relative humidity.

Transmission experiments were conducted by housing infected and uninfected guinea pigs together in an environmental chamber. Transmission of infection was most effective at humidities of 20 to 35% and blocked at a humidity of 80%. In addition, transmission occurred with greater frequency when guinea pigs were housed at 5°C than at 20°C. The authors conclude that low temperature and humidity, conditions found during winter, favor influenza virus spread. The dependence of influenza virus transmission on low humidity might be related to the nature of the droplets produced by coughing and sneezing (see figure).

Curiously, at 30°C, no transmission of infection took place, an observation at odds with the fact that influenza occurs all year in tropical climates. One explanation of these findings is that transmission in the tropics might occur by contact.



Model for the effect of humidity on transmission of influenza virus. Transmission efficiency at 20°C (dashed line) or 5°C (solid line) is shown as a function of percent humidity. At 20°C transmission is highest at low humidity, conditions which would favor conversion of exhaled droplets into droplet nuclei (defined as droplets less than 5 μm in diameter and which remain airborne). Reduced virion stability at intermediate humidity is the cause of poor transmission. At high humidity, the conversion from droplets to droplet nuclei is inhibited, and the heavier droplets fall from the air, reducing transmission. At 5°C transmission is more efficient than at 20°C, but there is a gradual loss of transmission with increasing humidity, presumably also as a consequence of reduced formation of droplet nuclei. Adapted from A. C. Lowen et al., *PLoS Pathog.* **3:**1470–1476, 2007, with permission.

Annual variations in viral disease may also be caused by changes in the susceptibility of the host. Such changes might be linked to circadian rhythms, and could be governed by alterations in mucosal surfaces, epithelial receptors, immune-cell numbers, and responsiveness. If annual changes in host resistance contribute to the seasonality of viral disease, then the gene products that regulate such changes should be identified to provide therapeutic targets for intervention.

Viral Virulence

Once infected, a host may develop a wide range of disease symptoms depending on several variables. **Virulence** refers to the capacity of infection to cause disease. It is a quantitative statement of the degree or extent of pathogenesis. In general, a **virulent virus** causes disease whereas an **avirulent virus** does not. In populations, viral virulence may be manifested as efficiency of spread from individual to individual.

From the earliest days of experimental virology, it was recognized that viral strains often differ in virulence. Virologists thought that the study of viruses with reduced virulence (**attenuated**) would answer the question of how viruses cause disease. This understanding has stood the test of time; indeed, the study of attenuated viruses is still a common strategy. We have learned how to alter viral virulence by direct and indirect methods, and have produced viruses of such limited virulence that they can be used as live vaccines (Chapter 8). Today, the methods of recombinant DNA technology permit a more systematic analysis, by introducing defined mutations into viral genomes so that virulence genes can be identified. The goal of these studies is to understand the mechanisms by which viral and cellular gene products control virulence.

Measuring Viral Virulence

Virulence can be quantified in a number of ways. One approach is to determine the amount of virus that causes

death or disease in 50% of the infected animals. This parameter is called the 50% lethal dose (LD_{50}), the 50% paralytic dose (PD_{50}), or the 50% infectious dose (ID_{50}), depending on the parameter that is measured. Other measurements of virulence include time to death (Fig. 2.3A) or appearance of symptoms, and degree of fever or weight loss. Virus-induced tissue damage can be measured directly by examining histological sections or blood (Fig. 2.3B). The safety of live, attenuated poliovirus vaccine is determined by assessing the extent of pathological lesions in the central nervous system in experimentally inoculated monkeys. The reduction in the concentration of $CD4^+$ lymphocytes in blood as a result of human immunodeficiency virus type 1 infection is another example. Indirect measures of virulence include assays for levels of liver enzymes (alanine or aspartate aminotransferases) that are released into the blood following virus-induced liver damage.

It is important to recognize that the virulence of a single virus strain may vary dramatically depending on the dose and the route of infection, as well as on the species, age, gender, and susceptibility of the host. The effect of inoculation route on virulence is illustrated in Table 2.8. Clearly, virulence is a relative property. Consequently, when the degree of virulence of two very similar viruses are compared, the assays must be identical. Quantitative terms such as LD_{50} cannot be used to compare virulence among different viruses.

Genetic Determinants of Virulence

A major goal of animal virology is to identify viral and host genes that control virulence. Once such genes have been identified, defined mutations can be made, gene products can be purified, and mechanistic hypotheses can be tested. If the molecular mechanisms by which viruses cause disease are known, drugs that block these processes may be synthesized. In addition, the contribution of virulence genes to the characteristic patterns of viral infections can be determined. It may be possible to test the hypothesis that virulence influences the long-term survival of viruses in nature.

Alteration of Viral Virulence

To identify viral virulence genes, it is necessary to compare viruses that differ only in their degree of virulence. Before the era of modern virology, several approaches were used to attain this goal. Occasionally, avirulent viruses were isolated from clinical specimens. For example, although wild-type strains of poliovirus type 2 readily cause paralysis after intracerebral inoculation into monkeys, an isolate from the feces of healthy children was shown to be completely avirulent after inoculation by the same route. A second approach to isolate viruses with reduced virulence was to serially passage viruses either in animal hosts or in cell culture (Chapter 8).

Although these approaches were useful, they were unpredictable. To overcome this limitation, viral genomes were often mutagenized, as described in Volume I, Chapter 2,

Figure 2.3 Two methods for measuring viral virulence. (A) Measurement of survival. Mice (5 per virus) were inoculated intracerebrally with either type 1 or type 2 poliovirus, and observed daily for survival. **(B)** Measurement of pathological lesions. Monkeys were inoculated intracerebrally with different viruses, and lesions in different areas of the central nervous system were assigned numerical values. C, cerebrum; B, brain stem; S, spinal cord. (A) Adapted from V. Racaniello, *Virus Res.* **1**:669–675, 1984, with permission. (B) Adapted from N. Nathanson (ed.), *Viral Pathogenesis and Immunity* (Academic Press, London, United Kingdom, 2007) with permission.

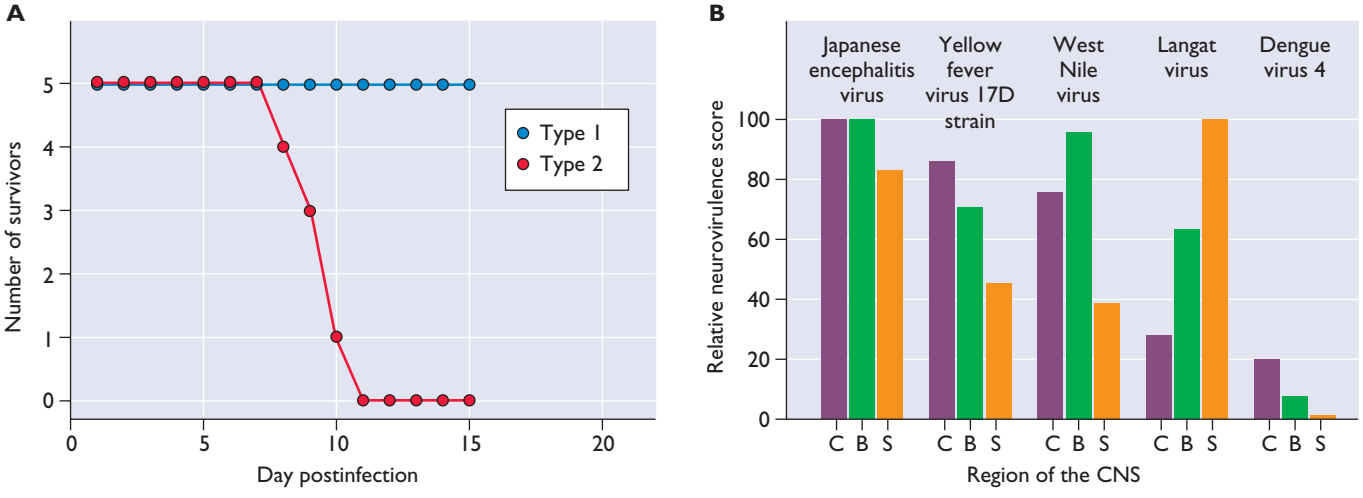


Table 2.8 Effect of route of inoculation on viral virulence[a]

Virus	No. of virions needed to kill 50% of animals			
	Suckling mice		Adult mice	
	Intracerebral infection	Subcutaneous infection	Intracerebral infection	Subcutaneous infection
Wild-type La Crosse virus	~1	~1	~1	~10
Attenuated La Crosse virus mutant	~1	$>10^5$	$>10^6$	$>10^7$

[a]Adapted from Table 9.1 of N. Nathanson, *Viral Pathogenesis and Immunity*, 2nd ed. (Academic Press, London, United Kingdom, 2007), with permission.

and the altered viruses were assayed for virulence in animals. However, controlling the degree of mutagenesis was difficult, and multiple mutations were often introduced. Until the advent of recombinant DNA technology, the ability to identify mutations in a specific gene was limited. More recently, rapid sequencing of entire viral genomes, polymerase chain reaction (PCR) amplification of selected genomic segments, and site-directed mutagenesis have become routine procedures in the quest to identify viral virulence genes and their products (Fig. 2.4).

Viral Virulence Genes

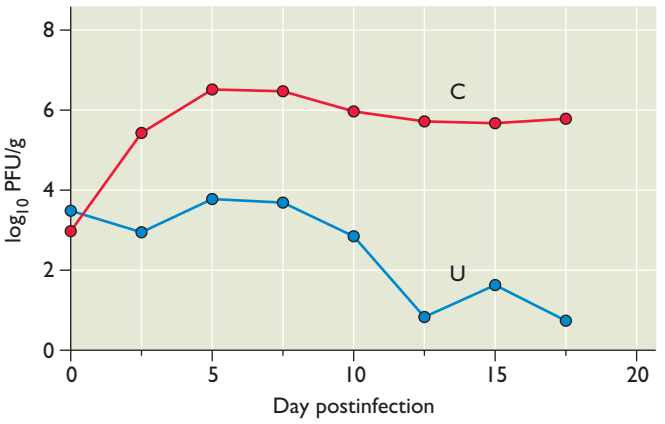
Despite modern technological advances, the identification and analysis of virulence genes in a systematic way have not been straightforward. Part of the problem is that there are no simple tissue culture assays for virulence. Many of the pathogenic effects promoted by viruses are a result of action of the intrinsic immune defense systems, and it is not possible to reproduce their complex actions in a tissue culture dish. Another problem confronting investigators is the simple fact that it is not obvious *a priori* by inspection of viral genomes what comprises a virulence gene. Consequently, most studies begin with the premise that a virus that causes reduced or no disease in an animal host harbors a defective virulence gene. The genomes of attenuated viruses obtained by empirical efforts (Chapter 8) were often found to harbor multiple mutations, and the contributions of the individual mutations to the attenuation phenotype was often difficult to ascertain. However, by reversion of point mutations, repair of deletions, and crossing of mutants and wild-type strains, many relevant defects were identified. Some mutations reduced, eliminated, or augmented protein function, while others affected the binding of transcription, translation, or replication proteins.

A significant drawback to studies of virulence phenotypes of viruses that infect humans is that relevant animal models of disease are not always readily available. Nevertheless, considerable progress has been made in recent years. In the following sections, we discuss examples of viral virulence genes that can be placed in one of four general classes (Box 2.7). It should be understood, however, that the vast majority of known virulence genes have not been studied sufficiently to be placed in one of these categories.

Although this discussion focuses on producing viruses that are less virulent, the opposite approach, producing viruses that are **more** virulent than the wild type, is possible. The approach is rarely used simply because of the unknown risks involved. However, there are some cases of inadvertent production of a more virulent pathogen. Perhaps the best example is the production of a recombinant ectromelia virus containing the gene encoding interleukin-4 (IL-4) (Box 2.8).

Figure 2.4 Attenuation of viral virulence by a point mutation. Mice were inoculated intracerebrally with two strains of poliovirus which differ by a single base change at nucleotide 472. **(A)** The dose of virus causing death in 50% of the animals (LD_{50}) was determined. The change from C to U is accompanied by a large increase in LD_{50}. **(B)** Viral replication in mice was determind by plaque assay of spinal cord homogenates. The change from C to U decreases viral replication in the spinal cord. Adapted from N. La Monica, J. W. Almond, and V. R. Racaniello, *J. Virol.* **61**:2917–2920, 1987, with permission.

Virus	Base at 472	LD_{50}
PRV7.3	U	$>2 \times 10^7$
PRV8.4	C	9×10^3



Gene products that alter virus replication. Genes that encode proteins affecting both viral replication and virulence can be placed in one of two subclasses (Fig. 2.5).

BOX 2.7 TERMINOLOGY

Four classes of viral virulence genes

In general, virulence genes are defined by mutations that reduce virulence. For reasons of safety and ethics, experiments to increase virulence are rarely done.

The viral genes affecting virulence can be sorted into four general classes (and some may be included in more than one). The genes in these classes specify proteins that

- affect the ability of the virus to replicate
- modify the host's defense mechanisms
- facilitate virus spread in and among hosts
- are directly toxic

As might be expected, mutations in these genes often have minimal or no effect on replication in cell culture and, as a consequence, are often called "nonessential genes," an exceedingly misleading appellation.

Virulence genes require careful definition, as exemplified by the first general class listed above (ability of the virus to replicate). **Any** defect that impairs virus reproduction or propagation often results in reduced virulence. In many cases, this observation is not particularly insightful or useful. The difficulty in distinguishing an indirect effect due to inefficient replication from an effect directly relevant to disease has plagued the study of viral pathogenesis for years. There is an adage in genetics that says, "You always get what you select, but you may not get what you want."

Classification is accomplished by analyzing mutants defective in the gene of interest. Viral mutants with alterations in one subclass of genes exhibit reduced or no replication in the animal host and in many cultured cell types. Reduced virulence results from failure to produce sufficient numbers of virus particles to cause disease. Such a phenotype may be caused by mutations in any viral gene.

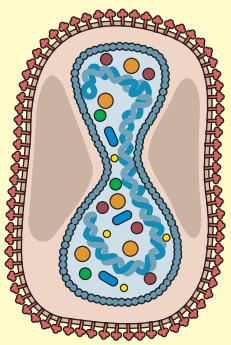
Mutants of the second subclass exhibit impaired virulence in animals, but no replication defects in cells in culture (except perhaps in cell types representative of the tissue in which disease develops). Such host range mutants should provide valuable insight into the basis of viral virulence, because they identify genes specifically required for disease. Host range mutations in a wide variety of viral

BOX 2.8 EXPERIMENTS

Inadvertent creation of a more virulent poxvirus

Australia had a wild-rodent infestation, and scientists were attempting to attack this problem with a genetically engineered ectromelia virus, a member of the *Poxviridae*. The idea was to introduce the gene for the mouse egg shell protein zona pellucida 3 into a recombinant ectromelia virus. When the virus infects mice, the animals would mount an antibody response that would destroy eggs in female mice. Unfortunately, the strategy did not work in all the mouse strains that were tested. It was decided to incorporate the gene for IL-4 into the recombinant virus. This strategy was based on the previous observation that incorporation of this gene into vaccinia virus boosts antibody production in mice. The presence of IL-4 was therefore expected to increase the immune response against zona pellucida.

To the researchers' great surprise, the recombinant virus replicated out of control in inoculated mice, destroying their livers and killing them. Moreover, mice that were vaccinated against ectromelia could not survive infection with the recombinant virus; half of them died. "This was a complete shock to us," said one researcher. Essentially, they had shown that the common laboratory technique of recombinant DNA technology could be used to overcome the host immune response and create a more virulent poxvirus.



Those who conducted this work debated whether to publish their findings, but eventually did so. Their findings raised alarms about whether such technology could be used to produce biological weapons, and the incident was widely reported in the press. Although the result was a surprise to the investigators, analysis of previously published data suggests that increased virulence of the recombinant virus could have been predicted. This incident emphasizes the need to consider carefully one's experimental design, and to be aware of possible dangers that might arise from the inappropriate use of genetic engineering.

Jackson, R. J., A. J. Ramsay, C. D. Christensen, S. Beaton, D. F. Hall, and I. A. Ramshaw. 2001. Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox. *J. Virol.* **75:**1205–1210.

Müllbacher, A., and M. Lobigs. 2001. Creation of killer poxvirus could have been predicted. *J. Virol.* **75:**8353–8355.

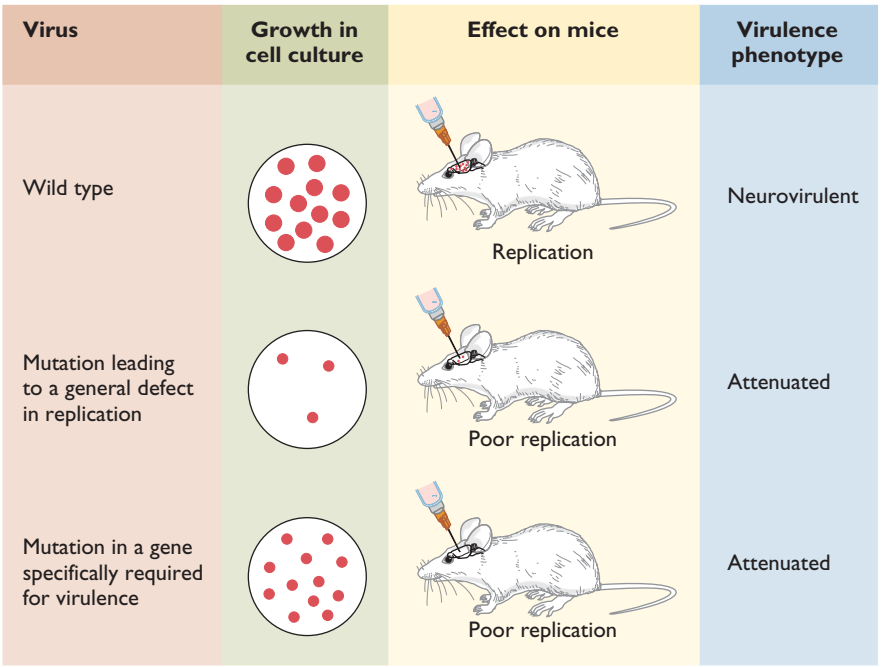


Figure 2.5 Different types of virulence genes. Examples of virulence genes that affect viral growth, using intracerebral neurovirulence in adult mice as an example. Wild-type viruses grow well in cell culture; after inoculation into the mouse brain, they replicate and are virulent. Mutants with defects in replication do not grow well in cultured cells, or in mouse brain, and are attenuated. Mutants with a defect in a gene specifically required for virulence replicate well in certain cultured cells, but not in the mouse brain, and are attenuated. Adapted from N. Nathanson (ed.), *Viral Pathogenesis* (Lippincott-Raven Publishers, Philadelphia, PA, 1997), with permission.

genes encoding proteins that participate in many of the steps in viral replication have been described. We discuss three gene families found in alphaherpesviruses as specific examples of this type of mutant.

A primary requirement for the replication of DNA viruses is access to large pools of deoxyribonucleoside triphosphates. This need poses a significant problem for viruses that replicate in terminally differentiated, nonreplicating cells such as neurons. The genomes of many small DNA viruses encode proteins that alter the cell cycle, such that the cellular substrates for DNA synthesis are produced. Another solution, exemplified by alphaherpesviruses, is to encode enzymes that function in nucleotide metabolism, such as thymidine kinase and ribonucleotide reductase. Mutations in these genes often reduce the neurovirulence of herpes simplex virus because the mutants cannot replicate in neurons or in any other cell unable to complement the deficiency.

Several attenuated herpesvirus strains harbor viral DNA polymerase gene mutations that alter virus replication in neurons but not in other cell types. Such mutants are attenuated after direct inoculation into the brains of mice, yet replicate well if introduced in the periphery. In another example, point mutations that affect the helicase activity of the helicase-primase complex result in a virus that cannot replicate in neurons and so is attenuated. These observations imply that neuron-specific proteins cooperate with the viral DNA replication machinery to promote DNA synthesis. Analogous attenuating mutations are also found in the genomes of members of other virus families.

Deletion of the herpes simplex virus gene encoding ICP34.5 protein produces a mutant virus so dramatically attenuated that it is difficult to determine an LD_{50}, even when it is injected directly into the brain. Such mutants can replicate in some, but not all, cell types in culture and in the brain (Box 2.9). Notably, they are unable to grow in postmitotic neurons. The molecular basis for the cell type specificity of ICP34.5 mutants has yet to be determined. Their lack of virulence is related to the interaction of this protein with components of the interferon-activated Pkr pathway (Chapter 3).

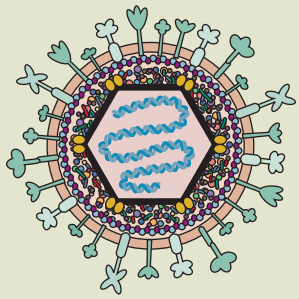
Noncoding sequences that affect virus replication. The attenuated strains that comprise the live Sabin

BOX 2.9 DISCUSSION

The use of attenuated herpes simplex viruses to clear human brain tumors

Malignant glioma, a common brain tumor, is almost universally fatal, despite advances in surgery, radiation, and chemotherapy. Patients rarely survive longer than a year after diagnosis. Several groups have proposed the use of cell-specific replication mutants of herpes simplex virus to kill glioma cells *in situ*. One such virus under study carries a deletion of the ICP34.5 gene and the gene encoding the large subunit of ribonucleotide reductase. These mutant viruses replicate well in dividing cells, such as glioma cells, but not in nondividing cells, such as neurons. The theory is that attenuated virus injected into the glioma will replicate and kill the dividing tumor cells, but will not replicate or spread in the nondividing neurons.

This idea works in principle: studies of mice have indicated that direct injection of this mutant virus into human gliomas transplanted into mice causes clearing of the tumor. The virus is attenuated and safe: injection of 1 billion virus particles into the brain of *Aotus nancymai* (a monkey highly sensitive to herpes simplex virus brain infections) had no pathogenic effect on the animal. This degree of attenuation is remarkable.



Several human trials are in progress to test safety and dosage. In one study, up to 10^5 PFU was inoculated directly into the brain tumors of nine patients. No encephalitis, adverse clinical symptoms, or reactivation of latent herpes simplex virus were observed. Higher concentrations will be used until a therapeutic effect is attained.

Mineta, T., S. D. Rabkin, T. Yazaki, W. D. Hunter, and R. L. Martuza. 1995. Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. *Nat. Med.* **1:** 938–943.

Rampling, R., G. Cruickshank, V. Papanastassiou, J. Nicoll, D. Hadley, D. Brennan, R. Petty, A. MacLean, J. Harland, E. McKie, R. Mabbs, and M. Brown. 2000. Toxicity evaluation of replication-competent herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma. *Gene Ther.* **7:**859–866.

poliovirus vaccine are examples of viruses with mutations that are not in protein-coding sequences (Chapter 8). Each of the three serotypes in the vaccine contains a mutation in the 5′ noncoding region of the viral RNA that impairs replication in the brain (Fig. 2.4). They also reduce translation of viral messenger RNA (mRNA) in cultured cells of neuronal origin, but not in certain other cell types. An interesting finding is that attenuated viruses bearing these mutations apparently do not replicate efficiently at the primary site of infection in the gut. Consequently, many fewer virus particles are available for hematogenous or neural spread to the brain. Mutations in the 5′ noncoding regions of other picornaviruses also affect virulence in animal models. For example, deletions within the long poly(C) tract within the 5′ noncoding region of mengovirus reduce virulence in mice without affecting viral replication in cell culture.

Gene products that modify host defense mechanisms. The study of viral virulence genes has identified a diverse array of viral proteins that sabotage the body's intrinsic, innate, and adaptive defenses. Some of these viral proteins are called **virokines** (secreted proteins that mimic cytokines, growth factors, or similar extracellular immune regulators) or **viroceptors** (homologs of host receptors). Mutations in genes encoding either class of protein affect virulence, but these genes are **not** required for growth in cell culture (Fig. 2.6). Most virokines and viroceptors have been discovered in the genomes of large DNA viruses (Box 2.10).

As discussed in Chapter 3, many viral infections induce apoptosis, an intrinsic response that contributes significantly to viral pathogenesis. For example, the pattern of infection of Sindbis virus changes from nonlethal persistent to lethal acute, depending on whether the infected cell can mount an apoptotic response. After infection, apoptosis can be either indirect (uninfected cells die) or direct (infected cells die), and both responses influence subsequent pathogenesis. African swine fever, a highly contagious disease of pigs that is caused by a double-stranded DNA virus transmitted by insects, is an example of a disease caused by indirect apoptosis. The severe lesions and hemorrhages are striking, but intense destruction of lymphoid tissue is characteristic of the disease. This destruction is caused by apoptosis of uninfected lymphocytes induced by cytokines and apoptotic mediators released from infected macrophages. Direct pathogenic effects of virus-induced apoptosis have been suggested in a model of herpes simplex virus-mediated fulminant hepatitis, in which massive hepatocyte death occurs within 24 h of virus injection into the bloodstream. In this disease model, virions in the blood are

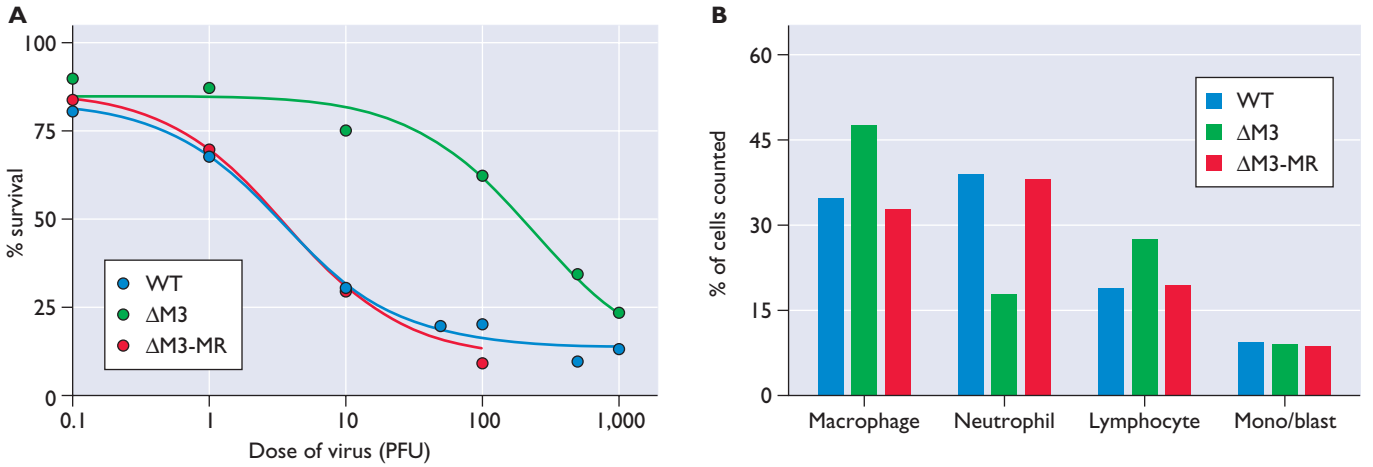


Figure 2.6 Role of a herpesviral chemokine in pathogenesis. (A) Survival of mice after intracerebral inoculation with wild-type gammaherpesvirus type 68, with a mutant virus lacking the M3 gene (ΔM3), which encodes a protein that binds CC chemokines, or with the mutant virus to which the M3 gene has been restored (ΔM3-MR). **(B)** Cellular infiltration in meninges of mice after intracerebral inoculation with the three viruses described in panel A. Adapted from V. van Berkel et al., *J. Clin. Investig.* **109:**905–914, 2002, with permission.

removed rapidly by cells of the reticuloendothelial system, primarily those found in the liver. If the phagocytic activity of macrophages is impaired, virus replication ensues in the liver and is followed by massive apoptotic death throughout the organ.

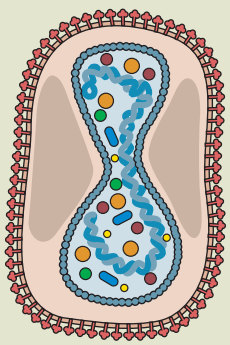
Gene products that enable the virus to spread in the host. The mutation of some viral genes disrupts spread from peripheral sites of inoculation to the organ in which disease is manifested. For example, after intramuscular inoculation in mice, reovirus type 1 spreads to the central

BOX 2.10 DISCUSSION

Variola virus virulence: a highly efficient inhibitor of complement encoded in the genome

Variola virus, which causes the human disease smallpox, is the most virulent member of the *Orthopoxvirus* genus. The prototype poxvirus, vaccinia virus, does not cause disease in immunocompetent humans, and is used to vaccinate against smallpox. Both viral genomes encode inhibitors of the complement pathway. The vaccinia virus complement control protein is secreted from infected cells and functions as a cofactor for the serine protease factor I. The variola virus homolog, called smallpox inhibitor of complement, differs from the vaccinia virus protein by 11 amino acid substitutions. Because the variola virus protein had not been studied, it was produced by changing the 11 codons in DNA encoding the vaccinia virus homolog. The variola virus protein produced in this way was found to be 100 times more potent than the vaccinia virus protein at inactivating human complement. This finding provides an explanation for the virulence of variola virus.

These findings suggest that the virulence of variola virus, and the avirulence of vaccinia virus, might be controlled in part by complement inhibitors encoded in the viral genome. Furthermore, if smallpox should reemerge, the smallpox inhibitor of complement might be a useful therapeutic target.



Rosengard, A. M., Y. Liu, N. Zhiping, and R. Jimenez. 2002. Variola virus immune evasion design: expression of a highly efficient inhibitor of human complement. *Proc. Natl. Acad. Sci. USA* **99:**8808–8813.

nervous system through the blood, while type 3 spreads by neural routes. Studies of viral recombinants between types 1 and 3 indicate that the gene encoding the viral outer capsid protein s1, which recognizes the cell receptor, determines the route of spread. Only 1 plaque-forming unit (PFU) of La Crosse virus (a bunyavirus) causes lethal encephalitis after intracerebral injection into mice. However, subcutaneous inoculation of over 10^7 PFU causes no disease because it cannot produce a viremia and spread to the brain (Table 2.8).

Viral membrane proteins have been implicated in neuroinvasiveness. For example, the change of a single amino acid in the gD glycoprotein of herpes simplex virus type 1 blocks spread to the central nervous system via nerves after footpad inoculation. Similarly, studies of neuroinvasive and nonneuroinvasive strains of bunyaviruses indicate that the G1 glycoprotein is an important determinant of entry into the central nervous system from the periphery. Although it is tempting to speculate that these viral glycoproteins, which participate in entry, facilitate direct ingress into nerve termini, the mechanisms by which they govern neuroinvasiveness are unknown. These glycoproteins may also influence the ability of host antibodies to clear virus from the primary site of infection.

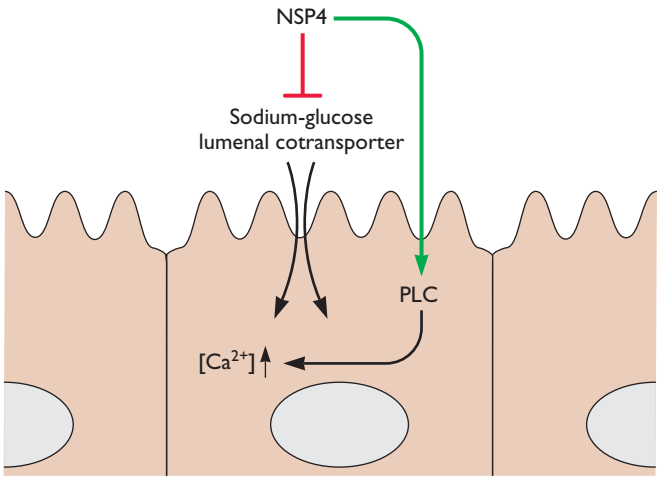


Figure 2.7 Model for rotavirus-induced diarrhea. nsP4, produced during rotavirus replication in intestinal epithelial cells, inhibits the sodium-glucose lumenal cotransporter. Because this transporter is required for water reabsorption in the intestine, its inhibition by nsP4 could be one mechanism of diarrhea induction. nsP4 also induces a phospholipase C (PLC)-dependent calcium signaling pathway. The increase in the concentration of intracellular calcium could induce calcium-dependent chloride secretion. Adapted from M. Lorrot and M. Vasseur, *Virol. J.* **4**:31–36, 2007, with permission.

Toxic viral proteins. Some viral gene products cause cell injury directly, and alterations in these genes reduce viral virulence. Evidence of their intrinsic activity is usually obtained by adding purified proteins to cultured cells, or by synthesis of the proteins from plasmids or viral vectors. The most convincing example of a viral protein with intrinsic toxicity relevant to the viral disease is the nsP4 protein of rotaviruses, which cause gastroenteritis and diarrhea. nsP4 is a nonstructural glycoprotein that participates in the formation of a transient envelope as the particles bud into the endoplasmic reticulum. When nsP4 is fed to young mice, it causes diarrhea. It is thought that in cultured cells, the protein induces a phospholipase C-dependent calcium signaling pathway that leads to chloride secretion (Fig. 2.7). nsP4 therefore acts as a viral enterotoxin, and triggers a signal transduction pathway in the intestinal mucosa.

The SU and TM glycoproteins of human immunodeficiency virus are toxic to cultured cells. TM causes death of cultured cells, most probably as a result of alterations in membrane permeability. The addition of SU to cells results in a high influx of calcium. The contribution of this toxicity to pathogenesis in humans remains untested.

Targets of viral virulence gene products. Mutagenesis can also be used to identify the cellular target of a viral virulence gene product. Mutation of the ICP34.5 gene of herpes simplex virus dramatically reduces neurovirulence in mice. It was hypothesized that the protein product of this gene inhibits the antiviral effects of the cellular protein Pkr (Chapter 3). In support of this hypothesis, the ICP34.5 mutant virus was found to be neurovirulent in mice lacking the gene encoding Pkr. Similar experiments have been conducted with a variety of viruses, allowing identification of specific cellular pathways that are altered by viral virulence gene products.

Cellular Virulence Genes

Disruption of cellular genes that encode proteins required for innate and adaptive immune responses may have enormous effects on viral infection. In some cases viral disease becomes more severe, while in others the disease severity is lessened. Such cellular genes can therefore be considered virulence determinants. A consideration of immune response genes can be found in Chapter 4.

Host proteins required for viral translation, genome replication, and mRNA synthesis are prime candidates for virulence determinants. Tissue-specific differences in such proteins could in principle influence virus replication. A common approach in studying viral pathogenesis is to construct mice with specific gene disruptions (Box 2.1). It may be possible to derive mice lacking such proteins as further tools with which to study viral virulence. Few studies have been done in this largely uncharted territory of animal

virology, an area that may be one of the most interesting yet to be explored in viral pathogenesis.

Host Susceptibility to Viral Disease

When populations of humans or other animals are infected, many different responses are possible. Some hosts may be highly resistant to infection, some may become infected, and still others may fall in the spectrum between the two extremes. Those who become infected may develop disease ranging from asymptomatic to fatal. Susceptibility to infection and susceptibility to disease vary independently. Understanding the basis for such variation is important, because it may suggest methods for preventing viral disease. The results of such studies demonstrate that both host genes and nongenetic parameters control how populations respond to viral infections (see further discussion in Chapter 10 on emerging viral diseases).

Intrinsic and Immune Defenses

Epidemiologists divide human populations into two groups: susceptible and immune (or resistant). Individuals who have been infected in the past are immune and are not likely to transmit infection. Susceptible individuals can develop disease and spread the virus to others. Persistence of a virus in a population depends on the presence of a sufficient number of susceptible individuals. How efficiently infection occurs determines this number. Immunization against viral infection, by natural infection or vaccination, reduces the number of susceptible people, and therefore limits viral persistence and spread (discussed in more detail in Chapters 5 and 10). For example, epidemics of poliomyelitis were self-limiting, because the asymptomatic spread of the virus immunized the population. The competence of the immune response also determines the speed and efficiency with which the infection is resolved, and the severity of symptoms.

Genetic Determinants of Susceptibility

Several examples of susceptibility genes were first identified in animal systems. The *mx* gene of mice, which confers resistance to infection with influenza A virus, is a well-characterized example. The *mx* gene encodes a guanosine triphosphatase (GTPase) that inhibits influenza virus replication (Chapter 3). Resistance of mice to flavivirus disease has been mapped to the *flv* gene. Flavivirus titers in mice with an *flv* mutation are 1,000- to 10,000-fold lower than in susceptible animals, and the infection is cleared before disease symptoms develop. The product of the *flv* locus is 2′-5′-oligo(A) synthetase, an interferon-induced enzyme that activates ribonuclease L (RNase L), leading to degradation of host and viral mRNAs (Chapter 3). *mx* and *flv* genes have not been shown to play roles in human infections.

It is rare to find single human genes that influence susceptibility to viral infections. The few that do influence susceptibility have been found to encode components of the intrinsic and innate immune systems, e.g., the Toll-like receptors and the chemokine receptors that serve as cofactors for entry of human immunodeficiency virus type 1 into cells. Some genes may influence susceptibility to more than one virus infection. Remarkably, a mutation in the gene encoding the CCr5 chemokine that is protective against infection with human immunodeficiency virus type 1 increases susceptibility to lethal encephalitis caused by West Nile virus.

Recently, researchers found two different mutations in humans that predispose carriers to herpes simplex virus encephalitis. The mutations were in the gene encoding Tlr3 or in the gene expressing the protein Unc-93B. Both gene products affect the production of alpha/beta interferon. The *TLR3* mutation is autosomal dominant (a single copy of the mutant gene increases susceptibility to herpes simplex virus encephalitis), while the *UNC-93B* mutation is recessive (two copies of the mutated gene are required for the susceptibility phenotype). One might expect these mutations to result in broad sensitivity to many viral pathogens, because similar defects in mice are not pathogen specific. As far as could be determined, these patients did not have increased susceptibility to other microbial pathogens. The implication is that in addition to being a general response to all pathogens, intrinsic and innate defenses may evolve to be targeted directly toward a single pathogenic process in a particular species.

Proteins that mediate the humoral and cellular immune responses are other well-known determinants of susceptibility to viral infections. The class I and class II major histocompatibility complex proteins, for which there are many genes, present foreign peptides to T cells. The ability of these proteins to interact with peptides derived from viral proteins determines, in part, how efficiently the infection is cleared. Diversity in major histocompatibility protein genes makes it more likely that there will be a suitable molecule to present peptides for any given infectious agent. Populations from isolated islands have less polymorphism in these genes, a property that may account for their greater susceptibility to infection.

Other Determinants of Susceptibility

The age of the host plays an important role in determining the result of viral infections. Very young and very old humans are most susceptible to disease (Fig. 2.8). The increased susceptibility of infants and young children is likely to be explained by the immaturity of their immune responses. Although young animals may have an immature immune response and become infected more frequently,

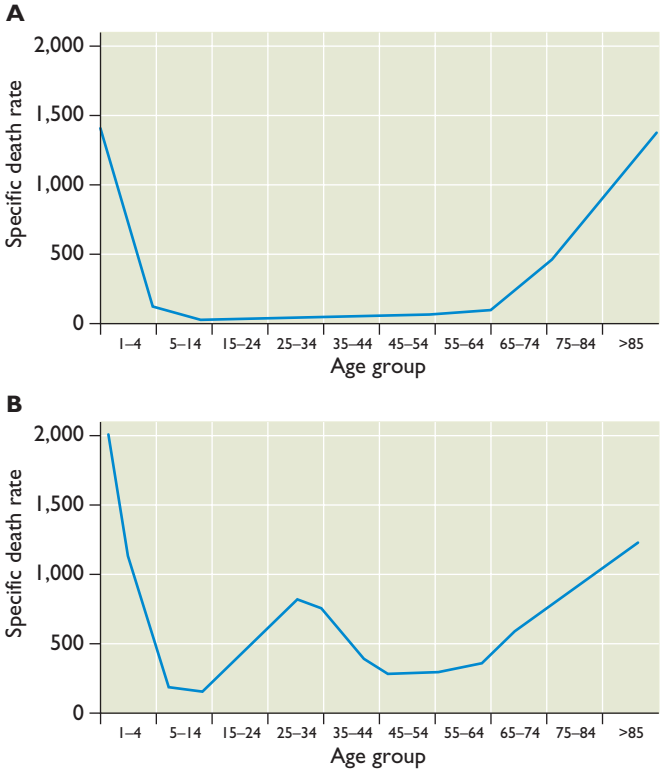


Figure 2.8 Age dependence of influenza pneumonia. Influenza pneumonia deaths per 100,000 in the United States from 1911 to 1915 **(A)** and 1918 **(B)** are shown. Adapted from R. Ahmed et al., *Nat. Immunol.* **8:**1188–1193, 2007, with permission.

they also have greater freedom from immunopathology. Intracerebral inoculation of lymphocytic choriomeningitis virus in adult mice is lethal, because of the T-cell response, while infant mice survive because of their weaker response (Box 2.11).

Other physiological differences may explain age-dependent variation in susceptibility. Infection of human infants with enteric coronaviruses is severe because the alimentary canal is not fully active and presents a particularly hospitable niche for infection. In these individuals, the gastric pH tends toward neutrality, and digestive enzymes are not available. Infection with rubella virus during the first 11 weeks of human gestation results in severe damage to the fetus. Infants that survive have abnormalities in the heart, eyes, and central nervous system. Infection at later times during gestation results in fewer congenital abnormalities, but babies who survive may have hearing loss, mental retardation, and growth deficits.

A major reason for the increased susceptibility of the very old to infection is immune senescence. Furthermore, as animals age, their alveoli become less elastic, the respiratory muscles weaken, and the cough reflex is diminished. These changes may explain in part why elderly people have increased susceptibility to respiratory infections. However, there are notable exceptions to this general trend. Respiratory syncytial virus causes severe lower respiratory tract infections in infants, but only mild upper tract infections in adults. It is not known whether the difference is due to

BOX 2.11

DISCUSSION

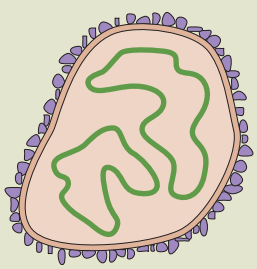
Congenital brain infections: the lymphocytic choriomeningitis virus model

The fetal brain is one of the most vulnerable organs during development: viral infections of the fetus often result in severe brain injury. Unfortunately, many animal models of congenital brain infection do not mimic human disease, for a variety of poorly understood reasons.

In contrast, the neonatal rat model for congenital lymphocytic choriomeningitis virus (LCMV) infection reproduces virtually all of the neuropathological changes observed in congenitally infected humans.

Within the developing rat brain, the virus selectively infects mitotically active neuronal precursors, a fact that explains the variation in pathology with time of infection during gestation.

Importantly, LCMV infection results in delayed-onset neuronal loss after the virus has been cleared by the immune system. Accordingly, many researchers think that this model can be used to study neurodegenerative or psychiatric diseases associated with loss of neurons or their function.



Bonthius, D., and S. Perlman. 2007. Congenital viral infection of the brain: lessons learned from lymphocytic choriomeningitis virus in the neonatal rat. *PLoS Pathog.* **3:**1541–1550.

host defenses or to variations in the susceptibility of cells to viral infection. The 1918 influenza pandemic was particularly lethal, not only for the very young and the very old, but unexpectedly also for young adults, 18 to 30 years of age (Fig. 2.8B). It has been suggested that the increased lethality in young adults occurred because they lacked protective immunity that would be conferred by previous infection with a related virus.

Some viral infections, including those caused by poliovirus, mumps virus, and measles virus, are less severe in children than in adults. The basis for this property is not known, but one possibility is that the protective and pathogenic immune responses are better balanced in children.

Males are more susceptible to viral infections than females, but the difference is slight and the reasons are not understood. Hormonal differences, which affect the immune system, may be partly responsible. Pregnant women are more susceptible to infectious disease than nonpregnant women, probably for similar reasons. Hepatitis A, B, and E are more lethal, and paralytic poliomyelitis was more common, in pregnant women than in others.

Malnutrition increases susceptibility to infection because the physical barriers to infection, as well as the immune response, are compromised. An example is the increased susceptibility to measles in children with protein deficiency. For this reason, measles is 300 times more lethal in developing countries than in Europe and North America. When children are malnourished, the small red spots in the buccal mucosa that are pathognomic for measles (Koplik spots) become massive ulcers, the skin rash is much worse, and lethality may approach 10 to 50% (Chapter 5). Such severe measles infections are observed in children in tropical Africa and in aboriginal children in Australia.

Many other parameters influence susceptibility to infection. Corticosteroid hormones have large effects, because they are essential for the body's response to the stress of infection. These hormones have an anti-inflammatory effect, which is thought to limit tissue damage. Cigarette smoking increases susceptibility to respiratory infections in some situations. Increased susceptibility is correlated with a poor mental state, such as occurs in stressful life situations (e.g., a death in the family, injury, or the loss of a job). Epidemiological studies indicate that there is a link between air pollution and hospital admissions for viral respiratory diseases. Our mothers told us that exposure to changes in temperature increases susceptibility to infection. However, comprehensive studies with rhinovirus have failed to reveal any relationship between exposure to low temperature and the common cold. Fortunately for some mothers, hot chicken soup still works wonders.

Perspectives

A fundamental principle of virology is that to perpetuate and maintain any given viral population, virions must be released from one infected host and infect another. This process of serial infection, while simple in principle, is surprisingly difficult to study in natural systems. Nevertheless, local clusters of infection in our homes, more widespread epidemics in our communities, and global pandemics of epic proportions all arise from serial transmission of virions. Epidemiology, the study of this process, is evolving rapidly as new technology for working with populations, as well as tracking and identifying infectious agents, progresses. Advances in genetics have now opened doors to identification of human susceptibility genes that were inconceivable a decade ago. Similarly, rapid progress is being made in our understanding of the molecular biology of viral replication and pathogenesis. Despite all this progress, predicting and derailing the transmission of **any** viral disease remains a challenge (Table 2.9). The simple fact is that individual hosts and their viruses are exceptionally diverse. Moreover, the environment in the real world is a kaleidoscope of variability compared to the stable confines of the virology laboratory.

Our current understanding of the fundamental principles of viral pathogenesis comes largely from studies with animal models. For example, the large number of genetically identical strains of mice has led to the identification of at least 25 loci conferring susceptibility to particular viral infections. It is noteworthy that most of these gene products affect a single virus or virus family and many target a particular step in virus replication (Chapter 3). Some gene products that have broad effects on susceptibility to infection affect intrinsic and innate immune pathways (e.g., interferon).

At one time, it seemed impossible to do similar studies with humans, because genetic techniques were not powerful enough to detect rare susceptibility mutations in outbred human populations. However, recent studies have identified several human genes whose products are involved in intrinsic or innate defense for particular viral

Table 2.9 Fundamental questions of viral pathogenesis

How does a virion enter the host?
What is the initial host response?
Where does primary replication occur?
How does the infection spread in the host?
What organs and tissues are infected?
Is the infection cleared from the host or is a persistent infection established?
How is the virus transmitted to other hosts?

infections. It will be exciting to read about how the modern techniques of genetics and genomics reveal the genes and perhaps selection pressures that drove the evolution of these remarkable viral defenses in different species.

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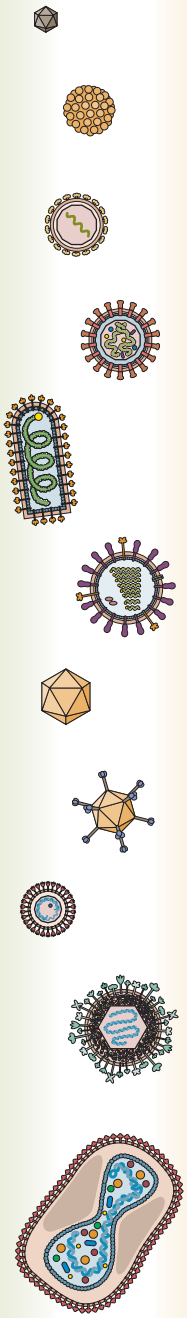


3

Virus Offense Meets Host Defense: Early Actions

Keep a-knockin' but you can't come in!
Come back tomorrow night and try again!
Louis Jordan and His Tympany Five
Vocal by Louis Jordan, recorded 29 March 1939

Introduction

We live and prosper in a literal cloud of viruses. The numbers of potentially infectious particles that impinge on us daily are astronomical. Seasonal "colds," "flu," childhood rashes, measles, chicken pox, and mumps, as well as acquired immunodeficiency syndrome (AIDS) and Ebola fever, all serve notice of our vulnerability. Despite the fact that multicellular organisms evolved in close association with microbes, our understanding of how the defense systems of metazoans recognize and deal with these organisms is incomplete. In this and the next two chapters, we consider the powerful primary and secondary defense systems that ensure our survival.

The labyrinthine complexity of the host defense system is bewildering, and the details can be overwhelming initially. We offer this piece of advice: in the beginning, do not be sidetracked by all the details, but rather try to grasp the big picture of host defense against viral infections (Table 3.1). The apparently distracting nuances of each viral infection can be appreciated **only** when taken in this admittedly oversimplified context. When a cell is infected, intrinsic defensive actions initiate almost immediately. The defensive actions, while initially cell autonomous, escalate in complexity and intensity, recruiting more and more cells if viral replication is not stopped. Control of this response is critical, as the final effector actions usually include the destruction of infected cells. When improperly stimulated or controlled, defensive responses can lead to collateral cellular damage or even host death.

Defense mechanisms, even in healthy hosts, are imperfect despite millions of years of evolution in the face of microbial infections, in large part because the genomes of successful pathogens encode gene products to modify, redirect, or block every step of host defense. Indeed, for every host defense, there will be a viral offense. As many viral family members express a small number of proteins (some genomes have only one or two open reading frames), we can be both amazed and daunted by the realization that the genome of every virus on the planet today **must** encode countermeasures to modulate the defenses of its

Table 3.1 Basic concepts: host defense systems against viral infections are multistep, sequential, and intercommunicating

First: physical and chemical defenses
The skin, surface coatings of tissues such as mucous secretions, tears, acid pH, and surface-cleansing mechanisms
Second: frontline defense
Cell-autonomous, intrinsic defense systems
Detection of altered cell metabolism
Detection of unusual macromolecules made only by invading parasites
Production of cytokines, induction of apoptosis, interference with early steps of viral replication
Third: attack and clean up
Innate and adaptive immune defense
Direct, amplified response by coordinated action of cytokines and lymphocytes.
Infection cleared by pathogen-specific antibodies, helper T cells, and cytotoxic T cells
Production and maintenance of B-cell and T-cell "memory" cells
"Immune" host, ready to respond instantly to the same infection that induced the memory response

host (Box 3.1). The challenge is to identify the key nodes in host defense that are targeted by viral proteins. Only then will we appreciate the foibles of our own vulnerabilities.

Primary Physical and Chemical Defenses

Most virions have no chance in the real world; almost all that impinge on prospective hosts never encounter a susceptible cell. This is so simply because of primary, but often underappreciated, host defenses. These defenses are extracellular and seemingly crude and primitive. They are the physical and chemical barriers of the body: the dead skin, surface coatings such as mucous secretions, tears, and surface-cleansing mechanisms (discussed in more detail in Chapter 1).

The skin is the largest organ of the body, weighing more than 5 kg in an average adult, and is a strong barrier to infection. It is impervious, unless broken by cuts, abrasions, or punctures (e.g., insect bites and needle sticks). Many virus particles that land on the skin are inactivated by desiccation, acids, or other inhibitors formed by indigenous commensal microorganisms. Surfaces exposed to the environment but not covered by skin are lined by living cells and therefore depend on other primary defenses for immediate protection. These surfaces are at risk for infection, despite the remarkable and continuous action of cleansing mechanisms.

The First Critical Moments of Infection

An infection can be initiated only when physical and chemical barriers are breached and virions encounter living cells that are susceptible and permissive. A central dictum for those who study viral pathogenesis is the following: "What happens early dictates what happens late." Indeed, the critical time is within the first hour or so when single cells are infected and spread of infection is local or nonexistent. During this time, various outcomes are in the balance, depending on which molecular switch is activated: will defensive actions be initiated? Which actions are appropriate? If the decisions are too late, the host may die. If the response is too strong or otherwise inappropriate, the host also may suffer. The decisions are based on two coupled, primary processes: intracellular recognition of the invader and subsequent, appropriate control responses. Once the invader is identified, the response must match the invader. Is it a DNA or RNA invader, an intracellular bacterium, a virus, or none of the above? The molecular coupling between the invader detectors and the response effectors is understood only in broad outline, but more details are emerging every day. As we will discuss, these two key processes are played out at each subsequent step in immune defense; the processes of recognition and control are monitored, adapted, and directed (or redirected) as the infection proceeds. Remarkably, the whole process begins with events initiated in a single infected cell.

BOX 3.1 TERMINOLOGY

Is it evasion or modulation?

From the online *Merriam-Webster Dictionary:*

Evade: to elude by dexterity or stratagem

Modulate: to adjust to or keep in proper measure or proportion

The phrase "immune evasion" is popular in the virology literature. It is meant to describe the viral mechanisms that thwart the host immune defense systems. However, in many cases, the phrase can be inaccurate, imprecise, and even misleading. The term "evasion" implies that host defenses are ineffective. In reality, defense and offense are matters of degree, not absolutes. If viruses really could evade the immune system, we might not be here discussing semantic issues.

Perhaps a more accurate term to describe viral gene products that engage immune defenses is "immune modulators." The strategic point is that given the speed of viral replication, an infection can be successful if defenses are only transiently suppressed.

Intrinsic Cellular Defenses

All cells have genetic programs that respond to various stresses, such as starvation, temperature extremes, irradiation, and infection. Some of these programs are designed to maintain homeostasis, and others have evolved to repel cellular invaders. These latter programs recognize and respond to alien nucleic acids and other microbial products, such as viral membrane proteins, bacterial cell wall and flagellum components, or other foreign protein modifications and lipids.

We define these cell-autonomous, protective programs as **intrinsic cellular defenses** to distinguish them from **immune defenses** (Chapter 4). Immune defenses tend to be more global (organism-wide) responses, whereas intrinsic defenses begin with a single cell and tend to be local. Intrinsic defenses arose very early in the evolution of cells. In contrast, immune defenses appeared later during the evolution of complicated multicellular organisms and depend on mobile lymphocytes and antibodies released in blood and secretions. Below, we consider several primary intrinsic defenses that are relevant to viral infection. It is important to note that while we make a clear distinction between intrinsic defense and innate immune defense, these two early-action arms of host defense are coupled by the action of cytokines (Fig. 3.1).

We first discuss what we understand about how an invader is recognized by a cell and how the coordination of recognition and appropriate defensive response is achieved. We then discuss cytokines, with a focus on the interferons, secreted proteins of early warning. Finally, we review six widely conserved processes that function in cellular defense against viral infections.

How Do Individual Cells Detect Foreign Invaders?

A major conundrum for many years was to understand how cells recognize microbial invaders as not "self." The problem in the case of viruses is obvious: the basic building materials are derived from self cells, the only difference being the way the materials are put together. We now understand that an infected cell differs from an uninfected one in at least two general properties. First, as soon as virions engage their receptors, new signals may flow through cellular signal transduction pathways. In these first minutes of infection, no nonhost proteins are made, but the cell may respond in many ways. For example, the dynamics of ion flow, membrane permeability, protein modification and localization, and even transcription of host genes may change. Second, soon after virions engage receptors, viral nucleic acid appears in the cytoplasm or the nucleus. As we shall see, the cytoplasm is a hostile environment for foreign nucleic acids. It is likely that the same holds true for the nucleus, but much less is known about this organelle's defenses. As soon as viral genomes are exposed to the cytoplasm, host proteins can bind to the foreign nucleic acid (recognizing structures that are not commonly found in cellular nucleic acids). These protein-nucleic acid complexes engage signal transduction pathways that stimulate synthesis of cytokines such as the type I interferons (IFN-α and IFN-β) (Fig. 3.2).

We have only a minimal understanding of nuclear defenses against foreign nucleic acids. For example, it is clear that if viral DNA or RNA enters the nucleus, a DNA damage response may ensue as a result of detection of single-stranded nucleic acid or double-strand ends of DNA. There is some evidence that nuclear proteins may bind the incoming foreign DNA and block transcription. After infection by retroviruses, the host genome invariably suffers direct damage: proviral DNA is always integrated in the cell genome. The process of integration depends on activation of DNA repair pathways, and the integrated proviral DNA may affect the expression or integrity of a cellular gene, a process known as **insertional mutagenesis** (Chapter 6; also see Volume I, Chapter 7).

As soon as viral proteins are produced, the cell is permanently changed, sometimes in dramatic ways (Table 3.2). For example, infection may result in visible changes in the cells, collectively called **cytopathic effect**. The consequences of viral infection also may include cessation of essential host processes such as translation, DNA and RNA synthesis, and vesicular transport. These deviations from the norm are detected by two general systems that monitor cell health. First, intracellular molecules monitor cell integrity and homeostasis. Second, extracellular proteins (cytokines) are produced and secreted in response to infection and bind to surface receptors on nearby cells. These cytokines announce the infection to neighboring uninfected cells. Cells that bind the cytokines initiate defensive actions. When homeostasis is altered or when signaling cytokines bind to their receptors, a common output pathway that results in cell death is activated. The integration of detection and response is one of the crucial features of intrinsic cellular defense. Viral evolution in the face of these responses has resulted in viral gene products that counter, modulate, or even bypass intrinsic cell defenses.

Receptor-Mediated Recognition of Pathogen-Associated Molecules

Pathogens are distinguished by the presence of unique molecules including bacterial and fungal cell wall and membrane materials (lipopolysaccharide, peptidoglycans, lipotechoic acids, glucans), bacterial and viral RNA and DNA, *N*-formylmethionine, and lipoproteins. Such pathogen-associated molecules are recognized by certain cellular proteins called **pattern recognition receptors**. These

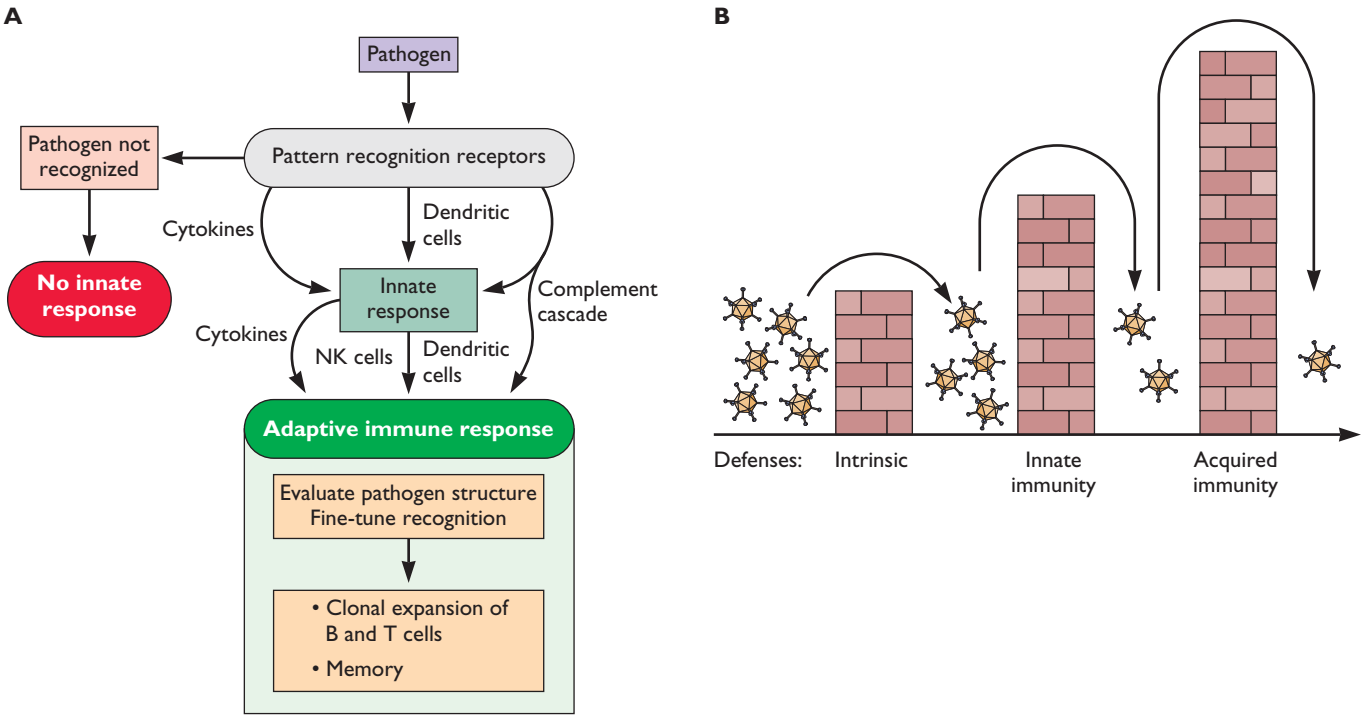


Figure 3.1 Integration of intrinsic defense with the innate and adaptive immune response. (A) An invading pathogen is first detected by molecular interactions that depend on pattern recognition receptors of the intrinsic defense system (see also Fig. 3.10). Molecules of microbial origin are usually detected, resulting in a variety of responses including cytokine production and release of stimulators of inflammation. As infection proceeds in the face of intrinsic defenses, the innate immune response comes into play. Complement proteins, natural killer cells (NK cells), dendritic cells, and other phagocytic cells act to contain the infection. During this phase, migratory sentinel cells (e.g., dendritic cells) take packets of ingested proteins to lymph nodes, where they contact and stimulate cells of the adaptive immune system. Molecules of the invading microbe and the effector molecules of the innate immune defenses then interact further with the adaptive immune system, often causing clonal expansion of distinct classes of lymphocytes. Highly specific effectors such as antibodies produced by B cells, and cytotoxic T cells, are released into the circulation to recognize the invading microbe and the infected cells. This adaptive response enables recognition of foreign proteins with a high degree of structural specificity. The intrinsic and innate immune defenses are essential, not only for immediate deployment, but also for reconnaissance and transfer of information to the adaptive immune system. **(B)** The sequential nature of host defenses depicted as the breaching of successive barriers by viral infection. Most infections are blocked by intrinsic defenses. If intrinsic defenses are breached, then innate defenses come into play to contain the infection. Activation of acquired immune defenses is usually sufficient to contain and clear any infections that escape intrinsic and innate defense. In rare instances, host defenses may be absent or inefficient and severe or lethal pathogenesis occurs. Adapted from D. T. Fearon and R. M. Locksley, *Science* **272:**50–54, 1996, with permission.

receptors also detect endogenous stress signals such as uric acid and some heat shock proteins. Pattern recognition receptors have been selected over evolutionary time to be highly pathogen specific. Our first insights into the nature of these receptors came from *Drosophila* developmental genetics (Box 3.2). We now understand that all intrinsic and innate defense systems arose early in the evolution of multicellular organisms, and remain absolutely essential for survival.

Several receptors detect specific motifs characteristic of invading microbial pathogens in single cells (Table 3.3). Four of these are the DEXD/H box RNA helicases (e.g., RigI and Mda5) capable of detecting foreign RNA in the cytoplasm (Box 3.3), the Dai protein (also known as the Z-DNA-binding protein) that binds foreign DNA in the cytoplasm, the Toll-like receptors (Tlrs) (Box 3.4) that detect a wide variety of microbial products including RNA and DNA, and the complement lectin C1q family members (see "Complement" below) (Table 3.4). All these receptors likely detect not only microbial but also nonmicrobial danger signals. Different cell types and tissues vary in the distribution and concentration of these receptors. Most cells

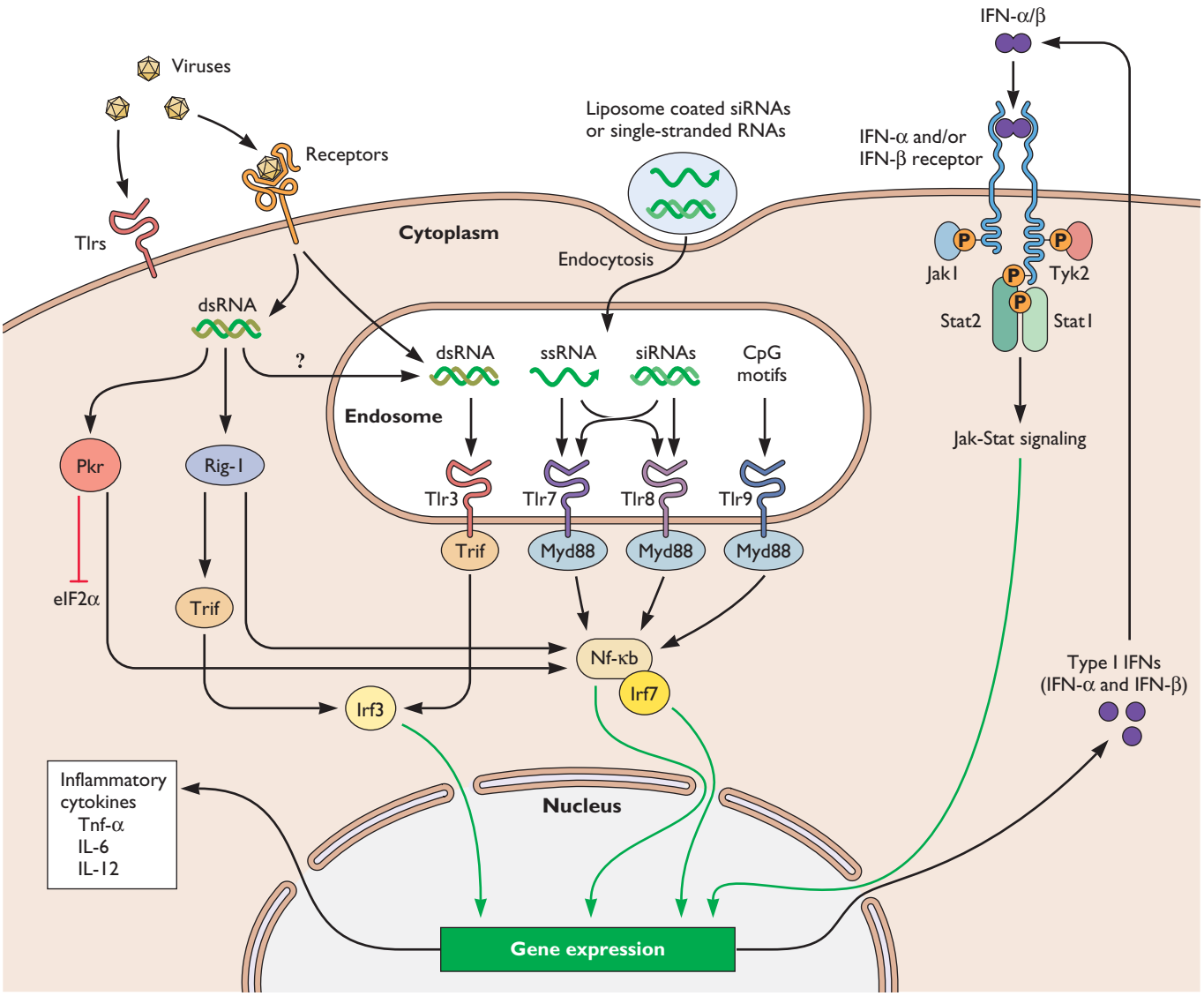


Figure 3.2 Recognition of foreign nucleic acids in mammalian cells. The Toll-like receptors (Tlrs), RigI, and protein kinase R (Pkr) all contribute to detection of pathogen-specific nucleic acids including single-stranded RNA, dsRNA, RNA nucleotides, siRNAs, and unmethylated CpG-containing oligonucleotides. As the receptor's cognate nucleic acid is bound on the cell surface, in the cytoplasm, or in the lumen of endosomes, signal transduction events lead to activation of Nf-κb, Irf3, or Irf7 to induce expression of inflammatory cytokines and IFN-α/β. Important cytoplasmic proteins in the signal transduction cascade, including Trif and Myd88, bind the cytoplasmic tails of endosomal Tlr proteins after they have engaged their cognate ligand. Viral RNA and DNA may be exposed in the lumen of endosomes after degradation or uncoating events. Pkr is autophosphorylated when dsRNA is bound, leading to phosphorylation of its substrates. One such substrate is the a subunit of the eukaryotic translation initiation factor 2α. Phosphorylation of this protein blocks protein synthesis. Many IFN-inducible genes, including Pkr, are induced when IFN-α and IFN-β bind the IFN receptor (autocrine pathway).

synthesize all these, but the Tlrs are critical for the function of cells in the immune system, particularly dendritic cells and macrophages (Table 3.3).

The pattern recognition receptor proteins are localized strategically to the sites at which the earliest stages of viral infections begin. For example, while the RNA helicase receptors tend to be found in the cytoplasm, certain Tlrs are present on the cell surface or in endosomes, where entering viral proteins and nucleic acids first appear (Figure 3.2). It is likely that all processes of viral uncoating

Table 3.2 Host alterations are early signals of infection

Alteration	Virus	Viral protein	Target
Inhibition of transcription	Poliovirus	3C	TBP-TFIIIC complex
Blocking accumulation of host mRNA in cytoplasm	Adenovirus	E1B-55K	Cellular protein involved in mRNA transport?
		E4-34K	
Inhibition of 5′-end-dependent translation	Poliovirus	$2A^{pro}$	eIF-4G
	Adenovirus	?	eIF-4E (dephosphorylation)
Inhibition of ER[a]-to-Golgi protein traffic	Poliovirus	2B, 3A	?
Membranous-vesicle accumulation	Poliovirus	2C, 2BC	?
Alteration of MAP4	Poliovirus	3C	MAP4
Increased plasma membrane permeability	Sindbis virus	?	Na,K-ATPase
Fusion of cell membranes, syncytium formation	Paramyxovirus	F protein	Plasma membrane
Inhibition of transport and processing of host RNA	Herpes simplex virus	ICP27	SR splicing proteins
Depolymerization of cytoskeleton	Many viruses	?	Actin filaments, microtubules, intermediate filaments

[a]ER, endoplasmic reticulum.

and replication have evolved to bypass detection by these receptors.

When pattern recognition receptors bind their particular ligand, a signal transduction cascade ensues that results in activation of cytoplasmic transcription regulatory proteins such as Nf-κb and interferon regulatory factors (Irfs). These regulatory proteins in turn stimulate expression of cytokine genes including those encoding (IFN-α and IFN-β) and other proinflammatory cytokines. IFN-α and IFN-β play important roles in amplifying intrinsic cellular defenses. For example, the expression of the RigI and Mda5 genes, as well as that of many Tlr genes, is stimulated by IFN-α and IFN-β. Indeed, the interferons are critical cytokines of early warning, as we will see below.

The protein detectors of viral infection must have amazing discriminatory power. It is clear that some of this power comes from activation of not one but rather sets of pattern recognition receptors after infection by particular pathogens. The unleashing of powerful cytokines in response to infection is a double-edged sword: intrinsic and immune defenses often are damaging to uninfected tissues. An inappropriate reaction in which "self" proteins or "self" nucleic acids are recognized will lead to a variety of immune dysfunctions including autoimmune disorders.

Discrimination occurs at several levels including sequestration of receptors in compartments or surfaces where self-ligands can be excluded (e.g., DNA-binding proteins like Tlr9 and RNA-binding proteins like Tlr3 are localized in

BOX 3.2 BACKGROUND

Toll receptors: pattern recognition of invaders

- The Toll signaling pathway was defined initially as being essential for the establishment of the dorsal-ventral axis in *Drosophila* embryos. Eric Wieschaus and Christiane Nüsslein-Volhard discovered the first Toll mutants. When Wieschaus showed the unusual mutant *Drosophila* embryos to Nüsslein-Volhard, she exclaimed, "Toll!" (a German slang term comparable to "far out" or "awesome").
- Toll signaling also initiates the response of larval and adult *Drosophila* to microbial infections.
- Toll-like receptors bind to a variety of microbe-specific components and trigger a defensive reaction in both flies and mammals via signal transduction pathways and activation of new gene expression.
- Insect Toll receptors are activated by an endogenous protein ligand produced indirectly by exposure to microbes. Vertebrate Toll receptors bind microbial ligands directly.

Anderson, K. V. 2000. Toll signaling pathways in the innate immune response. *Curr. Opin. Immunol.* **12:**13–19.

Gay, N., and M. Gangloff. 2007. Structure and function of Toll receptors and their ligands. *Annu. Rev. Biochem.* **76**:141–165.

Mushegian, A., and R. Medzhitov. 2001. Evolutionary perspective on innate immune recognition. *J. Cell Biol.* **155:**705–710.

Table 3.3 Intracellular detectors of viral infection[a]

Receptor	Cellular compartment	Ligand(s) detected	Virus infection(s) detected
RigI	Cytoplasm	Double-stranded RNA; single-stranded RNA with 5′ phosphate	Influenza virus
Mda5	Cytoplasm	Double-stranded RNA	Encephalomyocarditis virus, measles virus
Tlr2	Plasma and endosomal membranes	Measles virus HA protein	Human cytomegalovirus
Tlr4	Plasma and endosomal membranes	Mouse mammary tumor virus envelope protein	Respiratory syncytial virus
Tlr3	Plasma and endosomal membranes	Double-stranded RNA	Murine cytomegalovirus, reovirus, West Nile virus
Tlr 7 and Tlr8	Plasma and endosomal membranes	Single-stranded RNA	Human immunodeficiency virus, influenza virus
Tlr9	Plasma and endosomal membranes	Double-stranded DNA; synthetic, unmethylated CpG DNA	Herpes simplex virus types 1 and 2

[a]Data from T. Saito and M. Gale, Jr., *Curr. Opin. Immunol.* **19:**17–23, 2007; G. Trinchieri and A. Sher, *Nat. Rev. Immunol.* **7:**179–190, 2007; and N. Gay and M. Gangloff, *Annu. Rev. Biochem.* **76:**141–165, 2007.

endosomes). The RigI and Mda5 RNA helicases recognize chemical modifications on viral RNA that do not appear on cellular RNA. These modifications include 5′ phosphates as well as stem-loop structures (e.g., IRES elements). Similarly, Tlr3 recognizes double-stranded RNA(dsRNA), but appears to signal only when unmethylated nucleic acid is bound. Our current understanding of this high-fidelity molecular discrimination by the pattern recognition receptors is rudimentary.

Finally, the simple fact is that every virus existing today survives in the face of host defenses. Clearly, viral gene products can bypass or modulate the intracellular detectors of infection to the extent that the viral genome can be maintained in a host population. As will be discussed below, a common strategy is to confound the signal transduction pathways activated by nucleic acid receptors or Tlrs.

Cytokines, the Primary Output of Intrinsic Cell Defense

Cytokine production is the primary response when a single cell detects a foreign nucleic acid, or when a Tlr is occupied. In turn, locally released cytokines will activate a more global innate immune response should viral replication continue unabated. The presence of cytokines in the blood is one of the first indications that a host has been infected and that immune defenses have been activated. In general, the cytokines can be divided into three functional groups: the **proinflammatory cytokines**, the **anti-inflammatory cytokines**, and the **chemokines** (Table 3.5). Cytokines are potent molecules, capable of inducing a response at nanogram-per-milliliter concentrations. More than 80 cytokines are known, and the list is growing. Some of the more important in antiviral defense are listed in Tables 3.6 and 3.7.

Infected cells produce and secrete cytokines after stimulation by a variety of initiating cues (Fig. 3.2 and 3.3). All stimulated pathways converge, often by means of Nf-κb activation, to the same regulatory regions of cytokine genes to promote their transcription (Fig. 3.4). In general, several overlapping positive and negative regulatory sequences reside upstream of cytokine genes. In unstimulated cells, inhibitory proteins bind and repress transcription. In infected cells, proteins that increase transcription replace these repressors. Secreted cytokines engage receptors on sentinel dendritic cells, macrophages, and adjacent uninfected cells, which then synthesize a new burst of cytokines, amplifying the initial response. The first cytokines to appear in high concentrations are IFN-α and IFN-β, followed by tumor necrosis factor alpha (Tnf-α), interleukin-6 (IL-6), IL-12, and IFN-γ.

Cytokines initially function locally in antiviral defense. When they are produced in large quantities and enter the circulation, they also evoke global responses that are both essential and familiar to all who experience viral infections (Fig. 3.5). For example, they act directly on cells of the nervous system to produce many of the characteristic behaviors and responses exhibited by infected individuals, including sleepiness (somnolence), lethargy, muscle pain (myalgia), appetite suppression, and nausea. Proinflammatory cytokines (Table 3.5) stimulate the liver to synthesize characteristic **acute-phase proteins**, many of which are required to repair tissue damage and to clear the infection. Members of the colony-stimulating factor class of cytokines, which are made in the bone marrow after an inflammatory response, control the growth and maturation of lymphocytes and other cells essential in antiviral defense.

The adage we quoted earlier, "what happens early dictates what happens late in infection," is based on the long-term effects of the immediate cytokine response. The

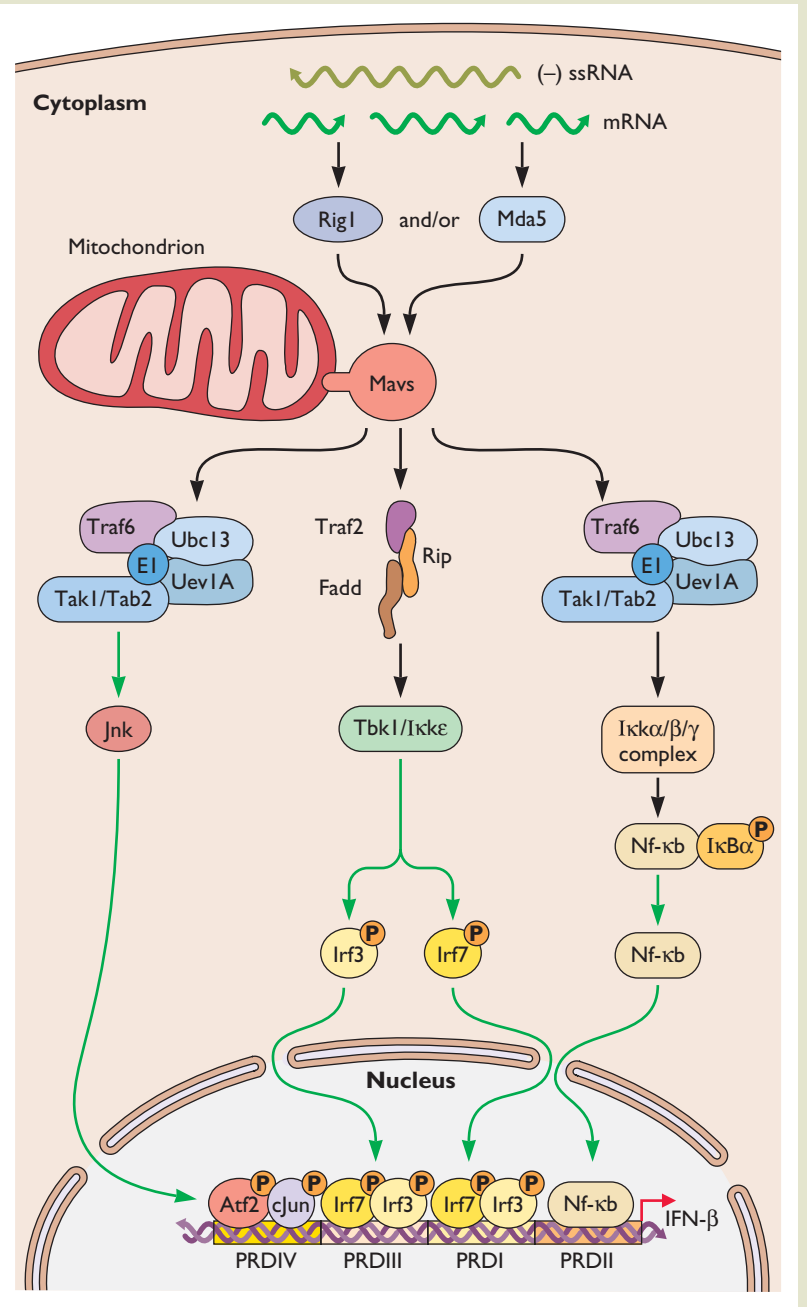
BOX 3.3 DISCUSSION

Detecting viral invaders

A fundamental problem solved over eons of evolution is the detection by individual cells of invading viral RNA or DNA. How an invading nucleic acid is distinguished from cellular RNA and DNA remained an enigma for decades. However, in the last few years, scientists have found a veritable treasure trove of powerful systems that detect alien nucleic acid and activate cellular alarm systems like the IFN response. The Toll-like receptors (Tlrs) are one class of frontline microbial sensors that bind to a variety of unique microbial products, while retinoic acid-inducible protein I (RigI) and melanoma differentiation-associated protein (Mda5) represent another class that recognizes RNA in the cytoplasm. RigI and Mda5 are RNA helicases of the DEXD/H box family. A distinguishing property of these two helicases is their tandem caspase activation and recruitment domains (CARDs). These modular domains interact with other CARD-containing proteins during the apoptotic response. After binding their ligand, RigI and Mda5 signal using a unique interaction of their CARD domains with an adapter protein. This adapter is an outer mitochondrial membrane protein (Mav) and, when bound to the CARD domain of either RigI or Mda5, activates Irf3 and Nf-κb by the pathways shown. Mav binds to Traf6 and induces its polyubiquitination by E1 ligase and Ubc12/Uve1A. In turn, Tak1 kinase and Tab2 adapter protein bind and phosphorylate Jnk kinases and the Iκκ complex. The common adaptor Mav integrates two different interactions with a common output. The coordination of these three signal transduction pathways leads to the assembly of a multiprotein enhancer complex in the nucleus, which drives expression of the IFN-β gene.

A crucial question is how RigI and Mda5 can distinguish viral RNA from cellular RNA. It was clear for some time that the two receptors had different specificities and actions *in vivo*. Use of mice that lacked either of these two RNA detectors showed that RigI is required for the *in vivo* response to paramyxoviruses and flaviviruses, as well as influenza virus. The Mda5 detector seems to be essential for the antiviral response to encephalomyocarditis virus and measles virus.

In 2006, papers by Hornung et al. and Pichlmair et al. provided new insight into how viral RNA can be distinguished from cellular RNA. It was widely held that discrimination was achieved by recognition of dsRNA produced during viral replication or by unique secondary structures. These two papers provided evidence that RigI binds to RNAs with a 5′ phosphate group, in hindsight an obvious discriminator. Unlike cellular mRNAs that have 5′ cap structures, viral RNAs are often uncapped and carry a 5′ triphosphate group. While certainly an important



finding, the 5′ phosphate recognition must be just the tip of the iceberg for discrimination. Some anomalies are apparent. Picornaviral RNAs do not have cap structures or triphosphates on their 5′ ends, yet infection can be detected by Mda5 (but not RigI). Similarly, the abundant human 7SL RNA has a 5′ triphosphate group yet does not activate an interferon response. Other aspects of RNA structure are certain to be involved.

Hornung, V. J. Ellegast, S. Kim, K. Brzózka, A. Jung, H. Kato, H. Poeck, S. Akira, K. Conzelmann, M. Schlee, S. Endres, and G. Hartmann. 2006. 5′-Triphosphate RNA is the ligand for RIG-I. *Science* **314:**994–997.

McWhirter, S., B. tenOever, and T. Maniatis. 2005. Connecting mitochondria and innate immunity. *Cell* **122:**645–658.

Pichlmair, A., O. Schulz, C. Tan, T. Näslund, P. Liljeström, F. Weber, and C. Sousa. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. *Science* **314:**997–1001.

Saito, T., and M. Gale, Jr. 2007. Principles of intracellular viral recognition. *Curr. Opin. Immunol.* **19:**17–23.

Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizume, M. Miyagishi, K. Taira, S. Akira, and T. Fujita. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA induced innate antiviral response. *Nat. Immunol* **5:**730–737.

constellation and concentration of each cytokine constitute a major means of communication between cells, and the innate and adaptive arms of the immune system. Indeed, cytokines participate in almost every phase of the host response to viral infection, including control of inflammation, induction of an antiviral state in uninfected cells, and regulation of immune responses.

Many viral gene products can mimic or modulate cytokine responses. These proteins have been called **virokines** if they mimic host cytokines, or **viroceptors** if they mimic host cytokine receptors. The arsenal includes remarkable proteins such as soluble IL-1 receptors, a variety of chemokine antagonists, and functional homologs of IL-10 and IL-17. Viral DNA genomes encode most of the well-known virokines and viroceptors, but viral RNA genomes contain some surprises. For example, the envelope protein of respiratory syncytial virus is a mimic of fractalkine, the only known chemokine that is a membrane protein. The viral envelope protein competes with fractalkine binding to its receptor, which also functions as a viral receptor.

Interferons, Cytokines of Early Warning and Action

The **interferons**, cytokines synthesized by mammals, birds, reptiles, and fish, are critical signaling proteins of the host frontline defense (Box 3.5). The discovery of interferon was first reported almost simultaneously in the 1950s by two groups of investigators. One group observed that chicken cells exposed to inactivated influenza virus contained a substance that interfered with the infection of other chicken cells by live influenza virus. The second group made their discovery using vaccinia virus infections. We now know that most cells synthesize new interferon when infected, and the released interferon inhibits replication of a wide spectrum of viruses. The broad spectrum of interferon action was a puzzle that was not resolved for more than 50 years.

There are three classes of interferons (Table 3.8). In following sections, we use the abbreviation IFN to mean both IFN-α and IFN-β (also called type I interferons). We will refer specifically to IFN-γ, which is induced only when certain lymphocytes are stimulated to replicate and divide after binding a foreign protein. In contrast, IFN-α and IFN-β are induced directly by viral infection of almost any cell type.

IFN Is Made by Infected Cells and by Immature Dendritic Cells

Virus-infected cells produce IFN when foreign nucleic acid is detected by RigI or Mda5 RNA helicase receptors, or by Tlr receptors (Fig. 3.2; Box 3.4). Other signals unique to viral infection can also lead to IFN production. For example, structural proteins of some viruses stimulate IFN synthesis upon binding of virions to cells. In other cases, virus-induced degradation of the inhibitor of Nf-κb (Iκbα) leads to activation of transcription of IFN genes (Fig. 3.4).

Virus-infected cells invariably produce IFN, but the uninfected macrophages and dendritic cells that patrol tissues, (sentinel cells; see Chapter 4) also make this cytokine when their Tlrs bind products released from infected cells. Such products include viral proteins, viral nucleic acids, and cellular stress proteins (e.g., heat shock proteins). The sentinel cell response, which is essential for amplification of the subsequent immune response, is discussed in the next chapter. If the infection is not contained and spreads to more cells, large quantities of IFN may be synthesized by specialized dendritic cell precursors in the blood called plasmacytoid cells. Such systemic production of IFN leads to many of the general symptoms of viral infection.

Production of IFN by infected cells and uninfected, immature dendritic cells at the site of infection is rapid, but transient; it occurs within hours of infection and declines in less than 10 h. Regulation of IFN synthesis

BOX 3.4 DISCUSSION

Pattern recognition receptors: the Toll-like receptors

Toll-like receptors (Tlrs) are the prototypical pattern recognition molecules. They are synthesized predominately by the sentinel cells (dendritic cells and macrophages), but can be found on other cells. Toll-like receptors are type I transmembrane proteins that are conserved from insects to humans. At least 12 members of this receptor family have been identified in mammals. Their ligands have been difficult to identify, because they are structurally diverse and vary among pathogens. However, some ligand-receptor pairs are known (Table 3.10). Ligands that might identify viral infections include CpG-containing DNA, dsRNA, and ssRNA. Unmethylated CpG tracts are present in bacterial and most viral DNA genomes, while dsRNA and ssRNA are commonly found in virus-infected cells.

After binding their unusual ligands, these Toll-like receptors, like many cytokine receptors, aggregate in the membrane, an event that stimulates binding of adapter proteins. Many downstream signaling steps in pattern recognition and inflammation are mediated by common components. For example, when ligands bind Tlrs, the IL-1 receptor-associated kinase (Irak) binds adapters such as Myd88, which engage the Tlr cytoplasmic domains to initiate signal transduction through Traf6 and then to protein kinase cascades to activate NF-κB. With the exception of Tlr3, all Tlrs engage Myd88. As indicated in the figure, the Tir domains of the Tklr and Myd88 adapter are important interfaces for complex formation. The DD domains of Myd88 and Irak act in a similar fashion. These complexes then can engage the Nf-κb, Erk, Jnk, and p38 mitogen-activated protein kinase signal transduction pathways to activate transcription of genes encoding inflammatory cytokines, IFN, and T-cell costimulatory molecules. The p38 pathway can lead to stabilization of short-lived mRNA and increased production of various cytokines. Short-lived mRNAs often have AU-rich pentameric elements (AREs) in their 3′ untranslated regions.

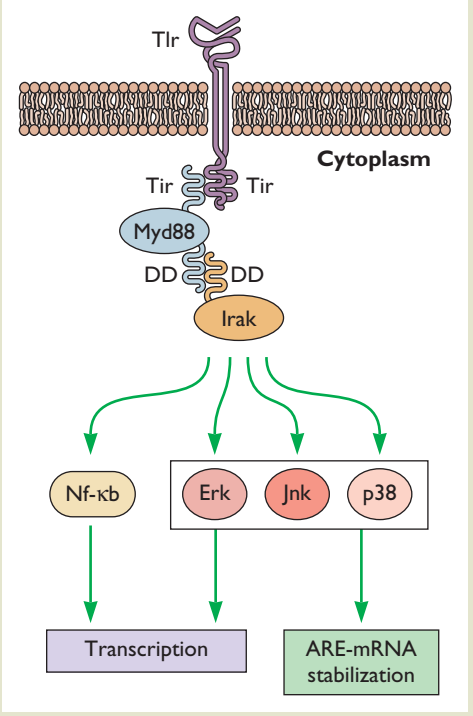
Tlrs can recognize extracellular as well as intracellular microbial ligands. Endocytosed proteins and virus particles end up in dendritic cell lysosomal compartments, where they can be digested. Some Tlrs, including Tlr3 and Tlr9, are located in endosomes and lysosomes, perfectly placed to bind these unusual viral products.

Tlrs are critical players in antiviral defense. Respiratory syncytial virus persists longer in the lungs of infected *tlr4*-null mice than in wild-type mice. NK cell responses and IL-12 synthesis are also reduced after challenge of these mice with this virus. One interpretation of these observations is that the Tlr4 protein is important for recognition of the infection and production of an antiviral response. An alternative idea is that virus propagation is dependent on signaling from this receptor in the dendritic cell. Other clues concerning the contribution of Tlrs to viral pathogenesis come from the study of viral proteins with the potential to disrupt their functions. Two vaccinia virus proteins, A46R and A52R, are similar in sequence to segments in the cytoplasmic domain of Tlrs and IL-1 receptors. These two viral proteins can inhibit IL-1- and Tlr4-mediated signal transduction, respectively. Vaccinia virus may modulate host immune responses by competing with this domain-dependent intracellular signaling.

Meylan, E., J. Tschopp, and M. Karin. 2006. Intracellular pattern recognition receptors in the host response. *Nature* **442:**39–44.

Saito, T., and M. Gale, Jr. 2007. Principles of intracellular viral recognition. *Curr. Opin. Immunol.* **19:**17–23.

Sioud, M. 2006. Innate sensing of self and non-self RNAs by Toll-like receptors. *Trends Mol. Med.* **12:**167–176.



and secretion is complex, and much remains to be discovered. For example, we know that many *IFN-α* genes are differentially expressed after infection (Box 3.6). In addition, transcription of the human *IFN-β* gene is stimulated by infection, but only for a short period. The *IFN-β* enhancer possesses several remarkable properties that allow precise temporal control of transcription (Box 3.7). The biological significance of such a mix of IFN-α and IFN-β is unclear. Moreover, the quantity of IFN released from cells infected by different isolates of a given virus is astonishingly

Table 3.4 Toll-like receptors recognize microbial macromolecular patterns[a]

Toll-like receptor	Pattern recognized
Tlr1	Triacyl lipoproteins
Tlr2	Lipoproteins, viral glycoproteins; gram-positive peptidoglycan
Tlr3	Double-stranded RNA
Tlr4	Lipopolysaccharide, viral glycoproteins
Tlr5	Flagellin
Tlr6	Diacyl lipoproteins
Tlr7	Single-stranded RNA
Tlr8	Single-stranded RNA
Tlr9	CpG DNA; unmethylated CpG oligonucleotides
Tlr10	Unknown
Tlr11	Profilin

[a]Data from G. Barton and R. Medzhitov, *Curr. Opin. Biol.* **14:**380–383, 2002; S. Akira and K. Takeda, *Nat. Rev. Immunol.* **4:**499–511, 2004.

variable. In the case of vesicular stomatitis virus infection, the released IFN concentration can vary over a 10,000-fold range, depending on the serotype of the infecting virus. As discussed later, many viral proteins affect the quantity of IFN, as well as its action.

IFN Affects Only Cells with IFN Receptors

IFN functions only when it occupies its receptor on the surfaces of cells. A cell without IFN receptors may synthesize IFN, but cannot be affected by this cytokine. Binding of IFN to its receptor initiates a signal transduction cascade that culminates in increased transcription of many genes. A simplified outline of this signaling pathway is shown in Fig. 3.6.

The Jak/Stat pathway contains proteins that respond to binding to appropriate receptors of not only IFN, but also IL-6 and other cytokines. There are four known Jak kinases and seven structurally and functionally related *stat* genes. Their targeted disruption in mice has revealed much about their functions. For example, a mouse in which the *stat1* gene has been inactivated has no innate response to viral or bacterial infection, whereas deletion of *stat4* and *stat6* abrogates specific functions of the adaptive response. *stat* gene homologs are encoded in the genomes of *Drosophila melanogaster* and *Dictyostelium discoideum*, underscoring the ancient evolutionary origin of this pathway. Signaling via Jak/Stat activates transcription dependent on specific DNA sequences (Fig. 3.6). These sequences are found in the promoters of the more than 300 IFN-activated genes. Regulation is also exerted through suppressors of cytokine signaling proteins, phosphatases, and proteases.

IFN-γ Signaling

Unlike the common signaling cascades induced by IFN-α/β, IFN-γ signaling is mediated by several pathways (Box 3.8). The best-understood pathway leads to formation of the transcriptional activator called gamma-activated factor (Gaf), which binds to specific promoter sequences of IFN-γ-activated genes. While cross talk between pathways does occur, the results of such mixed signals are neither predictable nor understood (even in principle). In the milieu of an infected tissue, multiple cytokines are produced and many signaling pathways are operating in both infected and uninfected cells. In theory, the orchestration of these multiple signaling pathways enables the whole (control of infection and appropriate host response) to be greater than the sum of its parts. How the integrated output is achieved remains a mystery.

IFN Action Produces an Antiviral State

As the name so aptly indicates, IFN interferes with the replication of a wide variety of viruses in cultured cells and animals. Shortly after infection, newly made IFN released from infected cells and local immature dendritic cells can be found circulating in the body, but its concentration is highest at the site of infection, where it is bound by any cell with the appropriate receptor. Cells that bind and respond to IFN are unable to support propagation of many different viruses; they are said to be in an **antiviral state**.

What does IFN do to a cell to make it inhospitable for the replication of almost any virus? We now know that IFNs can induce the synthesis of more than 300 cellular proteins, but their mix and concentrations vary according to cell type and specific IFN. Which subset of the hundreds of IFN-inducible proteins establishes the antiviral state in any given cell remains an open question. It is possible that many antiviral constellations exist, depending on the cell type, virus, and cocktail of IFN and other cytokines sensed by that cell. Many of the products of IFN-inducible genes possess potent broad-spectrum antiviral activities, but the relevant molecular mechanisms of only a few are understood. IFN not only induces death of the infected cells, but also ensures that uninfected cells in the vicinity are induced to kill themselves should they become infected. Such a local cauterizing response has led some to characterize IFN action as a **firebreak** to infection.

The IFN-induced proteins are functionally diverse and participate in signal transduction, chemokine action, antigen presentation, regulation of transcription, the stress response, and control of apoptosis. Some of these proteins are induced by other stimuli, including dsRNA, bacterial lipopolysaccharides, Tnf-α, or IL-1.

Because IFN induces the synthesis of many deleterious gene products and is potentially lethal to any cell that has

Table 3.5 Three primary classes of cytokines[a]

Functional group	Selected members	Activity
Proinflammatory	IL-1, Tnf, IL-6, IL-12	Promote leukocyte activation
Anti-inflammatory	IL-10, IL-4, Tgf-β[b]	Suppress activity of proinflammatory cytokines; return system to basal "circulate and wait" state
Chemokines	IL-8	Recruit immune cells during early stages of immune response

[a]The terms "lymphokines" and "monokines" were originally used to denote secreted proteins produced by activated lymphocytes or monocytes, respectively. Similarly, the term "interleukin" identified proteins such as IL-2 that communicated signals between different populations of white blood cells and other nonhematopoietic cells.

[b]Tgf-β, transforming growth factor β.

specific receptors (most cells in our bodies), the production of large quantities of IFN in an infected individual has dramatic physiological effects. These responses include such common symptoms as fever, chills, nausea, and malaise. All infections lead to IFN production, one reason why these "flu-like" symptoms are so common.

Soon after its discovery, IFN was touted as a broad-spectrum antiviral drug. However, as was quickly discovered, the side effects often are worse than the infection. Nevertheless, IFN has been effective in the treatment of some persistent infections, particularly those caused by hepatitis B and C viruses.

Table 3.6 Some cytokines that function in the immune response to viral infection

Cytokine	Source	Target or action[a]
IFN-α/β	Immature dendritic cells; many types	Induces antiviral state; inhibits cell proliferation; stimulates growth and cytolytic function of NK cells; increases expression of MHC class I and decreases expression of MHC class II molecules
IFN-γ	T cell, NK cell	Activates macrophages; promotes adhesion of Th cells to vascular endothelium; inhibits IL-6 effects; induces antiviral state
Tnf-α	T cell, macrophage	Activates neutrophils; induces inflammatory response and fever, and initiates catabolism of muscle and fat (cachexia); induces adhesion molecules on vascular endothelial cells; potentiates lysis of some virus infected cells
Gm-Csf	T cell, macrophage, fibroblast, endothelial cell	Induces myelomonocytic cell growth and differentiation; important maturation protein for dendritic cells
Mip-1α	Macrophage, T cell, B cell, neutrophil, Langerhans' cell	Chemoattractant for T and B cells, monocytes, and eosinophils
Mip-1β	T cell, macrophage, B cell	Chemoattractant for T cells and monocytes
IL-1	Macrophage, T cell, B cell, epithelial cell	Costimulator of T cells; initiates inflammatory T-cell and B-cell response; affects brain to produce fever; causes metabolic wasting (cachexia); induces acute phase protein synthesis in the liver
IL-2	T cell	Induces proliferation of T cells; stimulates growth and cytolytic function of NK cells; induces antibody synthesis in B cells
IL-4	Mast cell, bone marrow	Induces B-cell proliferation and differentiation; required for production of IgE; inhibits macrophage function/blocks effects of IFN-γ on macrophages; stimulates growth and differentiation of Th2 cells; stimulates synthesis of adhesion molecules on vascular endothelium
IL-6	Macrophage, T cells, vascular endothelial cell, fibroblast	Made in response to IL-1 and Tnf; stimulates B-cell growth and costimulates T cells; stimulates hepatocytes to synthesize acute-phase proteins
IL-10	T cell, B cell	Promotes growth and differentiation of B cells; inhibits macrophage function
IL-12	Macrophage, monocyte	Potent stimulator of NK cell growth and killing activity; promotes differentiation of Th cells to Th1 subset; stimulates differentiation of immature $CD8^+$ T cells to functionally active CTLs; stimulates expansion and activation of autoreactive $CD4^+$ T cells

[a]MHC, major histocompatibility complex; IgE, immunoglobulin E; CTLs, cytotoxic T lymphocytes.

Table 3.7 Some chemokine receptors and their ligands[a]

Receptor[b]	Chemokine ligand
CCr1	Mip1α, Rantes
CCr2	Mcp1, Mcp3, Mcp4
CCr5	Mip1α, Mip1β
CCr6	Mip3α
CCr7	Mip3β
CXCr1	IL-8
CXCr2	IL-8
CXCr3	Ip-10

[a]Information in this table taken from C. R. Mackay, *Curr. Biol.* **7:**R384–R386, 1997.

[b]The four families of chemokine receptors are distinguished by the pattern of cysteine residues near the amino terminus and are abbreviated CXC, CC, C, and CX3C. Only two types are listed in this table. The CXC family has an amino acid between two cysteines; the CC family has none; the C family has only one cysteine; and the CX3C family has three amino acids between two cysteines. Subfamilies of these major four groups also exist.

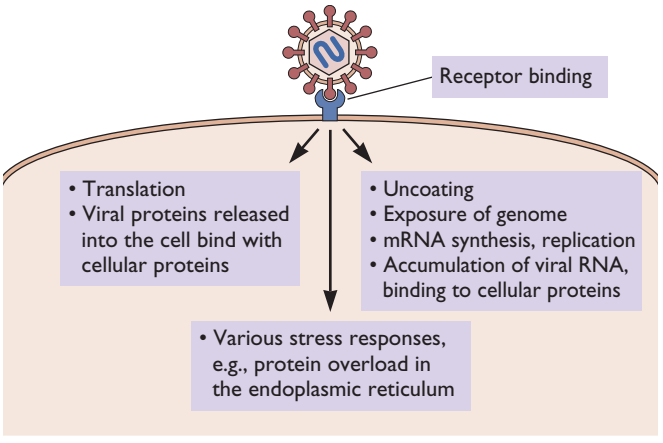
Selected IFN-Induced Gene Products and Their Antiviral Actions

dsRNA-activated protein kinase. Often viral and cellular protein synthesis in infected cells stops abruptly. In many cases, this lethal defense is mediated by a cellular dsRNA-activated protein kinase (Pkr) (also described in Volume I, Chapter 11). Establishment of the Pkr-mediated antiviral state is a two-step process, in which IFN promotes the increased production and accumulation of an inactive protein that subsequently can become activated only when the cell is infected.

All mammalian cells contain low concentrations of inactive Pkr, a serine/threonine kinase with both antiviral and antiproliferative and antitumor activities. The signal transduction cascade initiated by IFN binding to its receptor leads to a dramatic increase in the concentration of inactive Pkr. If the cell is infected, this enzyme can be activated by binding viral dsRNA. The enzyme then phosphorylates the alpha subunit of the eIF2 translation initiation protein (eIF2α), effectively rendering it incapable of supporting protein synthesis in the cell (see Volume I, Chapter 11). Phosphorylated eIF2α also can trigger autophagy, an intrinsic cell defense, discussed below).

Many viral genomes encode proteins that can block the lethal actions of Pkr (Table 3.9), but our understanding of the biological significance of this phenomenon is incomplete. An exception is the herpes simplex virus type 1 ICP34.5 protein, which redirects the cellular protein phosphatase 1 to dephosphorylate eIF2α, after it has been inactivated by Pkr (Box 3.9). While wild-type virus is fully virulent in mice, ICP34.5-null mutants are markedly attenuated, particularly in brain infections. Significantly, this mutant regains wild-type virulence in mice lacking the *pkr* gene. This experiment provides convincing evidence that Pkr mediates defense against herpes simplex virus infection in mice.

Figure 3.3 Processes inherent in viral infection that activate cellular defenses, signal transduction, and host gene expression.



RNase L and 2′-5′-oligo(A) synthetase. Another well-studied antiviral system induced by IFN comprises two enzymes and dsRNA. Ribonuclease L (RNase L) is a nuclease that can degrade most cellular and viral RNA species. Its concentration increases 10- to 1,000-fold after IFN treatment, but the protein remains inactive unless a second enzyme is synthesized. This enzyme, 2′-5′-oligo(A) synthetase, makes oligomers of adenylic acid, but only when activated by dsRNA. These unusual nucleotide oligomers then activate RNase L, which in turn begins to degrade all host and viral mRNA. We now know from studies of mouse mutants defective in RNase L that this enzyme is important for the IFN-β response to viral infection. The products of RNase L cleavage amplify the production of IFN-β.

Mx proteins. Unlike the broad-spectrum antiviral effects of Pkr and RNase L, at least one IFN-induced mouse protein and two related human proteins appear to be directed against specific viruses. Mouse strains that have an IFN-inducible gene called *mx1* are completely resistant to influenza virus infection. The Mx1 protein is part of a small family of IFN-inducible guanosine triphosphatase (GTPases) with potent activities against various (–) strand RNA viruses. After IFN induction, this protein accumulates in the nucleus and inhibits the unusual influenza virus "cap-snatching" mechanism (Volume I, Chapter 6). It is likely that the Mx1 protein interferes with the function of the viral polymerase subunit PB2, for overproduction of this viral protein overcomes the antiviral effect of Mx1 protein. The significance of the *mx1* gene in the biology of

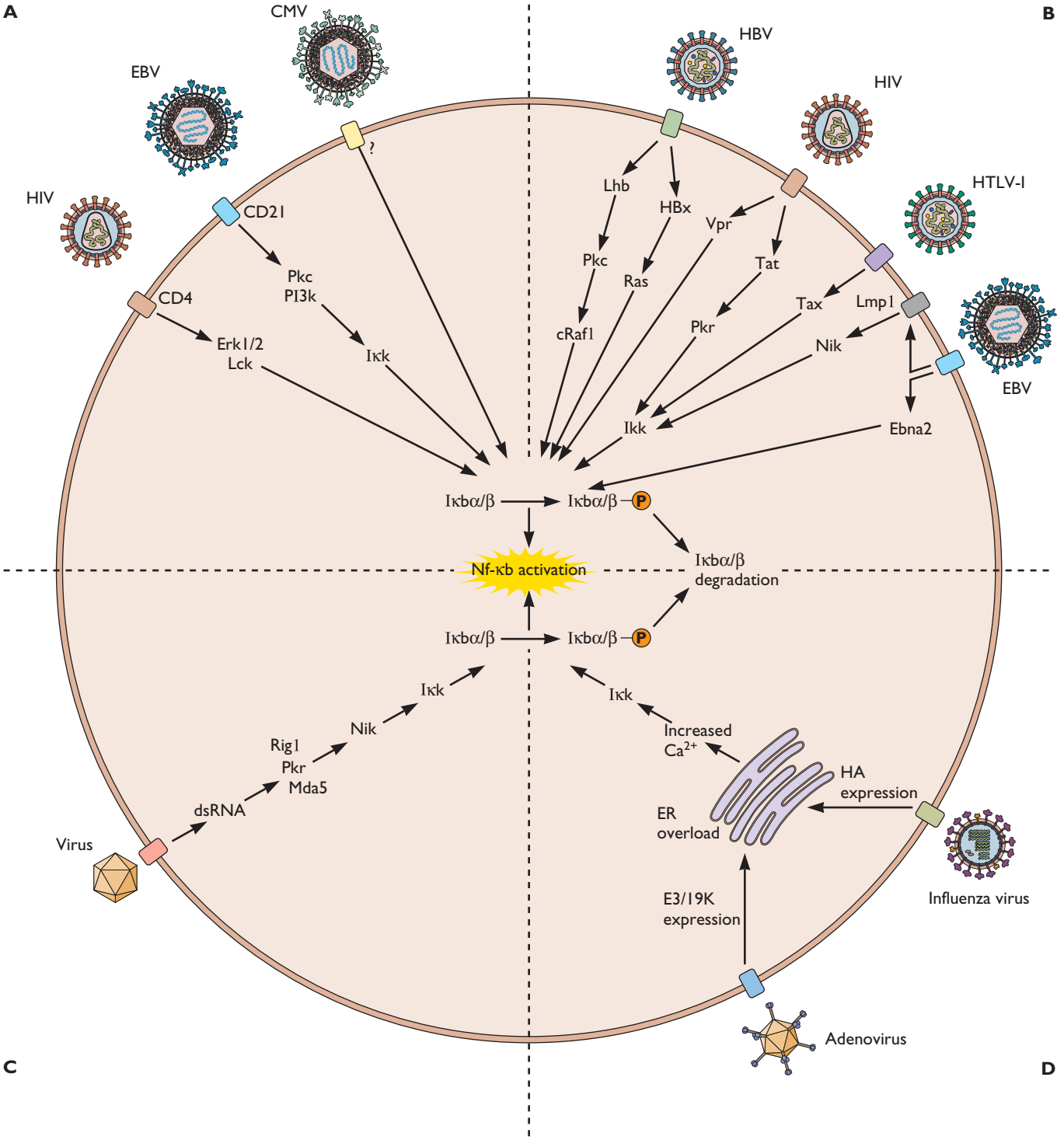


Figure 3.4 Activation of the transcription regulator Nf-κb by viral infection. Nf-κb is an important transcription control protein in the response to viral infection. Four diverse mechanisms that result in activation of Nf-κb, corresponding to the pathways described in Fig. 3.3, are illustrated. **(A)** Signal transduction pathways are activated on binding of a virus particle to its receptor; **(B)** viral proteins synthesized in the infected cell directly engage signal transduction pathways that culminate in Nf-κb activation; **(C)** Pkr binds double-stranded viral RNA, or RigI/Mda5 bind single-stranded viral RNA, leading to activation of Nf-κb; **(D)** overproduction of viral proteins in the endoplasmic reticulum (ER) leads to calcium release which, in turn, activates Nf-κb.

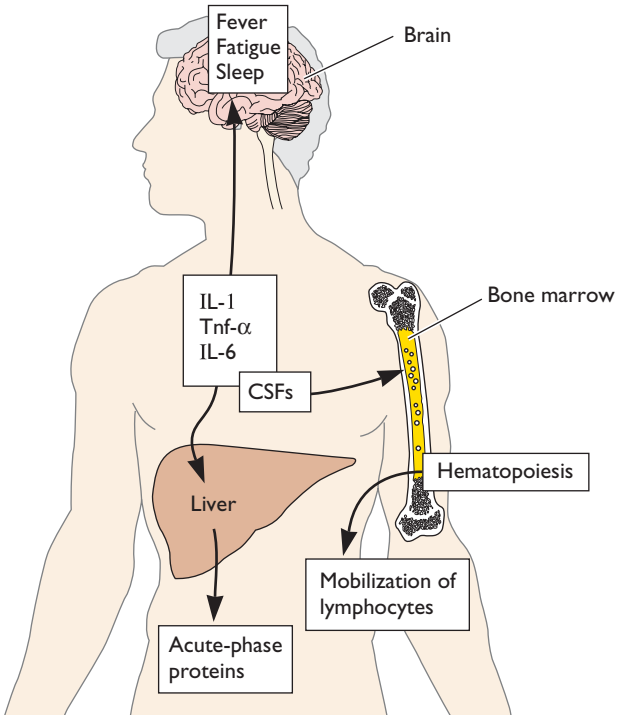


Figure 3.5 Systemic effects of cytokines in inflammation. A localized viral infection often produces global effects, including fever and lethargy, lymphocyte mobilization (swollen glands), and appearance of new proteins in the blood. The proinflammatory cytokines IL-1, IL-6, and Tnf all act on the brain (particularly the hypothalamus) to produce a variety of effects, including these typical responses to viral infection. These cytokines also act in the liver to cause the release of iron, zinc, and acute-phase proteins including mannose-binding protein, fibrinogen, C-reactive protein, and serum amyloid protein. Acute-phase proteins have innate immune defense capabilities: e.g., C-reactive protein binds phosphorylcholine on microbial surfaces and activates complement. The colony-stimulating factors (CSFs) activated by an inflammatory response have long-range effects in the bone marrow on hematopoiesis and lymphocyte mobilization. Adapted from A. S. Hamblin, *Cytokines and Cytokine Receptors* (IRL Press, Oxford, United Kingdom, 1993), with permission.

influenza virus or of mice is not at all clear, as influenza virus does not circulate among wild mice. Moreover, in these animals, about one-quarter of the population lacks a functional *mx1* gene with no clear consequences.

Two human genes related to the murine *mx1* gene are termed *mxA* and *mxB*. Expression of these genes is also induced by IFN, but unlike the murine protein, the human proteins reside in the cytoplasm. MxA, but not MxB, blocks replication of influenza virus. Interestingly, in contrast to murine Mx1, which inhibits only influenza virus, the human MxA protein also prevents replication of vesicular stomatitis virus, measles virus, and other (−) strand RNA viruses. The Mx proteins are related to members of the dynamin superfamily of GTPases, which regulate endocytosis and vesicle transport, but how this fact relates to their antiviral activities is unknown.

Interferon regulatory proteins. Members of the interferon regulatory (Irf) protein family are required for sustained IFN transcription after induction. Mice lacking the *irf1* gene are incapable of mounting an effective IFN response to viral infection. Other members of this gene family (*irf2* to *irf9*) were discovered because their protein products bound to the ISRE in promoters of IFN-regulated genes. The Irf2 protein is a repressor of transcription and cell growth. Irf4 is synthesized only in T and B cells, and Irf8 is made only in cells of the macrophage lineage. Mice defective for *irf8* gene expression are markedly more susceptible to infection and cannot synthesize proinflammatory cytokines. The protein Irf9 (also known as Isgf3 or p48) is the DNA-binding component of the transcriptional regulator Isgf3 (Fig. 3.6). Several viral Irf-like proteins that block IFN action have been identified (Table 3.9).

Nitric oxide synthase. Nitric oxide synthase is an IFN-γ-inducible protein with important antiviral activities. This enzyme exists as several isoforms, each of which has a distinctive tissue distribution. Nitric oxide synthase produces nitric oxide during the conversion of arginine to citrullene. Nitric oxide exerts a variety of antiviral effects, including inhibition of poxvirus and herpesvirus replication. Nitric oxide made by IFN-γ-activated NK cells accounts for much of their antiviral activity (see "NK cells" below).

Promyelocytic leukemia proteins. The promyelocytic leukemia (Pml) proteins are present in both the nucleoplasm and discrete multiprotein complexes known as nuclear bodies (Pml bodies, ND10 bodies, or PODs [discussed in Volume I, Chapter 9]). These structures are important in the intrinsic cellular response to infection because their components bind foreign DNA that enters the nucleus. Pml and other proteins present in the complexes are thought to exert their antiviral effects by transcriptional repression and nucleosome remodeling. Many viral infections promote disorganization of Pml bodies, in part as a measure to override global repression.

Ubiquitin-proteasome pathway components. The proteasome is a large multisubunit protease that destroys cytoplasmic and nuclear proteins targeted by polyubiquitination for proteolysis. This process is important for the destruction of abnormal or damaged proteins, in the turnover of short-lived regulatory proteins, and in the production of peptides for assembly of MHC-I complexes. All interferons induce transcription of a number of genes that

BOX 3.5 BACKGROUND

The interferon system is crucial for antiviral defense

Many steps in a viral life cycle can be inhibited by interferon, depending on the virus family and cell type. Binding of this cytokine to its receptor leads to increased transcription of more than 300 genes. The proteins so produced can inhibit viral penetration and uncoating, synthesis of viral mRNAs or viral proteins, replication of the viral genome, and assembly and release of progeny virions. More than one of these steps in a virus life cycle can be inhibited, providing a strong cumulative effect.

The contribution of interferon can be demonstrated in animal models in which the interferon response is reduced by treatment with anti-interferon antibodies, or in mice harboring mutations that delete or inactivate interferon genes, interferon receptor genes, genes that regulate the interferon response, or genes that are induced by interferons.

Animals with a defective interferon response exhibit a reduced ability to contain viral infections, and often show an increased incidence of illness or death. When the IFN-α/β response is abrogated, there is a global increase in susceptibility to most viruses. When the IFN-γ response is blocked, viral pathogenesis is modestly affected, at best.

These observations suggest that IFN-α/β is crucial as a general antiviral defense, whereas the IFN-γ response has other roles.

Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilcek, R. M. Zinkernagel, and M. Aguet. 1993. Immune response in mice that lack the interferon-gamma receptor. *Science* **259:**1742–1745.

Ryman, K., W. B. Klimstra, K. Nguyen, C. Biron, and R. Johnston. 2000. Alpha/beta interferon protects adult mice from fatal Sindbis virus infection and is an important determinant of cell and tissue tropism. *J. Virol.* **74:**3366–3378.

Stojdl, D., N. Abraham, S. Knowles, R. Marius, A. Brasey, B. Lichty, E. Brown, N. Sonenberg, and J. C. Bell. 2000. The murine double-stranded RNA-dependent protein kinase Pkr is required for resistance to vesicular stomatitis virus. *J. Virol.* **74:**9580–9585.

Zhou, A., J. Paranjape, S. Der, B. Williams, and R. Silverman. 1999. Interferon action in triply deficient mice reveals the existence of alternative anti-viral pathways. *Virology* **258:**435–440.

encode proteins of the ubiquitin-proteasome pathway. In fact, many interferon-stimulated genes encode ubiquitin ligases. Increased protein degradation may contribute to the antiviral response to some viruses. For example, proteasome inhibitors block the anti-hepatitis B virus action of IFN and IFN-γ. For this virus, activation of the proteasome may be **the** major antiviral effect, because the results of other experiments demonstrate that the Pkr and RNase L systems are completely ineffective. The hepatitis B virus X protein binds to various proteasomal subunits and may modulate protease activity.

Other IFN-induced proteins. The most strongly induced IFN gene, *isg56*, is one member of a gene family encoding the structurally related proteins p54, p56, p58, and p60. The p58 protein interacts with the p48 subunit of the translation initiation protein eIF3 and blocks the initiation of protein synthesis. Expression of intracellular nucleic acid detectors such as RigI and Mda5 is induced after IFN treatment. The expression of other intrinsic defense gene products, such as Trim5α, is increased as well. Other proteins with antiviral effects certainly remain to be discovered among the 300-plus IFN-induced proteins. For example, the IFN response is required to clear human cytomegalovirus infections, but Pkr, Mx, and RNase L proteins are not. Similarly, uncharacterized IFN-induced proteins block penetration and uncoating of simian virus 40 and some retroviruses. Others

Table 3.8 The interferons: antiviral cytokines

Interferon[a]	Producer Cells	Inducers
IFN-α	Most if not all nucleated cells	Viral infection
IFN-β	Most if not all nucleated cells	Viral infection
IFN-γ	T cells, NK cells	T-cell receptor activation, IL-2, IL-12

[a]IFN-α and IFN-β are sometimes called type I interferons. IFN-γ is often called type II or immune interferon. The molecular mass of all IFNs is approximately 20 kDa. Human IFN-α is produced from more than 24 closely related, intronless genes. Human IFN-β is produced from a single intronless gene with about 30 to 45% homology to IFN-α genes. IFN-γ is produced from a single-copy gene with three introns. There is little homology to IFN-α and IFN-β.

BOX 3.6 BACKGROUND

Differential induction of IFN-α *genes by viral infection*

- Most cells produce IFN-α from more than 20 genes, depending on the species.
- *IFN-α* genes are expressed differentially. Different members of this gene family are induced by specific viruses. For example, transcription of *IFN-A4* but not *IFN-A1* is induced by infection of mice with Newcastle disease virus. The function of such mixtures of potent IFNs is not understood and not well studied.
- Differential expression occurs as a result of specific binding of the transcriptional activator proteins Irf3 and Irf7 to particular *IFN-α* promoters.

Pitha, P. M., and W. C. Au. 1995. Induction of interferon-alpha gene expression, p. 151. *In* P. M. Pitha (ed.), *Interferon and Interferon Inducers.* Academic Press, London, United Kingdom.

BOX 3.7

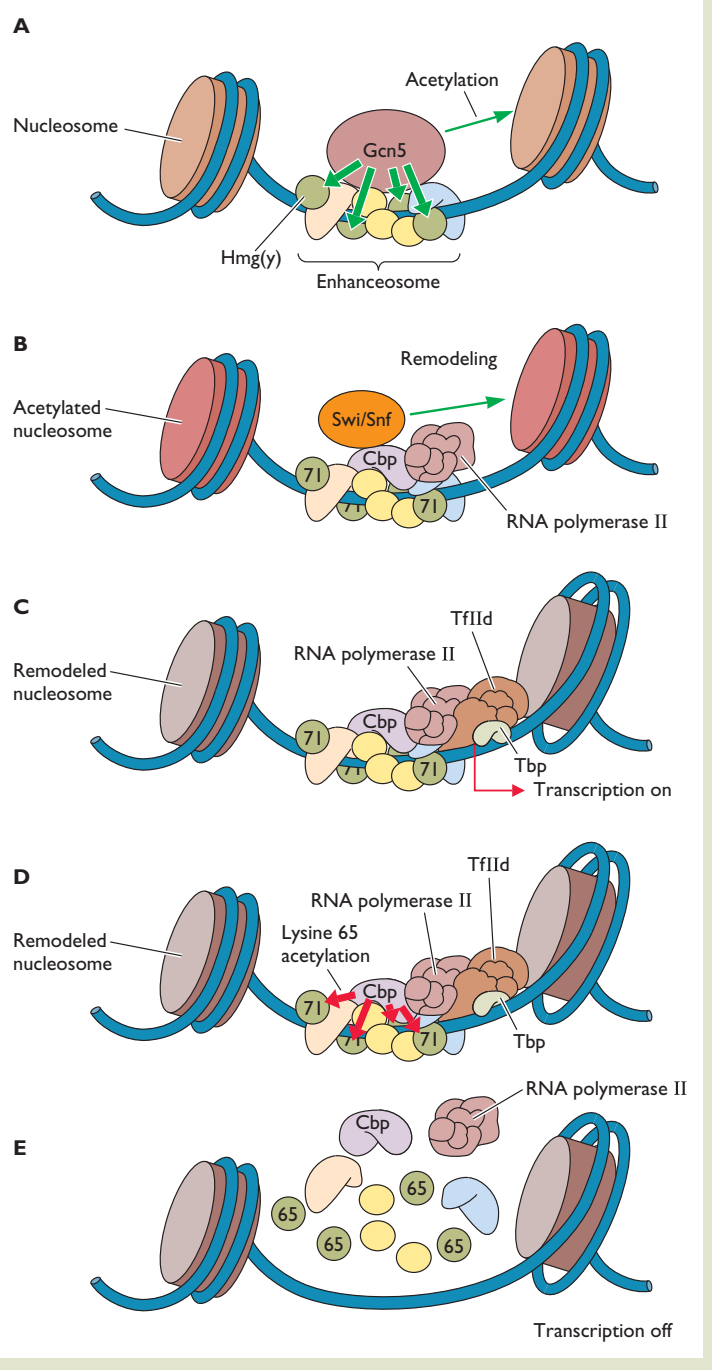
DISCUSSION

Switching IFN-β transcription on and off

Viral infection activates transcription of the human *IFN-β* gene, but only for a short period. This on-off response is controlled by an enhancer located immediately upstream of the core promoter. Like other enhancers, this regulatory sequence contains binding sites for multiple transcriptional activators, including Nf-κb and members of the Ap-1 and Atf families. However, the *IFN-β* enhancer possesses several remarkable properties that allow precise temporal control of transcription.

- The enhancer also contains four binding sites for the architectural protein Hmg(y), which alters DNA conformation to direct the assembly of a precisely organized nucleoprotein complex on the enhancer.
- In contrast to typical modular enhancers, all binding sites **and** their natural arrangement are essential for activation of *IFN-β* transcription specifically in response to viral infection.
- Formation of the complex takes place in stages, and is not complete until several hours after infection.
- Activation of transcription then requires sequential recruitment of the histone acetylase Gcn5, the coactivator Cbp and RNA polymerase II, and the chromatin-remodeling complex Swi/Snf.
- In addition to modifying nucleosomes, Gcn5 acetylates Hmg(A1) at Lys71. This modification stabilizes the complex.
- The Hmg(A1) protein is also acetylated by Cbp at Lys65. However, **this** modification impairs DNA-binding activity and results in disruption of the complex and cessation of *IFN-β* transcription.
- Remarkably, this inhibitory modification by Cbp is blocked for several hours by the prior Gcn5 acetylation of Hmg(A1). The "off" switch is delayed for a sufficient period to allow a burst of *IFN-β* transcription.

Munshi, N., T. Agalioti, S. Lomvardas, M. Merika, G. Chen, and D. Thanos. 2001. Coordination of a transcriptional switch by HMG1(Y) acetylation. *Science* **293:**1133–1136.



(A) Viral infection of human cells leads to assembly of a multiprotein complex on the *IFN-β* enhancer, which lies in a nucleosome-free region of the gene. The signals that direct binding of transcriptional activators (blue, yellow, and tan) and Hmg(A1) (green) are not fully understood. The precisely organized surface of the complex allows binding of Gcn5, which acetylates both histones in nearby nucleosomes and Lys71 of Hmg(A1) (green arrows). This modification stabilizes the enhanceosome. **(B)** A complex of Cbp and RNA polymerase II and the chromatin-remodeling protein Swi/Snf bind sequentially to the stabilized complex. The latter alters the adjacent nucleosome that contains the core promoter DNA (green arrow). **(C)** Such alteration allows binding of TfIId and activation of transcription. Because Lys71 of Hmg(A1) is acetylated, Cbp cannot acetylate Lys65. **(D)** Eventually, Cbp does acetylate Lys65 of Hmg(A1) (red arrows), but how the inhibition induced by Lys71 acetylation is overcome is not yet clear. **(E)** Regardless, Hmg(A1) modification by Cbp disrupts the complex and switches off transcription. Adapted from K. Struhl, *Science* **293:**1054, 2001, with permission.

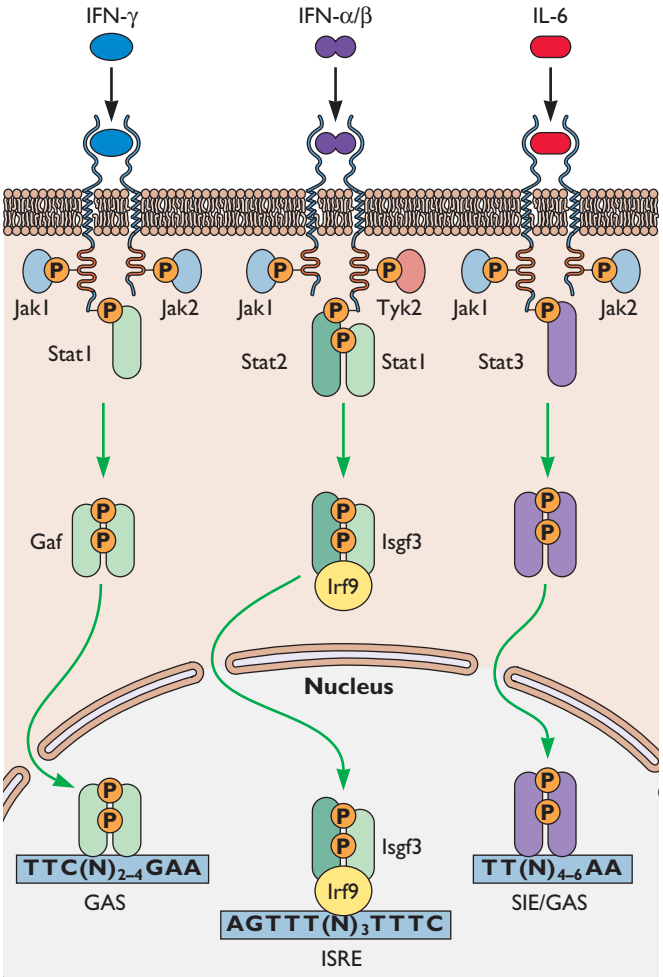


Figure 3.6 Overlapping signal transduction pathways for IFN-α/β, IFN-γ, and IL-6. IFN signals via the Jak/Stat pathway, characterized by a family of tyrosine kinases given the acronym Jak (Janus kinases; Janus, a Roman god, guardian of gates and doorways, is represented with two faces and therefore faces in two directions at once) and a set of transcription proteins named Stat (signal transduction and activators of transcription). The receptors for IFN-α/β, IFN-γ, and IL-6 are different, but all affect components of the Jak/Stat signal transduction pathway. All three interferon proteins and IL-6 bind to their receptors with high affinity (equilibrium dissociation constant [K_d] of about 10^{-10} M). Binding of IFN or IL-6 to the appropriate receptor leads to tyrosine phosphorylation of tyrosine kinases as well as of the receptor itself. These modifications are followed by tyrosine phosphorylation of the Stat proteins. In mammals there are seven Stat genes. The phosphorylated Stat proteins then form a variety of dimers that enter the nucleus. Within that organelle, Stat dimers bind, in some cases in conjunction with other proteins (e.g., Irf9), to specific transcriptional control sequences of IFN-α/β, IFN-γ, and IL-6-inducible genes called interferon-stimulated response elements (ISREs), gamma-activated site (GAS) elements, and Sis-inducible element (SIE), respectively. Later in the transcriptional response to IFN, a second transcriptional activator called Irf1 replaces Isgf3. For further information, see D. S. Aaronson and C. M. Horvath, *Science* **296:**1653–1655, 2002.

impair the maturation, assembly, and release of vesicular stomatitis virus, herpes simplex virus, and some retroviruses by unknown mechanisms.

Cellular micro-RNAs. Micro-RNAs (miRNAs) are single-stranded, noncoding host RNAs of 19 to 25 nucleotides that regulate gene function. While cellular miRNAs are active in the antiviral response in plants and invertebrates, their role in antiviral action in mammals is only now being analyzed. In one study, IFN-β treatment of the human hepatoma cell line Huh7 as well as freshly isolated primary murine hepatocytes, resulted in an induction of numerous cellular miRNAs. Moreover, eight of these miRNAs targeted hepatitis C virus genomic RNA: treatment of infected cells with synthetic miRNAs of the same sequence blocked virus replication. IFN-β treatment also reduces expression of liver-specific miR-122, an RNA known to be essential for hepatitis C virus replication. In another example, host miRNAs play central roles in shutting down human immunodeficiency virus type 1 transcription in blood mononuclear cells from infected donors. However, in other cell types, viral infection suppresses the host miRNAs that normally would repress proviral gene expression. Further work is in progress in many laboratories to determine if IFN induction of miRNAs is a general antiviral response in mammalian cells.

Viral Gene Products That Counter the IFN Response

The term "antiviral state" implies that the IFN response confers complete resistance to infection. However, it is misleading, because infections vary considerably in their sensitivity to the effects of this cytokine. The replication of some viruses, like vesicular stomatitis virus, is so sensitive to IFN that this property is used to titrate the cytokine. Other viruses can be more resistant to IFN. We now know that there are numerous viral mechanisms for confounding IFN production and action (Table 3.9). For example, viral soluble IFN receptors function as decoys, and viral regulatory proteins alter or block IFN-stimulated transcription.

Many viral genomes encode dsRNA-binding proteins that interfere with IFN induction. For example, the reovirus σ3 protein, the multifunctional influenza virus NS1 protein, and the hepatitis B virus core antigen are all well-characterized dsRNA-binding proteins with anti-IFN effects. The vaccinia virus E3L protein and the herpes simplex virus type 1 US11 protein also have dsRNA-binding properties that correlate with inhibition of IFN induction (Box 3.9). Interestingly, adenovirus VA-RNA I acts as a dsRNA decoy and blocks the activation of Pkr by directly binding to the enzyme.

BOX 3.8 BACKGROUND

IFN-γ: a powerful cytokine quite distinct from the type I interferons

- IFN-γ is produced primarily by NK and T cells, which are critical players in the innate and adaptive immune responses, respectively.
- Production of IFN-γ is stimulated by IL-1, IL-2, estrogen, and IFN-γ itself.
- IFN-γ synthesis is suppressed by glucocorticoids and the cytokines transforming growth factor β and IL-10.
- Transcription of *IFN-γ* is regulated by repressors and activators. A combination of positive and negative regulatory sequences in the *IFN-γ* promoter is essential for controlled expression, but the details remain to be elucidated.
- IFN-γ translation is also regulated in a remarkable fashion: a pseudoknot structure in the 5′ end of the mRNA activates Pkr, which, in turn, blocks translation of IFN-γ mRNA.

Ben-Asouli, Y., Y. Banai, Y. Pel-Or, A. Shir, and R. Kaempfer. 2002. Human IFN-γ mRNA autoregulates its translation through a pseudoknot that activates the interferon-inducible protein kinase Pkr. *Cell* **108:**221–232.

Goodbourn, S., L. Didcock, and R. E Randall. 2000. Interferons: cell signaling, immune modulation, anti-viral responses and virus countermeasures. *J. Gen. Virol*. **81:**2341–2364.

Table 3.9 Some viral modulators of the interferon response[a]

Type of modulation	Representative viruses	Viral protein, if known	Mechanism of action
Inhibition of IFN synthesis	Epstein-Barr virus	Bcrf1	IL-10 homolog, inhibits production of IFN-γ
	Vaccinia virus	A18R	Regulates dsRNA production
	Foot-and-mouth disease virus	L	Host protein synthesis block
IFN receptor decoys	Vaccinia virus	B18R	Soluble IFN-α/β decoy receptor
Inhibition of IFN signaling	Adenovirus	E1A	Decreases quantity of Stat1 and P48; blocks Isgf3 formation; interferes with Stat1 and CBP/P300 interactions
	Vaccinia virus	VH1	Viral phosphatase reverses Stat1 activation
	Human papillomavirus 16	E7	Binds p48
	Hepatitis C virus	NS5a	Blocks formation of Isgf3 and Stat dimers
	Nipah virus	V protein	Prevents Stat1 and Stat2 activation and nuclear accumulation
Block function of IFN-induced proteins	Adenovirus	VA-RNA I	Binds dsRNA, blocks Pkr
	Herpes simplex virus type 1	US11	Blocks Pkr activation
		γ34.5	Redirects protein phosphatase 1α to dephosphorylate eIF2α; reverses Pkr action
	Vaccinia virus	E3L	Binds dsRNA and blocks Pkr
		K3L	Pkr pseudosubstrate, decoy
	Human immunodeficiency virus type 1	TAR RNA	Blocks activation of Pkr
		Tat	Pkr decoy
	Hepatitis B virus	Capsid protein	Inhibits MxA
	Influenza virus	NS1	Binds dsRNA and Pkr; blocks action of ISG15
	Reovirus	σ3	Binds dsRNA, inhibits Pkr and 2′-5′ oligo (A) synthase

[a]For further examples and details, see B. B. Finlay and G. McFadden, *Cell* **124:**767–782, 2006.

BOX 3.9 EXPERIMENTS

Three herpes simplex virus proteins modulate the IFN response in the cytoplasm and the nucleus

Herpes simplex virus is only marginally sensitive to IFN in cultured cells, yet this cytokine plays a major role in limiting acute infection in animals. At least three viral proteins modulate the IFN response.

- ICP34.5 acts as a regulatory subunit of cellular protein phosphatase 1 and reverses Pkr-induced eIF2α phosphorylation. Consequently, this viral protein prevents the translational block and autophagy induction established by IFN.
- US11 is an RNA-binding protein that prevents Pkr activation.
- ICP0 functions in the nucleus to launch the replication cycle. This protein prevents the IFN-induced block to RNA polymerase II transcription.

Varicella-zoster virus, a closely related alphaherpesvirus, is similarly insensitive to IFN in cultured cells, but it has no counterparts to the ICP34.5 or US11 genes. It is unclear if the ICP0 homolog provides the only IFN defense for this common herpesvirus of humans.

Harle, P., B. Sainz, Jr., D. J. Carr, and W. P. Halford. 2002. The immediate-early protein, ICP0, is essential for the resistance of herpes simplex virus to interferon-alpha/beta. *Virology* **293:**295–304.

Mossman, K., and J. R. Smiley. 2002. Herpes simplex ICP0 and ICP34.5 counteract distinct interferon-induced barriers to virus replication. *J. Virol.* **76:**1995–1998.

An inescapable inference from the various countermeasures encoded by the genomes of diverse viruses is that IFN is an essential host defense component (Fig. 3.7). But numerous questions remain. For example, some infections (e.g., Newcastle disease virus) are inhibited only by IFN-α, while others (e.g., herpes simplex virus type 1) are inhibited primarily by IFN-β. IFN production is induced after infection by vaccine strains of measles virus, while little IFN is made after wild-type virus infection. When animals are infected, the IFN response varies depending on the route of infection (Box 3.10). It is clear that much remains to be learned about the antiviral effects of IFN and virus countermeasures.

Apoptosis (Programmed Cell Death)

Cell suicide is a potent intrinsic defense. The biochemical alterations initiated by infection can induce a process of controlled self-destruction called **apoptosis** (Fig. 3.8 and 3.9). Apoptosis normally functions to eliminate particular cells during development and differentiation. Cells in organs are in numerical equilibrium despite rather large changes in cell birth and death in response to physiological stimuli. Accordingly, apoptotic pathways are strictly controlled by a variety of mechanisms that monitor the processes of growth regulation, cell cycle progression, and metabolism. Death by apoptosis does not result in inflammation or activation of other defensive reactions. However, many diseases (including cancer) are associated with malfunctions in regulation of apoptosis. Survival signals from the cell's environment, and internal signals reporting on cell integrity, normally keep the apoptotic response in check. When these signals are perturbed by a variety of events such as viral infection, cell death invariably ensues. Cellular debris resulting from apoptosis is taken up by macrophages and dendritic cells, which are thereby stimulated to produce cytokines and migrate to local lymph nodes. Exogenous proteins are processed into peptides, and presented to T cells in the lymph nodes. If nonself peptides are detected, an immune response may be activated.

Controlled cell suicide, or apoptosis, can be activated by a large variety of both external and internal stimuli. Regardless of the nature of the initiation signal, all converge on common effectors, the **caspases.** Caspases are members of a family of *c*ysteine proteases that specifically cleave after *asp*artate residues. These proteases are first synthesized as precursors with little or no activity. A mature caspase with full activity is produced after cleavage by another protease (often another caspase). Alternatively, increasing the concentration of some caspase precursors results in cleavage-independent activation. These protease cascades are not unlike blood clotting or the complement cascade (see Chapter 4,"Complement"). The principle is similar: a modest initial signal can be amplified significantly, culminating in an all-or-none response.

Two convergent caspase activation cascades are known: the extrinsic and intrinsic pathways. The **extrinsic pathway** begins when a cell surface receptor binds a proapoptotic ligand (e.g., the cytokine Tnf-α). Binding changes the cytoplasmic domain of the receptor so that death-inducing signaling proteins are recruited (Figure 3.9B). This complex of proteins attracts pro-caspase 8, which is activated on binding. Caspase 8 cleaves and activates pro-caspase 3, the final effector for both extrinsic and intrinsic pathways.

The **intrinsic pathway**, often called the mitochondrial pathway, integrates stress responses, as well as internal developmental cues. Common intracellular initiators include DNA damage and ribonucleotide depletion. In

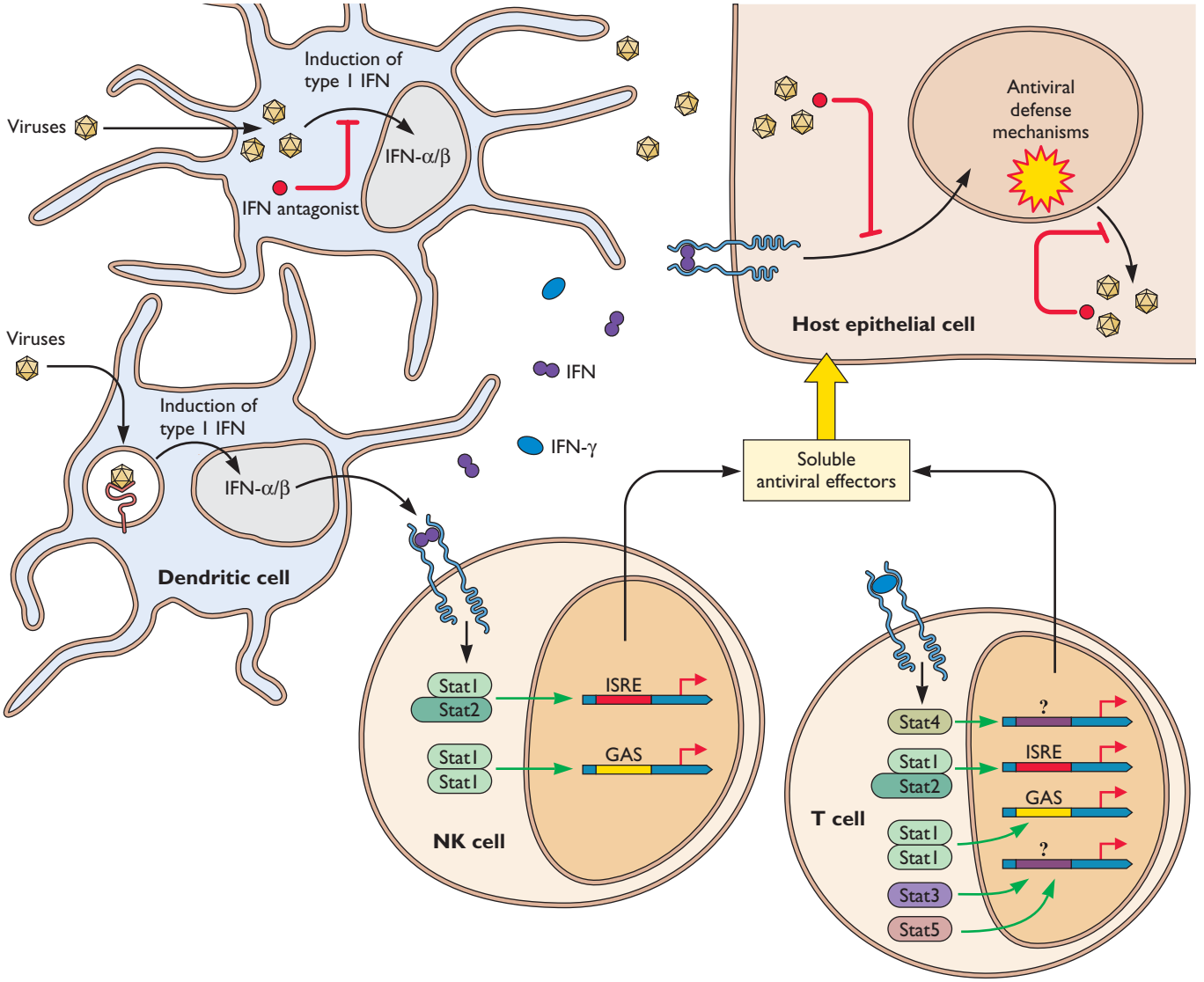


Figure 3.7 Virus-mediated modulation of IFN production and action. The pattern recognition receptors present in most cells, including dendritic cells, detect viral nucleic acid and viral proteins on the cell surface, in endosomes, and in the cytoplasm. IFN is produced as a consequence of these intrinsic defenses. Secreted IFN then binds to cells with IFN receptors and activates different but overlapping signal transduction cascades depending on the cell type. These cascades result in new gene expression including expression of IFN and other antiviral proteins. Viral gene products modulate most steps in the IFN response from the infected cell to the responding cell. This modulation changes the dynamics of cytokine production and action in ways that are not fully understood. For example, the dendritic cells (blue cytoplasm) detect viral infection or products of viral infection and produce IFN (purple ovals) and IFN-γ (blue circles). However, viral infection may lead to modulation of IFN production in these primary defense cells (red line, IFN antagonist). The IFN produced by dendritic cells can bind to receptors on innate immune cells (e.g., NK cells [yellow cytoplasm]) or T cells (yellow cytoplasm), leading to production of IFNs and other IFN inducible genes (indicated by the question mark). The combination of NK cell and T-cell action should produce soluble antiviral effectors leading to destruction of other infected host cells (e.g., epithelial cells, orange cytoplasm). However, viral gene products produced in these infected target cells can modulate IFN signaling or block recognition of the infected cell by NK cells or T cells. As a result, viral infected cells are exposed to a rapidly changing pallette of cytokines produced, not only by the infected cell, but also by innate and adaptive immune cells reacting to the infection. Adapted from A. Garcia-Sastre and C. Biron, *Science* **312:**879–882, 2006, with permission.

BOX 3.10 WARNING

Routes of infection make a difference

Most natural infections begin at mucosal surfaces. For ease of experimentation, many investigators resort to using rather unnatural routes, including injecting virus into the bloodstream or the peritoneal cavity. Can we assume that the innate defenses activated by unnatural infections are similar to those following infection by a natural route? Infections of transgenic animals lacking innate defense genes have provided some insight.

Mice are relatively resistant to infection by vesicular stomatitis virus no matter the route of infection. However, Pkr-null mutant mice become highly susceptible to this virus, **but only** after respiratory infection.

These findings show that the Pkr response in the respiratory tract is a primary defense against the virus. Defense after injection of virions into the bloodstream or the peritoneal cavity is mediated by some other mechanism.

The lesson is to be wary of generalizations about the innate immune response. Not all routes of infection are defended in the same manner.

Levy, D. E. 2002. Whence interferon? Variety in the production of interferon in response to viral infection. *J. Exp. Med.* **195:**F15–F18.

these situations, the cell cycle regulatory protein p53 is activated (see Volume I, Chapter 7) and apoptosis ensues. One single family of proteins, the Bcl-2 family, controls the process (Fig. 3.9A). These proteins are the master regulators that inhibit apoptosis. Their activity is regulated by proapoptotic proteins, which, curiously, are also Bcl-2 family members. The differential binding of antiapoptotic Bcl-2 proteins to BH3-only proteins enable tissue-specific regulation and stress-specific responses (Fig. 3.9A).

In general, apoptosis is held in check because the antiapoptotic Bcl-2 family members Bcl-xL and Mcl-1 directly block the translocation of proapoptotic Bax and Bak to the mitochondria. If these proteins reach mitochondrial membranes, they become permeable and internal stores of cytochrome *c* are released. Cytochrome *c* in the cytoplasm binds to a cellular protein (Apaf-1), which oligomerizes in the presence of deoxyadenosine 5′ triphosphate (dATP) or ATP. The oligomeric assembly then binds and cleaves pro-caspase 9, which in turn activates pro-caspase 3. The extrinsic and intrinsic signaling pathways can converge in other ways. For example, if the extrinsic pathway is activated, mature caspase 8 may cleave a proapoptotic protein called Bid that then translocates to the mitochondria to trigger the intrinsic pathway. There is ample evidence to suggest that the intrinsic pathway can serve to amplify the extrinsic pathway.

Once caspase 3 is activated (no matter what the initial signal), the end results are always the same: cell and organelle dismantling, vesicle and membrane bleb formation, phosphatidylserine exposure on the cell surface, and DNA cleavage to nucleosome-sized fragments (Fig. 3.8A).

Apoptosis as a Defense against Viral Infection

Because virions engage cell receptors, and because viral replication engages all or part of the host's transcription, translation, and replication machines, a variety of signals may activate the extrinsic and intrinsic pathways (Box 3.11). In many infections, the target cell is quiescent and hence unable to provide the enzymes and other proteins needed by the infecting virus. Consequently, viral proteins induce the cell to leave the resting state. However, cell cycle checkpoint proteins then respond to this unscheduled event by inducing apoptosis. Typically, viral early proteins activate the cell cycle (e.g., adenoviral E1A proteins or simian virus 40 large T protein). Not surprisingly, most viral infections trigger apoptosis. Accordingly, to ensure that infected cells survive long enough to produce progeny, viral genomes encode gene products that modulate this potentially lethal process.

Viral Gene Products That Inhibit Apoptosis

The discovery of viral proteins that modulate the apoptotic pathway proved exceedingly valuable in dissecting the complex pathways and regulatory circuits in normal cells. As noted above, apoptosis is normally held in check by regulatory proteins called inhibitors of apoptosis (IAPs). The prototype IAP gene was described in baculovirus genomes by the late Lois Miller and colleagues in 1993. This seminal work led to the discovery of cellular orthologs in yeasts, worms, flies, and humans. Mutant viruses unable to inhibit apoptosis were detected originally because the host DNA of infected cells was unstable, the cells lysed prematurely, and, as a consequence, viral yields were reduced, resulting in small plaques. Since then, we have discovered many viral proteins that regulate or block apoptosis (Table 3.10).

Remarkably, human cytomegalovirus encodes an abundant, 2.7-kb noncoding RNA (β2.7) that binds to and inhibits a mitochondrial protein complex that triggers apoptosis. Not only is apoptosis blocked, but mitochondrial function is maintained so that cells do not die quickly. The mitochondrial membrane potential is maintained, enabling

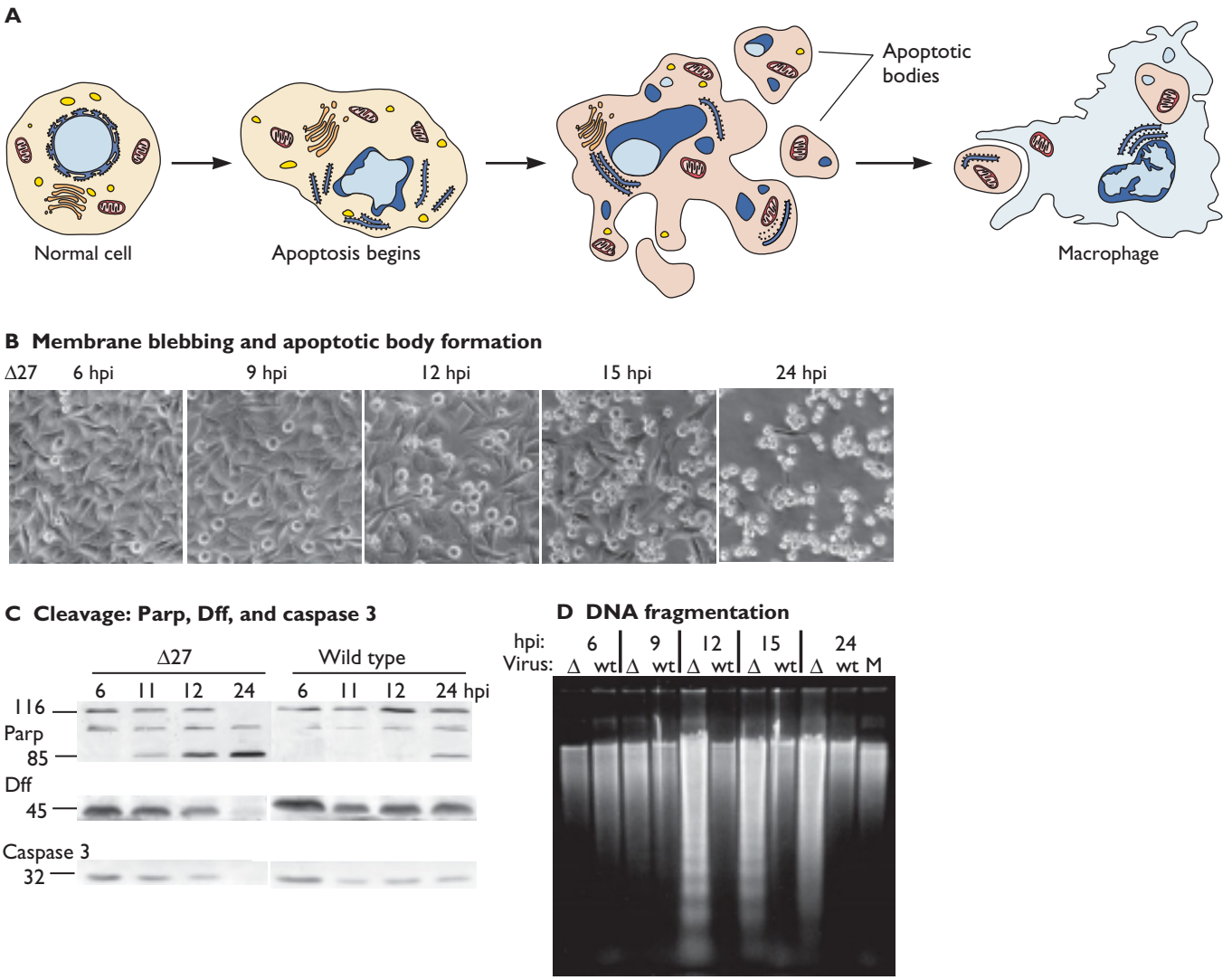


Figure 3.8 Apoptosis, the process of programmed cell death. (A) Apoptosis can be recognized by several distinct changes in cell structure. A normal cell is shown at the left. When programmed cell death is initiated, as indicated by the second cell, the first visible event is the compaction and segregation of chromatin into sharply delineated masses that accumulate at the nuclear envelope (dark blue shading around periphery of nucleus). The cytoplasm also condenses, and the outline of the cell and nuclear membranes changes, often dramatically. The process can be rapid: within minutes the nucleus fragments and the cell surface convolutes, giving rise to the characteristic "blebs" and stalked protuberances illustrated. These blebs then separate from the dying cell and are called apoptotic bodies. Macrophages (the cell at the right) engulf and destroy these apoptotic bodies. Adapted from J. A. Levy, *HIV and the Pathogenesis of AIDS,* 2nd ed. (ASM Press, Washington, DC, 1998), with permission. **(B)** Apoptosis in human epithelial cells infected with herpes simplex virus type 1. Infection by herpes simplex virus type 1 initiates apoptosis, but *de novo* synthesis of infected-cell proteins prevents cell death. The viral immediate-early ICP27 protein is one of several proteins involved in this inhibition: infection by a mutant virus with a deletion of the ICP27 gene (Δ27) induces apoptosis but does not block cell death. Phase-contrast microscopy of human epithelial cells infected with Δ27 is shown. Membrane blebbing and apoptotic-body formation typical of apoptosis increase with time after infection. hpi, hours postinfection. **(C)** Determination of apoptosis induction after infection by monitoring proteolytic processing of three proteins: DNA fragmentation factor (Dff), poly(ADP-ribose) polymerase (Parp), and caspase 3. Proteins are detected with specific antibodies. The processing of Parp, a 116-kDa protein, produces an 85-kDa product. Apoptosis-induced processing of Dff (45 kDa) and caspase 3 (32 kDa) results in the loss of reactivity with the specific antibodies. **(D)** DNA fragmentation after infection. Appearance of a ladder of short DNA fragments is indicative of apoptosis. The time course of DNA laddering in cells infected with Δ27 or wild-type (wt) herpes simplex virus type 1 is shown. Reprinted from M. Aubert and J. A. Blaho, *Microbes Infect.* **3:**859–866, 2001, with permission.

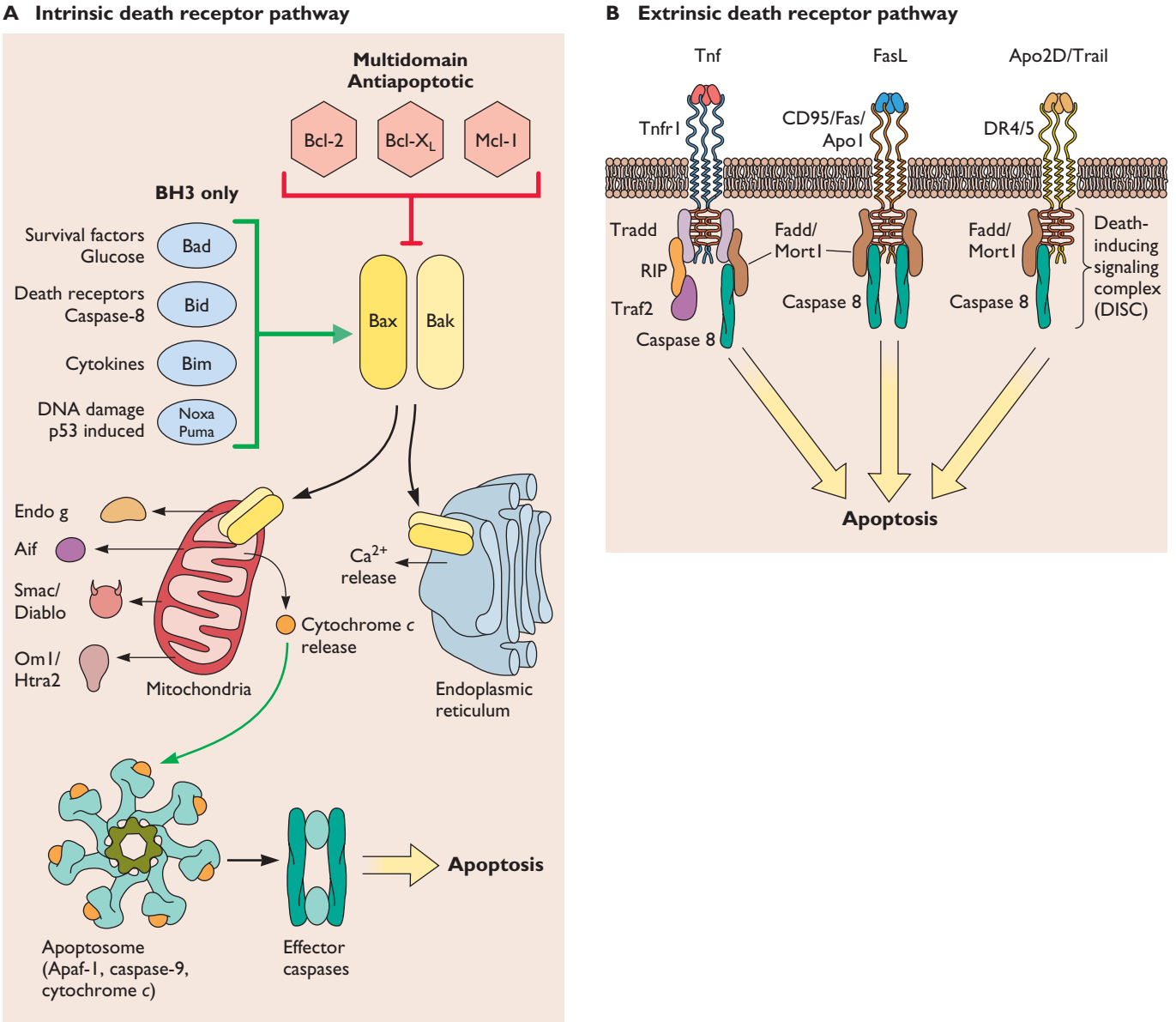


Figure 3.9 Pathways to apoptosis. (A) The process of apoptosis is controlled by the Bcl-2 family of proteins. The central antiapoptosis regulators are Bcl-2, Bcl-X_L, and Mcl-1 (pink hexagons). These proteins keep Bax and Bak (proapoptotic proteins [yellow]) from assembling on the mitochondrial or endoplasmic reticulum membranes and causing release of cytochrome *c* and calcium, respectively. A variety of other proteins are released from mitochondria after Bax/Bak action, as indicated. Four other classes of Bcl-2 regulatory proteins that also bind to different subsets of the Bcl-2 proteins are indicated at the top left (blue ellipses). These four classes act under conditions where survival is threatened (Bad), when the extrinsic pathway is stimulated (Bid), when certain cytokines are produced (Bim), and when DNA damage is detected and p53 is induced (Noxa and Puma). The oligomerization of Apaf-1, caspase 9, and cytochrome *c* forms a large structure called the apoptosome, which then activates effector caspases that produce the characteristic events of controlled cell suicide. **(B)** The extrinsic death receptors and their death-inducing signaling complexes. Three central receptors found on the surfaces of cells that can initiate the apoptosis pathway are illustrated. These are the Tnf receptor, the Fas ligand receptor (CD95), and the Apo2/Trail receptor (DR4/5). When these receptors engage their cognate ligand, the cytoplasmic domains of each protein complex forms a scaffold for assembly of the death-inducing signaling complex (DISC). Important cytoplasmic proteins in this complex are shown. Caspase 8 is activated when it binds these complexes, which initiates the apoptopic pathway. Adapted from N. Danial and S. Korsmeyer, *Cell* **116:**205–219, 2004, with permission.

BOX 3.11 BACKGROUND

The many ways by which virus infections perturb apoptotic pathways

At the Cell Surface

- Production of apoptosis-inducing cytokines after virions bind their receptors
- Alteration of membrane integrity or composition via membrane fusion or virion passage into the cytoplasm via receptor mediated endocytosis

In the Cytoplasm

- Production of metabolic inhibitors (e.g., arrest of host translation)
- Modification of cytoskeleton (e.g., disruption of actin microfilaments)
- Disruption of signal transduction pathways (e.g., death domain proteins and kinase- and phosphatase-binding proteins)

In the Nucleus

- Degradation of and damage to DNA
- Alteration of gene expression (e.g., increased expression of heat shock genes)
- Disruption of the cell cycle (e.g., inactivation of p53 or pRb)

Hay, S., and G. Kannourakis. 2002. A time to kill: viral manipulation of the cell death program. *J. Gen. Virol.* **83:**1547–1564.

Miller, L. K., and E. White (ed.). 1998. Apoptosis in viral infections. *Semin. Virol.* **8:**443–523.

continuing synthesis of ATP. It remains to be seen how many other viral RNA molecules are inhibitors of apoptosis.

Apoptosis Is Monitored by Sentinel Cells

Specialized phagocytes, called dendritic cells and macrophages, monitor most living tissues (see also Volume I, Chapter 4). These mobile phagocytes are critical players in early defense, as well as in activating a more global immune response. We call them **sentinel cells** because their function is to gather information (as packets of proteins) by taking up cellular debris and extracellular proteins released from dying cells. Then, after migrating to local lymph nodes, the sentinel cells bind to and present their collected peptide fragments to lymphocytes of the adaptive immune system (the T cells in particular). This cell-cell communication informs T cells about the nature of the insult that is killing cells in peripheral tissues, and the T cells respond accordingly. The sentinel cells, as well as the damaged and dying cells, produce cytokines that can induce apoptosis in nearby infected cells. For example, Tnf-α is a cytokine that can induce apoptosis when it binds to the Tnf receptor (a so-called "death receptor") (Fig. 3.9B).

Table 3.10 Some viral regulators of apoptosis[a]

Cellular target	Virus	Gene	Function
Bcl-2	Adenovirus	E1B 19K	Bcl-2 homolog
	Epstein-Barr virus	LMP-1	Increases synthesis of Bcl-2; mimics CD40/Tnf receptor signaling
Caspases	Adenovirus	14.7K	Inactivates caspase 8
Cell cycle	Hepatitis B virus	pX	Blocks p53 mediated apoptosis
	Human papilloma virus	E6	Targets p53 degradation
	Simian virus 40	Large T	Binds and inactivates p53
Fas/TNF receptors	Adenovirus	E3 10.4/14.5K	Internalizes Fas
	Cowpox	CrmB	Neutralizes Tnf and LT-α
	Myxoma virus	MT-2	Secreted Tnf receptor homolog
vFLIPs; DED box-containing proteins	Human herpesvirus 8	K13	Blocks activation of caspases by death receptors
Oxidative stress	Molluscum contagiosum virus	MC066L	Inhibits UV- and peroxide-induced apoptosis; homologous to human glutathione peroxidase
Transcription	Human cytomegalovirus	IE1, IE2	Inhibits Tnf-α but not UV-induced apoptosis

[a]Data from D. Tortorella, B. Gewurz, M. Furman, D. Schust, and H. Ploegh, *Annu. Rev. Immunol.* **18:**861–926, 2000; S. Redpath, A. Angulo, N. Gascoigne, and P. Ghazal, *Annu. Rev. Microbiol.* **55:**531–560, 2001; S. Hay and G. Kannourakis, *J. Gen. Virol.* **83:**1547–1564, 2002.

In a curious twist of molecular biology, the vaccinia virus envelope is derived from membranes of a dying cell. These membranes have cellular markers of apoptosis including phosphatidylserine. This phospholipid normally is available to bind to receptors that are present on the surface of phagocytic cells and that initiate the destruction of the dying cell and endocytosis of debris. When vaccinia virions, with their envelopes marked by apoptotic phospholipids, bind to the cell surface of a susceptible cell, they trigger an endocytic engulfment response normally appropriate for apoptotic debris and enter the cell (a viral mimic of the Trojan horse).

The Hostile Cytoplasm: Other Intrinsic Defenses

At least five other widely conserved cellular processes function in cellular defense against viral infections. These processes are autophagy, epigenetic silencing, RNA interference, cytosine deamination, and Trim protein interference. The first four tend to be general defenses activated upon infection, whereas Trim proteins function only against retroviruses, In addition, this mechanism is constitutive (not induced by infection), and is found only in some cells.

Autophagy

Cells can be induced to degrade the bulk of their contents by formation of specialized membrane compartments related to lysosomes. This process is called **autophagy,** and is evoked by stress such as nutrient starvation or viral infection. Infection by many viruses induces a state of metabolic stress that normally triggers intrinsic defenses. Such stress-induced alterations in translation are modulated in part by eIF2α kinases. It has been proposed that phosphorylated eIF2α triggers autophagy, which in turn leads to engulfment and digestion of cytoplasmic virions or other viral components. Two lines of evidence are consistent with the proposal. In one, virus replication can be blocked when autophagy is induced. For example, Sindbis virus replication in neurons is inhibited after induction of autophagy. In the other, some viral genomes encode gene products that actively block this process. For example, herpes simplex virus infection transiently activates Pkr-mediated autophagy, but a viral protein (ICP34.5) blocks the process by reversing the phosphorylation of eIF2α. In addition, this protein binds to and inhibits the mammalian autophagy protein beclin 1. This interaction is essential for viral neurovirulence.

Autophagy also may play a role in the initial sensing of viral invaders by providing a mechanism for transfer of viral nucleic acid from the cytoplasm to intracellular compartments containing Toll-like receptors. The idea is that autophagy integrates viral induced stress responses with the molecular detectors of viral nucleic acid (e.g., cytoplasmic RigI/Mda5; and endosomal receptors Tlr3, Tlr7, Tlr8, and Tlr9).

Epigenetic Silencing

Epigenetic silencing is an intrinsic defense against DNA viruses that replicate in the nucleus. It is thought that upon entering the nucleus, foreign DNA molecules are quickly organized into transcriptionally silenced chromatin. Silencing is mediated by DNA methylation or chromatin modifications including histone deacetylation. These modifications can persist for long periods, often over many cell divisions. Organized collections of proteins in the nucleus called **Pml bodies** may mediate such repression. These structures are implicated in intrinsic antiviral defense for many reasons, including the fact that interferon stimulates synthesis of the proteins that comprise them (see "Promyelocytic leukemia proteins" above). As might be predicted, viral proteins to counter epigenetic silencing have been identified. For example, the human cytomegalovirus protein pp71 binds to a cellular protein called Daxx that interacts with histone deacetylases to relieve transcriptional repression. The global repression of Pml-bound DNA can be relieved by viral proteins such as the ICP0 protein of herpes simplex virus type 1. This protein accumulates at Pml bodies and induces the proteasome-mediated degradation of several of their protein components. The human cytomegalovirus IE1 proteins, the Epstein-Barr Ebna5 protein, and the adenovirus E4 Orf3 protein all affect Pml protein localization or synthesis. The ways in which the components of Pml bodies associate with DNA and repress transcription are active areas of study.

Epigenetic silencing manifests itself in many ways, but those studying gene transfer with retrovirus vectors often find that expression of their favorite gene is low or completely off. We now understand that integrated retrovirus DNA is subject to reversible epigenetic silencing, a prominent process in embryonic or adult stem cells. Histone deacetylases associate with newly integrated proviral DNA soon after infection and act to repress viral transcription (Volume I, Chapters 8 and 9).

RNA Silencing

RNA silencing is a mechanism of sequence-specific degradation of RNA observed among diverse plants and animals. It is likely to have arisen early in the evolution of eukaryotes to detect and destroy foreign nucleic acids. RNA silencing is related to a process called RNA interference (RNAi) that was first found to occur in *Caenorhabditis elegans* and subsequently detected in fungi, insects, and algae (Volume I, Chapter 10).

Table 3.11 Some viral gene products that suppress RNA interference

Virus	Gene product	Mechanism
Human adenovirus type 5	VA-RNA I and VA-RNA II[a]	Competition for binding to exportin-5 and Dicer
Ebola virus	VP35 protein	Binding to dsRNA
Influenza A virus	NS1 protein	Binding to dsRNA
Vaccinia virus	E3L protein	Binding to dsRNA
Human immunodeficiency virus type 1	Tat	Inhibition of Dicer?

[a]Both RNAs are cleaved by Dicer, and the products are incorporated into RNA-induced silencing complexes.

We are learning more about RNA interference as an antiviral defense, as many viral genomes, including those of plant, insect, fish, and human pathogens, encode suppressors. Many of these suppressors are RNA-binding proteins without a preference for small interfering RNAs (siRNAs) (Table 3.11). Others sequester these RNAs, inhibit their production, or affect amplification of the process. Other examples are certain to be discovered.

RNA interference is now used routinely as a tool in the laboratory to block cellular gene expression as well as viral replication. Some hold hope that this intrinsic defensive response may be harnessed for development of highly specific and effective antiviral drugs (see Chapter 9).

As previously discussed, cellular miRNAs are induced by IFN treatment and may play an important role in antiviral defense. Probably a more intriguing outcome of this research was the discovery of miRNAs in DNA viral genomes (discussed in Volume I, Chapter 10). In human cytomegalovirus-infected cells, a viral miRNA reduces expression of the major histocompatibility complex class I polypeptide sequence B (MicB) protein. As a result, NK cells have reduced capacity to kill the infected cell. Similarly, a human herpesvirus 8-encoded miRNA mimics host miR-155 required for B-cell development. This viral miRNA may contribute to viral lymphomagenesis. The interplay of viral gene products and host miRNA defenses is only now being described for a number of DNA and RNA viruses.

Cytosine Deamination (Apobec [Apolipoprotein B Editing Complex])

All mammalian genomes contain *apobec*3 genes. The Apobec family of proteins play a variety of roles, including RNA editing of host genes (Chapter 6). Several members of the Apobec3 family are induced by IFN and are intrinsic antiretroviral proteins packaged into virions. After infection, such cellular enzymes affect the process of reverse transcription such that newly replicated retroviral DNA is degraded. When the viral reverse transcriptase begins to copy viral RNA into DNA, Apobec deaminates single-stranded DNA, specifically the nascent minus strand, which is synthesized first. The enzyme converts C's to U's with the consequence that when the deaminated minus DNA strand is copied, the U pairs with A, producing a G-to-A transition. The new proviral genome therefore is mutated in a very characteristic pattern (many GC pairs become AT pairs). However, the deamination event has two consequences: in one, uracil-containing DNA is quickly attacked by uracil DNA glycosidase, leaving an abasic site that is a target for endonucleases. If the uracil-containing DNA is copied and is integrated, viable progeny cannot be produced. For example, all tryptophan codons (TGG) could be converted into stop codons (TAA). One retrovirologist called Apobec a WMD—a weapon of mass deamination.

The action of Apobec should be lethal for retroviruses that incorporate this enzyme into their virions. However, human immunodeficiency virus counters this potential lethal defense by producing the Vif protein. Vif binds to the Apobecs as well as to a particular host ubiquitin ligase complex and promotes the ubiquitinylation and subsequent degradation of the enzymes by the proteasome.

Trim (Tripartite Interaction Motif) Proteins

A distinct class of intrinsic defense proteins prevent the cell from being infected by certain retroviruses (see Chapter 6). The hypothesis is that these genes evolved independently in various species to protect against endemic retroviruses. They are present in normal cells, and some have been recognized for years as mediators of processes called "restriction" or "exclusion." We know by the presence of large numbers of retroviral proviruses in vertebrate genomes that retroviruses have been around for millions of years. We also know that many cell types are quite resistant to infection by some retroviruses, despite carrying functioning receptors. Recently, human immunodeficiency virus infections were found to be restricted in some, but not all, cell types. The race was on to identify these constitutive inhibitors in hopes of finding new mechanisms to stop the AIDS pandemic.

The facts were simple: human immunodeficiency virus type 1 is unable to replicate in Old World monkeys, but virions can enter their cells. Infection is blocked soon after entry, but before reverse transcription. No proviral DNA is produced, and the infection is aborted. It was possible to introduce a rhesus macaque complementary DNA (cDNA) library into permissive cells and identify the dominant gene that blocked replication. The protein responsible for the inhibition of human immunodeficiency virus replication in

rhesus macaques is called Trim5α. Trim stands for "tripartite interaction motif." A critical fact is that human Trim5α does not restrict human immunodeficiency virus replication. If humans had the rhesus macaque Trim protein gene, we might not have the devastating AIDS pandemic today.

Rhesus macaque Trim5α targets the human immunodeficiency virus capsid protein, but not other retroviral capsid proteins. When synthesis of this protein was reduced using small interfering RNA molecules, the block against human immunodeficiency virus infection in rhesus cells was relieved, but had no effect on murine leukemia virus infection. Trim5α appears to block infection by binding to some retroviral capsids to disrupt an ordered uncoating process or sequester particles to a nonproductive infection pathway. Trim5α is now known to be a ubiquitin ligase and promotes ubiquitinylation of capsids and their subsequent degradation by the proteasome. IFN treatment increases Trim5α mRNA production in both human and rhesus cells.

The idea that a retroviral capsid can be a target for intrinsic defense is not new, but took some time for support to develop. Host restriction (or exclusion) of mouse retrovirus infection has been known for more than 30 years. The prototypical host gene blocking early retroviral replication events was identified using the Friend strain of murine leukemia retrovirus. The locus is called FV1 (Friend virus susceptibility). The FV1 protein blocks replication of murine leukemia virus soon after reverse transcription. Importantly, host Fv1 action depends on the infecting virus coat protein. A single residue at position 110 of the capsid coat determines sensitivity to FV1. Remarkably, the mouse Fv1 gene is the capsid gene of an endogenous retrovirus resident in the mouse genome! How Fv1 acts remains unclear, but restriction depends on a specific interaction with the capsid protein of the incoming virus. This finding is a remarkable demonstration of selection events turning endogenous retroviral gene expression against potential infection by other retroviruses.

Perspectives

Every moment of our lives, we encounter a myriad of virus particles. Our physical, chemical, and biological defenses ensure that the vast majority of these encounters are of no consequence. Nevertheless, and invariably, we all experience many viral infections during our lifetimes. Obviously, our defenses, while powerful, can be surmounted. For every host defense, there is a viral countermeasure.

Infections, whether successful or not, reflect initial probabilistic events that are not well understood. For example, we have limited information on the probability of infection by a single virion, the probability that a cell will detect and respond to the infection, or the probability that sentinel cells will detect the products of a single-cell infection. The difficulties of translating information derived from tissue culture models, to animal models and then to human infections are substantial. Nevertheless, from what we know today, we can articulate several principles of host-virus interactions that operate during the first minutes to hours of an infection.

First, all infections begin with events in a single cell. Many cells can be infected at one time, but the way in which each responds dictates the course of events from there on out. If the infection is curtailed by cell-autonomous defenses (the intrinsic defenses), secondary innate and tertiary adaptive immune responses will not be activated. If infection is not curtailed, the actions of these relatively few infected cells dictate subsequent events.

The second principle is that every cell has receptors on the surface and inside the cell that bind microbial proteins and nucleic acids. On binding their cognate ligand, these pattern recognition receptors initiate reactions leading to production of potent secreted proteins called cytokines. Cytokines are the primary response mediators that emanate from a single infected cell. The cytokine receptors and cells that carry them provide the integrating network that produces the appropriate effector action so characteristic of an individual host's response to infection (Fig. 3.10).

The third principle is that these early events are noisy. Individual cells do not produce the same amount of a given cytokine, or the same cytokine cocktail. One reason is that natural infections are not synchronous. In addition, some infected cells curtail the infection rapidly, while others are slower in responding. Some infected cells undergo apoptosis, while some do not. This rather large variation in single-cell responses to a single infectious agent is amplified or dampened, depending on the number of infected cells, the tissue containing the infected cells, and characteristics of the host (age and state of health, for example). Such potentially large variation at the earliest stages of infection influences the extent and duration of subsequent responses. Because many of the subsequent events in host defense are not reversible (such as killing of a cell, or activation of an antibody response instead of a cytotoxic T-cell response), extreme outcomes are possible for the host (life, death, uncomplicated acute infection that is cleared, or a persistent infection that lasts for the life of a host).

The remarkable fact is that while initially cell autonomous, the subsequent cytokine-initiated events escalate into a choreographed global response of ever-increasing complexity and intensity. The process recruits more and more effector cells, which in turn produce more cytokines and defensive proteins. If all works well, the infection is stopped, the host survives, and the host defenses stand down to fight another day.

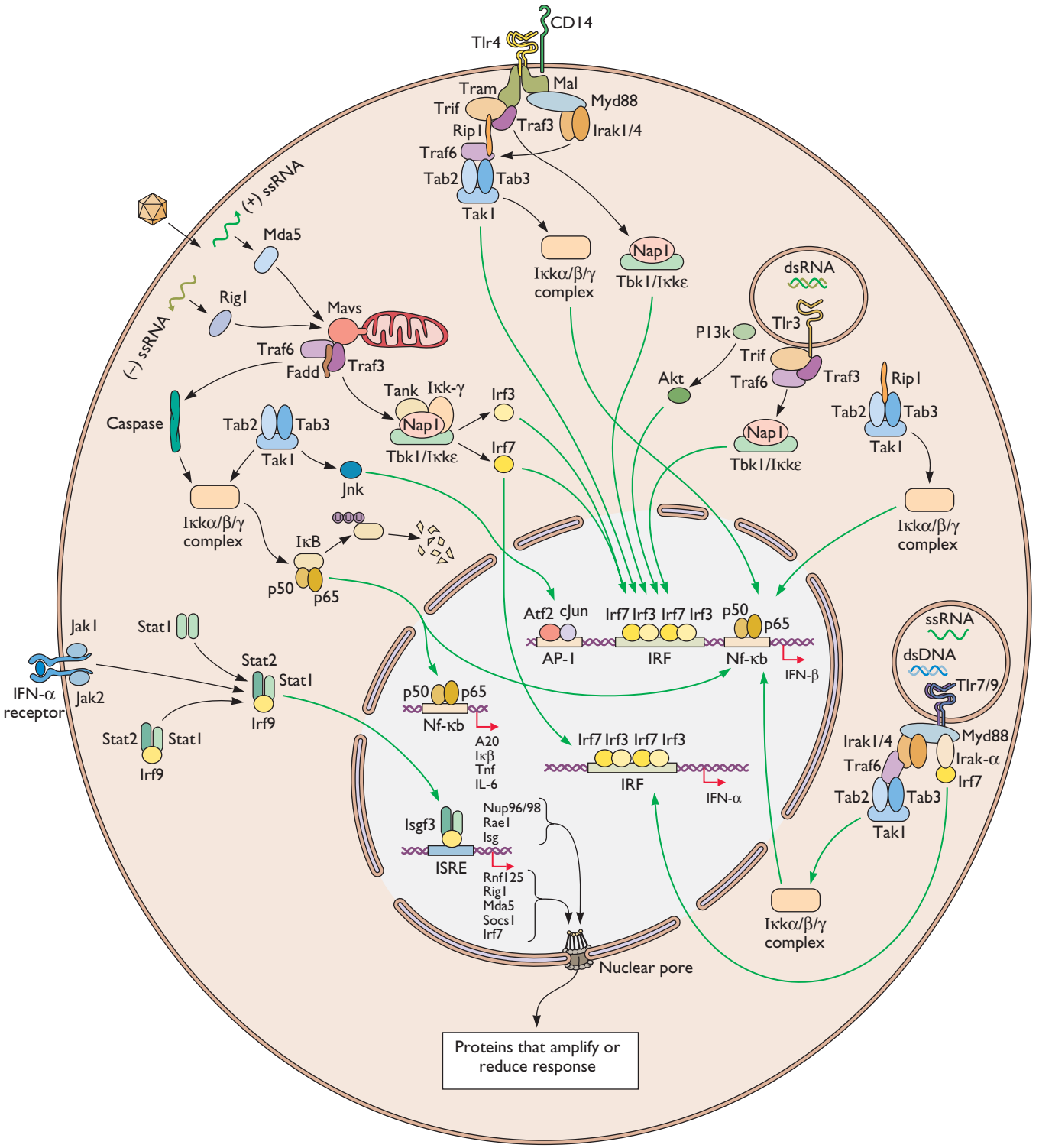


Figure 3.10 Summary of some established intrinsic defense responses. Every cell has receptors on the surface and inside that bind microbial proteins and nucleic acids. A generic cell is indicated here, but it should be clear that not all cells have the same constellation of responses. Upon binding their cognate ligand, these pattern recognition receptors initiate reactions leading to production of potent secreted proteins called cytokines. Cytokines, such as IFN, are the primary response mediators that emanate from a single infected cell. The cytokine receptors and cells that carry them provide the integrating network that produces the appropriate effector action so characteristic of an individual host's response to infection.

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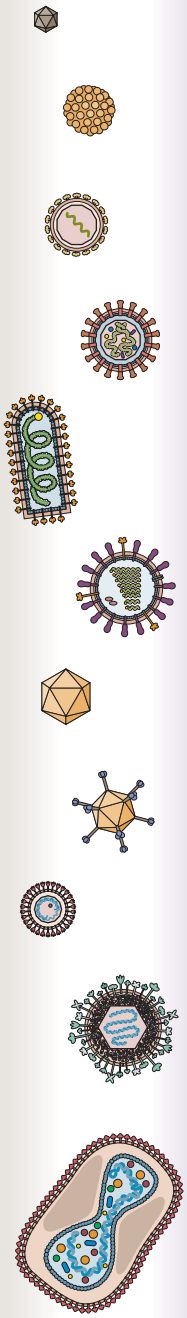


4

Immune Defenses

The trouble with facts is that there are so many of them.
ANONYMOUS

We have met the enemy and he is us.
WALT KELLY
Pogo

Introduction

The cascade of antiviral defense starts by the reactions of a single infected cell. If, despite single-cell defenses, viral replication continues unabated, a threshold that signals the need for more aggressive and global defenses is crossed. Almost immediately thereafter, a remarkable defense, called the **immune response**, begins. This highly coordinated response depends on the interplay of secreted proteins, receptor-mediated signaling, and intimate cell-cell communication. The immune response is to be contrasted with intrinsic defense, which is cell autonomous and not dependent on white blood cells. White blood cells participate at every level of immune defense (Table 4.1). Several remarkable life-or-death decisions are made quickly: the nature of the invader is established, and an appropriate mixture of soluble proteins and white blood cells is mobilized to remove the invader. Amazingly, infections of just a few cells can be detected in the context of billions of uninfected cells.

Three critical steps in immune defense are **recognition**, **amplification**, and **control**. A viral infection must be recognized early, defenses must be activated quickly and amplified if needed, and then all responses must be turned off when the infection has ceased. Pathologies and immune dysfunction can result if any of these processes is defective, modulated, or bypassed. The paramount importance of the immune system in antiviral defense is amply documented by the devastating viral infections observed in children who lack normal immune function, as well as in patients with acquired immunodeficiency syndrome (AIDS).

Innate and Adaptive Immune Defenses

The immune response to viral infection consists of an innate (nonspecific) and an adaptive (specific) defense (see Fig. 3.1). The **innate response** is the **first line of immune defense**, because it functions continually in a normal host without any prior exposure to the invading virus (Box 4.1). Indeed, most viral invasions are repelled by intrinsic defenses and the innate immune system

Table 4.1 White blood cells that participate in the innate and adaptive defense systems

Cell	Source/function
Lymphocytes	Responsible for specificity of immune responses; recognize and bind to foreign antigens; derived from bone marrow
T cells	Differentiate into Th cells that secrete cytokines and CTLs; regulatory or suppressor T cells are a type of Th cell; all have T-cell receptors
B cells	Produce antibody
NK cells	Natural killer cells; large granular cytolytic cells; do not have T-cell receptors
Mononuclear phagocytes	Responsible for phagocytosis and antigen presentation; derived from bone marrow; monocyte lineage; includes macrophages, Kupffer cells in the liver, alveolar macrophages in the lungs
Dendritic cells	Responsible for induction of immune response; antigen presentation to Th cells; migratory cells found in every tissue except the brain
Interdigitating dendritic cells	Bone marrow derived; present in most organs, lymph nodes and spleen; includes Langerhans cells in the skin
Follicular dendritic cells	Not bone marrow derived; present in germinal layers of lymphoid follicles in spleen, lymph nodes, and mucosal lymphoid tissue; trap antigens and antigen-antibody complexes for display to B cells in lymphoid tissue
Plasmacytoid dendritic cells	Class of immature dendritic cells found in the blood and T-cell zones of lymph nodes; capable of synthesizing large quantities of IFN to protect immune cells from viral infection
Granulocytes	Contain abundant cytoplasmic granules; inflammatory cells
Neutrophils	Polymorpholeukocytes; respond to chemotactic signals, phagocytose foreign particles; major leukocyte in the inflammatory response
Eosinophils	Function in defense against certain pathogens (like worms) that induce IgE antibody; not critical in antiviral defense; involved in hypersensitivity (allergic) reactions
Basophils	Circulating counterparts to tissue mast cells; mediate hypersensitivity caused by IgE antibody response

before viral replication outpaces host defense. However, once this threshold is passed, second-line defenses (the **adaptive response**) must be mobilized if the host is to survive.

The **adaptive defense** consists of the antibody response and the lymphocyte-mediated response, often called the **humoral response** and the **cell-mediated response**, respectively. This system is called "adaptive" because it not only differentiates infected from noninfected self, but also is tailored individually to the particular foreign invader such that an appropriate combination of soluble molecules (antibodies and cytokines) and lymphocytes (Table 4.1) participate in the action. The tailoring of the specific adaptive response requires close communication with cells that participate in the innate response. Such communication occurs by binding of cytokines and intimate cell-cell interactions among dendritic cells and lymphocytes in the lymph nodes. Considerable evidence indicates that the cells and cytokines of the innate response provide essential information to the adaptive immune system about the nature of the potential hazard confronting the host. In fact, it is possible to demonstrate that the adaptive response cannot be established without the innate immune system.

A defining feature of the adaptive defense system is **memory**; subsequent infections by the same agent are met almost immediately with a robust and highly specific response that usually stops the infection as soon as it starts, with minimal reliance on the innate defenses.

The cellular agents of the innate and adaptive immune responses are the **myelomonocytes** (monocytes, macrophages, dendritic cells, and a variety of granulocytes) and the **lymphocytes** (natural killer, T, and B cells)

BOX 4.1 BACKGROUND

Innate immune defense stands alone

- Other than intrinsic defenses of cells, it is the **only** immune defense available for the first few **days** after viral infection.
- It can discern the general nature of the invader (viruses, bacteria, protozoans, fungi, or worms).
- It can inform the adaptive response when infection reaches a dangerous threshold.

(Table 4.1). One fascinating aspect of the immune system is that its effector cells are dispersed throughout the body, yet the response can be directed quickly to the focal point of infection. It is the powerful cytokines that coordinate the activity of this dispersed cellular defense system.

The Innate Immune Response

General Features

When intrinsic cell defenses are unable to stop the spread of infection, the combination of cell death, local increasing concentrations of cytokines, and release of other stress-related molecules around the area of infection leads to activation of the next phase of host defense, the innate immune response (see Fig. 3.1). The innate immune defense system comprises **cytokines** released from infected cells as part of the intrinsic defense response, local **sentinel cells** (dendritic cells and macrophages), a complex collection of serum proteins termed **complement**, and cytolytic lymphocytes called **natural killer cells (NK cells)**. Neutrophils and other granulocytic white blood cells also play important roles in innate defense in response to the initial burst of cytokines from dendritic cells, macrophages, and infected cells.

The innate immune response is crucial in antiviral defense, because it can be activated quickly if intrinsic defenses are overwhelmed and can begin functioning within minutes to hours of infection. Such rapid action contrasts with the activation of the adaptive response, which is orders of magnitude slower than the replication cycles of some viruses. It takes days to weeks to orchestrate the effective response of antibodies and activated lymphocytes specifically tailored for the infecting virus. While the rapidity of the innate response is important, this response must also be transient, because its continued activity is damaging to the host. Below, we discuss crucial components of the innate response. Despite their apparent diversity, every component initiates an immediate action that, in turn, is detected and amplified by cells of the adaptive immune system. Viral genomes encode a surprising variety of gene products that modulate every step of innate defense.

Sentinel Cells

Dendritic cells and macrophages are crucial sentinel cells present in peripheral compartments (e.g., the skin and various mucosal surfaces). These cells are active very early in infection and play central roles in classifying the infecting agent, mounting a strong intrinsic response, and then communicating with cells of the immune system. Dendritic cells bind cytokines produced by infected cells and take up viral proteins from dead and dying cells (Fig. 4.1). Sentinel cells are specially equipped not only to initiate immediate immune defense, but also to convey information of the attack to the adaptive immune system. This latter ability is emphasized by their common name, **professional antigen-presenting cells**. Even if a viral protein is introduced by injection into the skin or muscle, local dendritic cells and macrophages will most likely bind some of the molecules and stimulate an immune response to that protein. Indeed, most vaccines would not be effective without dendritic cells.

Dendritic cells play two major roles in antiviral responses: they directly inhibit viral replication at the onset of infection by producing large quantities of cytokines such as alpha/beta interferon (IFN), and subsequently they trigger adaptive, T-cell-mediated immunity appropriate to the infection. Dendritic cells exist in two functionally distinct states called **immature** and **mature** (Fig. 4.1). The immature cells are found in the periphery of the body, around body cavities, and under mucosal surfaces. Several dendritic cell types can be identified by cell surface markers, and probably differ in function. In general, dendritic cells are proficient at endocytosis and can synthesize copious quantities of cytokines when stimulated. Soluble proteins are taken up avidly and retained in endosomes until the dendritic cell matures, which may be hours, if not days, after the actual uptake of proteins. Immature dendritic cells also capture proteins from dead or dying cells by taking up complexes containing heat shock proteins and unfolded proteins, as well as cellular debris and vesicles that are produced by apoptosis. As the cell matures, internalized proteins are processed into peptides and moved to the cell surface complexed to receptors that can be recognized by lymphocytes of the adaptive immune system (see "Antigen Presentation and Activation of Immune Cells" below).

Immature dendritic cells carry Toll-like receptors (Tlrs), the RigI and Mda5 RNA detector proteins, and receptors for various proinflammatory cytokines. They act as a bridge between intrinsic defenses and immune defenses. When these receptors bind the appropriate ligands, most immature dendritic cells undergo dramatic morphological and functional changes, and differentiate. The mature cells no longer have the capacity for endocytosis, and they display a new repertoire of cell surface receptors. Some of these are chemokine receptors that direct migration of the mature dendritic cell (loaded with stored viral proteins) to the local lymph nodes (often called **homing**). Other new receptors are T-cell adhesion receptors and T-cell costimulatory molecules, essential for binding to and activation of naive T cells on arrival in the lymph node. A remarkable change in morphology also occurs as mature cells extend long eponymous dendritic processes that increase their

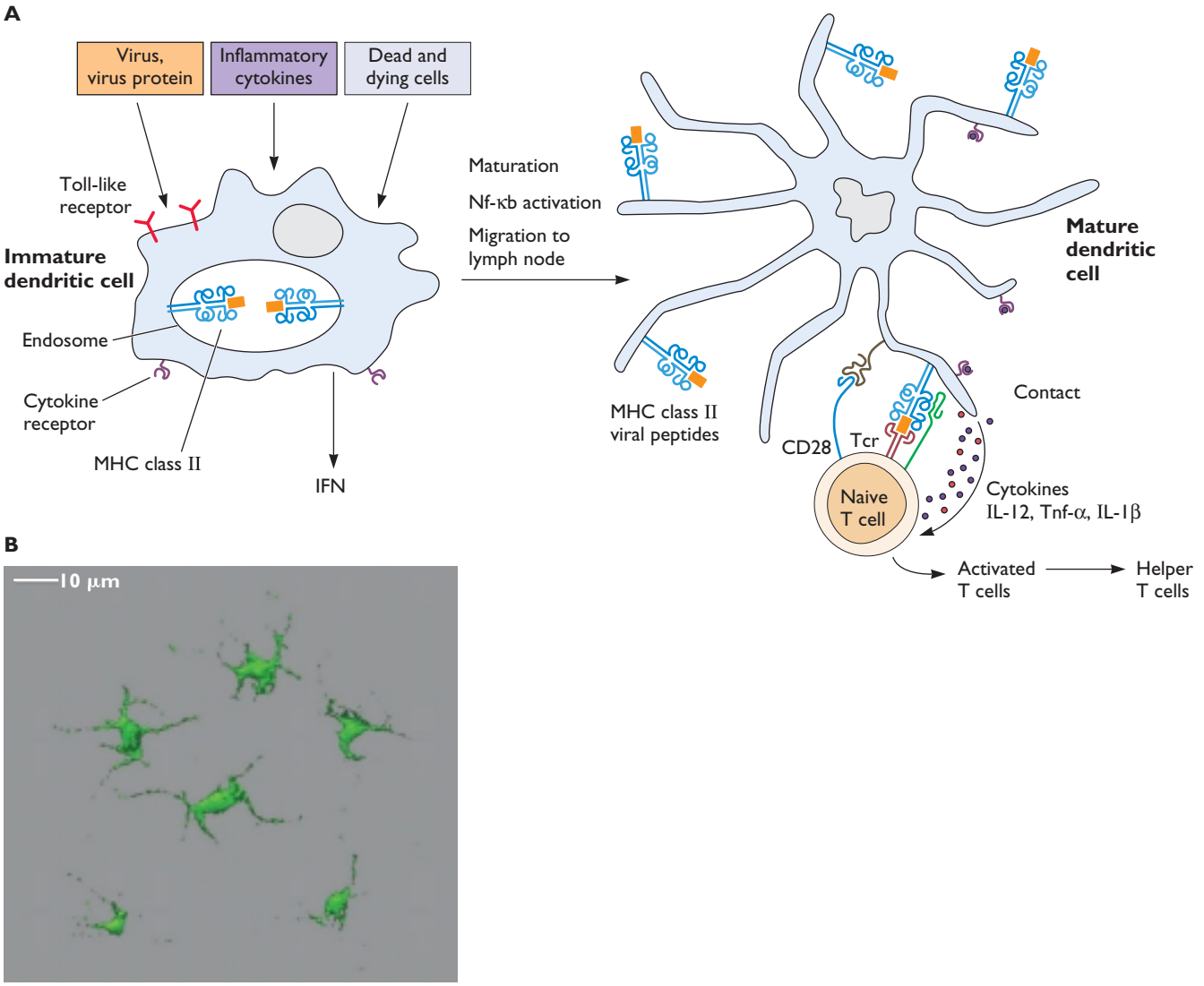


Figure 4.1 Dendritic cells provide cytokine signals and packets of protein information to naive T cells. **(A)** Immature dendritic cells actively take up extracellular proteins by endocytosis and store the proteins internally. They do not express MHC class II complexes on their surfaces. Binding of ligands to the Toll-like receptors or cytokine receptors induces differentiation into mature dendritic cells. These cells no longer have the capacity for endocytosis of proteins and display a new repertoire of cell surface receptors. Some of these are chemokine receptors that enable the dendritic cell to migrate to the local lymph nodes. The proteins ingested by the immature cell are now processed into peptides and loaded on to MHC class II proteins for subsequent transport to the cell surface. Mature cells extend long dendritic processes to increase surface area for binding of naive T cells in the lymph node. Mature dendritic cells release proinflammatory cytokines as indicated to stimulate T-cell differentiation. Naive but antigen-specific T cells bind to the MHC class II-peptide complexes via their T-cell receptors. The interaction is strengthened by the presence of increased costimulatory ligands (e.g., CD28) on the mature dendritic cell. The T cell is activated, begins the maturation process into its final effector state, and moves out of the lymph node into the circulation. **(B)** Langerhans cells are abundant in mouse ear epithelium and are visualized here by confocal microscopy in a live tissue preparation by their production of an MHC class II-enhanced green fluorescent protein (EGFP) fusion protein. Figure provided by Marianne Boes, Jan Cerny, and Hidde Ploegh (Harvard Medical School, Boston, MA).

surface area. Mature dendritic cells become potent mobile signaling centers, releasing proinflammatory cytokines that act locally and at a distance.

Once within lymphoid tissue, mature dendritic cells instruct the adaptive immune response by directly engaging and stimulating naive, antigen-specific T lymphocytes. The density of mature dendritic cells and the combination of cytokines they secrete in the lymph node dictate the type of adaptive response that ensue (see "Th1 and Th2 Cells" below). Dendritic cell maturation provides an essential link between innate and adaptive immunity.

While dendritic cells function by sampling proteins and cytokines found in the area of infection, they may also be infected by the virus that they detect (Box 4.2). One obvious outcome is that the mobile, infected cells travel to the lymph node where the virus can be transmitted to T and B cells. Indeed, human immunodeficiency virus type 1 virions can bind to a lectin (DC-Sign [dendritic-cell-specific, Icam-3-grabbing nonintegrin]) on immature dendritic cells with grave consequences. This protein is essential for establishing contact of a mature dendritic cell with a naive T cell in lymphoid tissue. Virus particles so bound do not replicate in the dendritic cell, but rather are retained just below the cell surface during migration to the lymph node, where they subsequently infect $CD4^+$ T cells (see Chapter 6). Two mosquito-borne viruses, Venezuelan equine encephalitis virus and dengue virus, replicate initially in immature dendritic cells at the site of inoculation. These infected cells then exhibit attributes of mature cells, in that they migrate to lymph nodes and propagate the infection with severe consequences to the host. Maturation appears to be triggered by viral RNA binding the RNA detectors, RigI and Mda5. This property, maturation of dendritic cells by infection, has inspired new approaches for vaccination in which attenuated versions of these viruses are used to deliver selected immunizing antigens directly to the dendritic cells, which then stimulate a strong immune response.

As might be expected, viral gene products modulate dendritic cell functions. For example, infection by either herpes simplex virus type 1 or vaccinia virus inhibits maturation of dendritic cells by blocking a signal transduction cascade, or by interfering with cytokine stimulation of maturation, respectively. In contrast, dendritic cells infected with murine cytomegalovirus or measles virus are fully capable of maturing, but the mature cells cannot stimulate T cells. Such interference with a critical component of the innate response is likely to contribute to the profound immunosuppression that follows infection by cytomegalovirus and measles virus. The contribution of dendritic cell infection to immunosuppression is a topic of considerable interest.

Natural Killer Cells

NK cells are in the immediate front line of innate defense: they are ready to recognize and kill virus-infected cells. Like dendritic cells, they recognize infected cells in the company of vast numbers of uninfected cells. However, the mechanism of recognition is completely different: NK cells recognize "missing self" or "altered self." NK cells are abundant lymphocytes (representing about 2% of circulating lymphocytes [Table 4.1]) that patrol the blood and lymphoid tissues. They are large, granular lymphocytes, distinguished from others by the absence of antigen receptors found on B and T cells (see below). When an NK cell binds an infected target cell, it releases a mix of cytokines (notably IFN-γ and tumor necrosis factor alpha [Tnf-α]) that contribute to a local inflammatory response and alert cells of the adaptive immune system. They also can produce prodigious quantities of interleukin-4 (IL-4) and IL-13, the major cytokines that stimulate antibody production. NK cells also participate later in adaptive defense by

BOX 4.2 BACKGROUND

Infection of the sentinels: dysfunctional immune modulation

When viruses infect the dendritic cells, the immune system's first command-and-control link is compromised. Some of the many possible consequences of sentinel cell infection, any one of which could suppress the immune response locally or systemically, include the following:

- interference with recruitment to peripheral sites of infection
- impairment of antigen uptake or processing
- infection and destruction of immature dendritic cells
- interference with maturation
- impairment of migration to lymphoid tissue
- interference with activation of T cells

López, C. B., J. S. Yount, and T. M. Moran. 2006. Toll-like receptor-independent triggering of dendritic cell maturation by viruses. *J. Virol.* **80:**3128–3134.

Mellman, I., and R. Steinman. 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell* **106:**255–258.

Raftery, M., M. Schwab, S. Eibert, Y. Samstag, H. Walczak, and G. Schonrich. 2001. Targeting the function of mature dendritic cells by human cytomegalovirus: a multilayered viral defense strategy. *Immunity* **15:**997–1009.

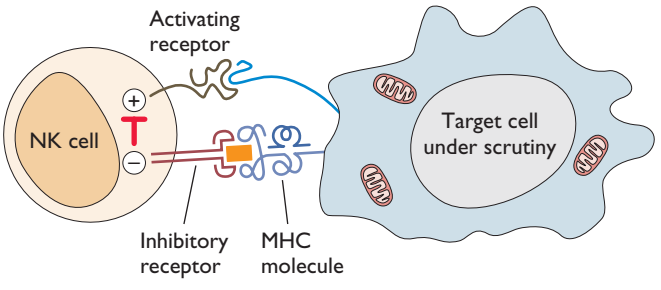
binding to infected cells coated with immunoglobulin G (IgG) antibody and killing them (see "Antibody-Dependent Cell-mediated Cytotoxicity" below).

The number of NK cells increases quickly after viral infection and then declines as the acquired immune response is established. These cells are stimulated to divide whenever infected cells and sentinel dendritic cells make IFN. NK cells kill after contact with the target by releasing perforins and granzymes that perforate membranes and trigger caspase-mediated cell death, respectively. In humans, NK cells are particularly important in controlling primary infection by many herpesviruses, as patients with NK cell deficiencies suffer from severe infections with varicella-zoster virus, human cytomegalovirus, and herpes simplex virus. While a role for direct NK cell-mediated killing in antiviral defense is difficult to establish experimentally, NK cell production of IFN-γ clearly provides significant antiviral action.

NK Cell Recognition of Infected Cells: Detection of "Missing Self" or "Altered Self" Signals

As discussed below, a collection of cell surface proteins called the major histocompatibility complex (MHC) proteins are important receptors in the adaptive immune response. MHC class I proteins are found on the surfaces of most cells of the body. The MHC class I molecules are the **self antigens** that, when missing, cause the NK cell to kill the target cell. A mechanism for detection of **missing self** is illustrated in Fig. 4.2. At least two receptor-binding interactions are required for such discrimination: one to activate the NK cell and the other to block this activation if the cell is not infected. The activating signal is delivered when an NK cell receptor binds a pathogen-specific ligand (e.g., virus-infected cells may present new glycoproteins on their surface). As a consequence, a signal transduction cascade is initiated and the NK cell is stimulated to secrete a burst of cytokines and kill the cell. However, a negative regulatory signal is produced when an MHC class I-specific receptor on the NK cell engages MHC class I molecules on the surface of the same target cell. Because many infected cells carry fewer MHC class I molecules on their surfaces (discussed in Chapter 5), they are prime NK cell targets. The unusual two-receptor recognition system employed by NK cells ensures that normal cells that synthesize MHC class I proteins are not killed by NK cells, even if they have distinct ligands on their surfaces. Much work is in progress to test this idea.

Figure 4.2 NK cells distinguish normal, healthy target cells by a two-receptor mechanism. Both positive (stimulating) and negative (inhibiting) signals may be received when an NK cell contacts a target cell. The converging signal transduction cascades from the two classes of receptor regulate NK cell cytotoxicity and release of cytokines. The inhibitory receptors dominate all interactions with normal, healthy cells. Their ligands are the MHC class I proteins. When NK cells contact MHC class I molecules on the surface of the target cell, signal transduction blocks the response of activating receptors.



NK MHC Class I Receptors Produce the Inhibitory Signals

Human NK cells synthesize two inhibitory MHC class I receptors of either the C-type lectin family or the immunoglobulin family (called killer cell immunoglobulin-like inhibitory receptors, or Kirs). NK cells also can recognize and spare target cells carrying HLA-E, an unusual MHC class I protein that binds peptides derived from the signal sequences of other MHC class I molecules. The presence of HLA-E protein complexed with signal peptide informs the NK cell that MHC class I synthesis is normal. An intriguing finding is that infection by human cytomegalovirus induces synthesis of HLA-E protein, thereby diverting potential NK cell recognition and lysis.

Viral Proteins Modulate NK Cell Actions

Many viral genomes encode proteins that block or confound NK cell recognition and killing (Fig. 4.3). At least five distinct categories of modulation can be described. These gene products are homologs of MHC class I, proteins that regulate MHC class I production, proteins that interfere with the NK activation receptor and cognate ligand interactions, and proteins that modulate cytokine pathways relevant to NK cell function. NK modulators have been identified in several virus families including *Flaviviridae*, *Papillomaviridae*, *Herpesviridae*, *Retroviridae*, and *Poxviridae*. Some viral genomes encode more than one distinct NK modulator. For example, human cytomegalovirus encodes at least seven gene products that modulate the NK cell response. One striking example of viral interference with NK cell activity is provided by the hepatitis C virus E2 envelope protein, which binds to CD81, a protein on the surface of NK cells, and blocks activation signals. As a result, the NK cell no longer recognizes infected cells. An example of variation of surface MHC class I expression on infected target cells features the Nef protein of human

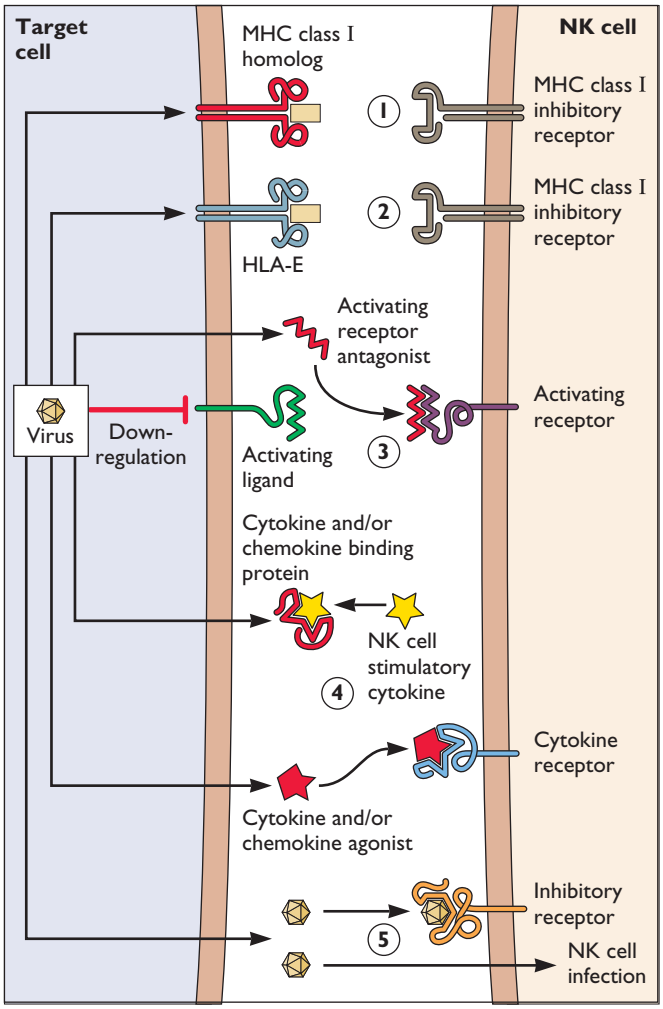


Figure 4.3 Virus-encoded mechanisms for modulation of NK cell activity. (Left) An infected target cell. **(Right)** An NK cell. The infected target cell should be lysed by an activated NK cell. However, five categories of NK-cell-modulating strategies are illustrated (circled numbers). Viral proteins produced in the infected cell are labeled in red. (1) Inhibition by a viral protein with homology to cellular MHC class I proteins. (2) Inhibition of expression or cell surface localization of host HLA-A or HLA-B (human MHC class I homologs) resulting in an increase in the amount of host HLA-E (or HLA-C) on the target cell surface. (3) Release of virus-encoded cytokine-binding proteins that block the action of NK cell-activating cytokines (also, viral proteins can reduce the amount of the activating ligand on the surface of the infected cell so that the NK cell is not activated). (4) Inhibition of action of NK cell-stimulating cytokines by binding these cytokines or by producing a chemokine antagonist. (5) Effect of newly produced virions, which can engage the NK cell, block an inhibitory NK cell receptor, or infect the NK cell itself to disrupt various effector functions or even kill the cell.

immunodeficiency virus type 1. Nef affects the cell surface expression of certain classes of MHC class I molecules, but not those involved in inhibition of NK cells (e.g., the HLA E proteins). Cells infected with ectromelia virus or molluscum contagiosum virus (both poxviruses), modulate the killing functions of NK cells by expressing proteins that bind IL-18, thereby inhibiting IFN-γ production by NK cells.

Complement

The **complement system** was identified in 1890 as a heat-labile serum component that lysed bacteria in the presence of antibody (it "complemented" antibody). The complement system in the blood is a major primary defense and a clearance component of **both** the innate and adaptive immune responses (Box 4.3). There are three distinct complement pathways: the **classical**, **alternative**, and **mannan-bindingpathways**. As part of the innate defense system, complement action can be initiated by direct recognition of a microbial invader by C1q or C3b proteins in the alternative pathway (Fig. 4.4). The mannan-binding lectin pathway triggers complement action upon binding of a lectin similar to C1q with mannose-containing carbohydrates on bacteria or viruses. Importantly, complement can also function as an effector of the adaptive defense system by the binding of C1q to antibody-antigen complexes on the surface of an invader or infected cell (the classical pathway [Fig. 4.4]).

The Complement Cascade

Complement comprises at least 30 distinct serum proteins and cell surface membrane proteins that act sequentially to produce a wide range of activities from direct cell lysis to the augmentation of adaptive immune responses (Fig. 4.4). Unfortunately, the nomenclature of the complement proteins is baroque because they were named in order of their discovery, but not in terms of their function.

In all three pathways, a cascade of protease reactions activates two critical proteases called **C3 convertase** and **C5 convertase** (note that the three pathways yield the same enzyme activity, but the proteins comprising each convertase are different). A crucial property of C3 and C5 convertase enzymes is that they are bound covalently to the surface of the pathogen or the infected cell. The action of surface-bound C3 convertase on its substrate yields C3b, the primary effector of all three complement pathways, and C3a, a potent soluble mediator of inflammation. C3b remains on the pathogen's surface, where it binds more complement components to stimulate a protease cascade that produces many other bioactive proteins. The protease cleavage products stimulate inflammation, attract lymphocytes, potentiate the adaptive response, and kill infected

BOX 4.3 TERMINOLOGY

The complement cascade has four major biological functions

Lysis

Membrane disruption and lysis occur when specific activated complement components (C6, C7, C8, and C9) polymerize on a foreign cell or enveloped virus, forming pores or holes that disrupt the lipid bilayer and compromise its function. The cell or virus is disrupted by osmotic effects.

Activation of Inflammation

Inflammation is stimulated by several peptide products of complement proteins produced during the complement cascade. These peptides (C3a, C4a, and C5a) bind to vascular endothelial cells and various classes of lymphocytes to stimulate inflammation and to enhance responses to foreign antigens.

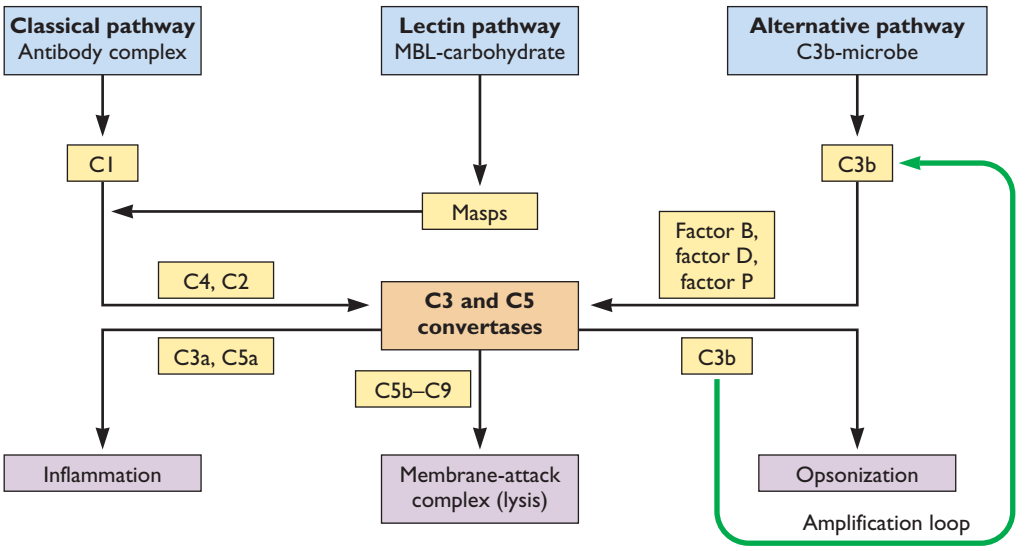
Opsonization

Complement proteins (typically C3b and C1q) can bind to virus particles so that phagocytic cells carrying appropriate receptors can then engulf the coated viruses and destroy them; this process is called opsonization. Complement receptors such as Cr1 present on phagocyte surfaces bind C3b-coated particles and stimulate their endocytosis.

Solubilization of Immune Complexes

Noncytopathic viral infections commonly result in pathological accumulations of antigen-antibody complexes in lymphoid organs and kidneys. Complement proteins can disrupt these complexes, by binding to both antigen and antibody, and facilitate their clearance from the circulatory system.

Figure 4.4 Activation and regulation of the complement system. The complement system can be activated through three pathways: classical, lectin, and alternative. Complement component 1 (C1) comprises C1q (a pattern recognition protein), C1r, and C1s. The complement cascade is activated when C1 binds an antigen-antibody complex on the surface of an infected cell or a virus particle; C1 also links the classical and lectin activation pathways by interacting with the mannose-binding lectin (MBL)-associated serine protease (MASPs). These complexes contain proteases that cleave complement proteins C2 and C4, which then form the C3 and C5 convertases for the classical and lectin pathways. The alternative pathway activates complement without going through the C1-C2-C4 complex. For the alternative pathway, factor B is the C2 equivalent. Factor B is cleaved by factor D. Factor P (properdin) stabilizes the alternative pathway convertases. All three pathways culminate in the formation of the C3 and C5 convertases (orange box) that produce the three primary actions of activated complement: inflammation, cell lysis, and coating of foreign antigens so that they can be taken up by phagocytes (opsinization). The C3a and C5a proteins are potent simulators of the inflammatory response (also called anaphylatoxins). The membrane attack complex is formed by the complement proteins C5b-C9 and forms a hole in membranes, leading to lysis of cells. The C3b (opsonin) coats bacteria and virions and also amplifies the alternative pathway. See C. Kemper and J. Atkinson, *Nat. Rev. Immunol.* **7:**9–18, 2007.



cells. C3b also stimulates phagocytic cells to take up the C3b-coated complex.

More than 90% of plasma complement components are made in the liver. However, these components also are produced elsewhere, including the major portals of pathogen entry. For example, the initiator complex C1 is synthesized mainly in the gut epithelium, and mannan-binding lectin is found in the respiratory tract. In addition, monocytes, macrophages, lymphocytes, fibroblasts, endothelial cells, and cells lining kidney glomeruli or synovial cavities all make most proteins of the complement system. Astrocytes in the brain can synthesize the full panoply of complement proteins when stimulated by inflammatory cytokines. As the brain is exquisitely sensitive to inflammation, it is not clear how the inflammatory action of peptides produced during the complement cascade is controlled.

One important consequence of complement cascade activation is the initiation of a local broad-spectrum defense (Box 4.3). Complement components released locally during the protease cascade aid in recruitment of monocytes and neutrophils (Table 4.2) to the site of infection, stimulate their activities, and also increase vascular permeability. The antiviral effects of complement are both direct and indirect. The membrane attack complex lyses infected cells and inactivates enveloped viruses, while phagocytes engulf and destroy virions coated with C3b protein. Complement components stimulate a local inflammatory response that can limit infection, and convey the nature of the invader to the adaptive immune system. The activated complement system "instructs" the humoral and T-cell responses much as activated dendritic cells communicate with T cells. Indeed, we now understand that the complement machinery plays a pivotal role in regulating both B- and T-cell-mediated immune responses. It represents a true bridge between frontline early defenses and adaptive immunity.

The molecular linkage between complement and adaptive defense is somewhat selective, as is evident by the antibodies made during the Th1 response (see "Th1 and Th2 Cells" below). These antibodies are predominantly of the IgG2a isotype, an isotype that can actively stimulate the complement cascade. In contrast, antibodies produced during the Th2 response typically are IgG1 (mice), IgG4 (humans), and IgE, which bind neither to complement nor to macrophage Fc receptors (Table 4.3). Curiously, many antibodies specific for viral envelope proteins cannot be recognized by C1q, a property likely to be a consequence of selective pressure to escape complement-mediated lysis.

"Natural Antibody" Protects against Infection

The classical complement pathway of humans and higher primates can be activated by a particular collection of antibodies present in serum prior to viral infection (historically called "natural antibody"). Synthesis of some of these antibodies is triggered by the antigen galactose α(1,3)-galactose (α-Gal) found as a terminal sugar on glycosylated cell surface proteins. Lower primates, most other animals, and bacteria synthesize the enzyme galactosyltransferase, which attaches α-Gal to membrane proteins. Importantly, humans and higher primates do not make this antigen, as they lack the enzyme. Because of constant exposure to bacteria producing α-Gal in the gut, human serum contains high levels of antibodies specific for this antigen; indeed, more than 2% of the IgM and IgG populations is

Table 4.2 Biological activities of proteins and peptides released during the complement cascade

Substance	Biological activity
C5b, C6, C7, C8, and C9	Lytic membrane attack complex
C3a	Peptide mediator of inflammation, smooth-muscle contraction; vascular permeability increase; degranulation of mast cells, eosinophils, and basophils; histamine release; platelet aggregation
C3b	Opsonization of particles and solubilization of immune complexes; facilitation of phagocytosis
C3c	Neutrophil release from bone marrow; leukocyte lysis
C3dg	Molecular adjuvant; profound influence on adaptive response
C4a	Smooth-muscle contraction; vascular permeability increase
C4b	Opsonin for phagocytosis, processing, and clearance of antibody-antigen immune complexes
C5a	Peptide mediator of inflammation, smooth-muscle contraction; vascular permeability increase; degranulation of mast cells, basophils, and eosinophils; histamine release; platelet aggregation; chemotaxis of basophils, eosinophils, neutrophils, and monocytes; hydrolytic enzyme release from neutrophils
Bb	Inhibition of migration and induction of monocyte and macrophage spreading
C1q	Opsonin for phagocytosis, clearance of apoptotic cells, and processing and clearance of antibody-antigen immune complexes

Table 4.3 The major cell-mediated and humoral immune responses to viral infections

Response	Effector	Activity
Cell mediated	IFN-γ secreted by Th and CTLs	Induces antiviral state
	CTLs	Destroys virus-infected cells
	NK cells and macrophages	Destroys virus-infected cells directly or by antibody-dependent cell-mediated cytotoxicity
Humoral	Primarily secretory IgA	Inhibits virion-host attachment
	Primarily IgG	Inhibits fusion of enveloped viruses with host membrane
	IgG and IgM antibody	Enhances phagocytosis (opsonization) after binding to virions
	IgM antibody	Agglutinates virions
	Complement activated via IgG and IgM	Lyses enveloped viruses; opsonization by C3b/antibody complex

directed against this sugar. It is this antibody that triggers the complement cascade and subsequent lysis of foreign cells and enveloped viruses bearing α-Gal antigens. The anti-α-Gal antibody-complement reaction is probably the primary reason why humans and higher primates are not infected by enveloped viruses of other animals, despite the ability of many of these viruses to infect human cells efficiently in culture. In support of this assertion, when such viruses are grown in nonhuman cells they are sensitive to inactivation by fresh human serum, but when grown in human cells they are resistant. Anti-α-Gal antibodies provide a mechanism for cooperation of the adaptive immune system and the innate complement cascade to provide immediate, "uninstructed" action.

Regulation of the Complement Cascade

Any amplified antiviral defense system as lethal as the actions of the complement cascade must be fail-safe and regulated with precision. Spontaneous activation of any one of the three pathways must be blocked, and triggering by minor infections (which occur regularly) must be avoided. Some regulation is intrinsic to the complement proteins themselves. For example, many are large and therefore cannot leave blood vessels to attack infected tissues unless there is local tissue damage that exposes cells directly to blood. Consequently, minor infections do not activate a substantial complement response. Many cascade intermediates are short-lived, with millisecond half-lives, and therefore do not exist long enough to diffuse far from the site of infection. Further control is maintained by complement-inhibitory proteins present in the serum and on the surface of many cells (e.g., the complement receptor type 1 protein [Cr1], decay-accelerating protein [Daf, or CD55], protectin [CD59], and membrane cofactor protein [CD46]). These proteins are the only regulators that can limit the alternative-pathway cascade by binding complement components such as C3b and C4b. Enveloped viruses that do not carry CD55 or Cr1 on their surfaces are susceptible to the action of complement, particularly via the alternative pathway. Others, such as human immunodeficiency virus type 1 and the extracellular form of vaccinia virus, incorporate CD46, CD55, and CD59 in their envelopes and are thereby protected from complement-mediated lysis.

Many viral genomes encode proteins that interfere with the complement cascade. For example, alphaherpesvirus glycoprotein C binds the C3b component, and several poxvirus proteins bind C3b and C4. The smallpox virus SPICE protein (smallpox inhibitor of complement enzymes) inactivates human C3b and C4b and is a major contributor to the high mortality of smallpox (see Box 2.11).

Several viral receptors, including those for measles virus and certain picornaviruses, are complement control proteins. Epstein-Barr virus particles bind to CD21 (the Cr2 complement receptor) with profound consequences for the host and virus. This interaction activates the Nf-κb pathway, which then allows transcription from an important viral promoter. Epstein-Barr virus binding to the complement receptor enables replication in resting B cells otherwise incapable of supporting viral transcription.

Pattern Recognition by C1q, the Collectins, and the Defensins

The action of the complement initiator protein C1q exemplifies a definitive property of intrinsic and innate defense: C1q can recognize molecular patterns characteristic of pathogens. It has properties of a pattern recognition receptor much like the Toll-like receptors. C1q is a calcium-dependent, sugar-binding protein (a **lectin**) in the collectin family of proteins. These proteins bind to polysaccharides on a wide variety of microbes and act as opsonins or activators of the complement cascade. Defensins represent another class of antimicrobial lectins. They are small (29- to 51-residue), cysteine-rich, cationic proteins produced by leukocytes and epithelial cells that are active against bacteria,

fungi and enveloped viruses. Collectins and defensins bind the glycoproteins of a number of enveloped viruses, including human immunodeficiency virus type 1, herpes simplex viruses, Sindbis virus, and influenza virus. Some collectins and defensins have antiviral activity in cells in culture. The basis for their antiviral activity appears to be inhibition of membrane fusion. An attractive hypothesis is that they function by cross-linking surface glycoproteins and blocking displacement of other proteins from the fusion site. While these interesting lectins display antiviral activity in the laboratory, their physiological contributions have not been well studied. Some have been modified for testing as antiviral compounds to be delivered systemically or topically.

The Inflammatory Response

As we have seen, during the earliest stages of infection, cells produce cytokines as various intrinsic defenses are activated. The rapid release of cytokines and the appearance of soluble mediators of the complement cascade at the site of infection initiate new responses with far-reaching consequences (Fig. 4.5). The multifunctional cytokine Tnf-α, one of the cytokines of early warning, is produced by activated monocytes and macrophages. The action of Tnf-α induces marked changes in nearby capillaries that attract, and facilitate entry of, circulating white blood cells to the site of infection. Tnf-α also can induce an antiviral response when it binds to receptors on infected cells. Within seconds, the combination of infection and binding of Tnf-α to its receptor initiates a signal transduction cascade that activates caspases, resulting in cell death (see Fig. 3.9B). Viral proteins that modulate the function of Tnf-α are well known.

One very visible response to Tnf-α is **inflammation**. The four classical signs of inflammation are redness, heat, swelling, and pain. These symptoms result from increased blood flow, increased capillary permeability, influx of phagocytic cells, and tissue damage. Increased blood flow occurs when vessels that carry blood away constrict, resulting in engorgement of the capillary network in the area of infection. This response produces redness (erythema) and an increase in tissue temperature. Capillary permeability increases, facilitating an efflux of fluid and cells from the engorged capillaries into the surrounding tissue. The fluid that accumulates has a high protein content, in contrast to that of normal fluid found in tissues, and contributes to the swelling. The cells that migrate into the damaged area are largely mononuclear phagocytes (Table 4.1). They are attracted by molecules synthesized by virus-infected cells, by cytokines elaborated by local defensive systems, and by secondary reactions that facilitate adherence of phagocytic cells to capillary walls near sites of damage. Neutrophils are found in abundance in the blood, but normally are absent from tissues. They are the earliest phagocytic cells to be recruited to a site of infection, and are classic cellular markers of the inflammatory response. Neutrophils also secrete a variety of cytokines and toxic products that can determine subsequent events. Some cytokines made by infected cells, dendritic cells, and macrophages, as well as soluble complement components, are chemokines that direct the migration of monocytes and granular cells to regions of cell damage (Fig. 4.5). Monocytes are also important in the healing reactions that take place after the infection is cleared. Not only does the inflammatory response control viral infections and contribute to the overt pathogenic effects that follow, but also, like most innate responses, it is essential for the initiation of adaptive immune defenses.

Noncytopathic viruses do not induce a strong inflammatory response and, as a consequence, have dramatically different interactions with the host. It is now understood that the early reactions in inflammation determine the type of immune response that will predominate, which in turn can influence the outcome of a viral infection.

Despite an incomplete understanding of the process, for many years scientists have induced inflammation deliberately by the use of adjuvants, such as Freund's adjuvant (killed mycobacterial cells in oil emulsion) or aluminum hydroxide gels. The adjuvant-induced inflammation mimics an infection to provide the environment for the induction of a strong immune response to injected viral proteins in vaccines. In fact, most vaccines would not work without adjuvants to stimulate the adaptive immune response (see Chapter 8 for a discussion of adjuvants and vaccines).

The nature and extent of the inflammatory response to viral infection depend on the tissue that is infected, as well as on the cytopathic nature of the virus. Many of the cells and proteins that participate in an inflammatory response come from the bloodstream and are directed by proinflammatory cytokines and soluble complement effectors to the site of infection. As a result, tissues that have reduced access to the circulatory system (e.g., the brain and the interior of the eyeball) normally avoid the destructive effects of the inflammatory response. However, as a consequence of their so-called "privileged" state, the kinetics, extent, and final outcome of viral infections of these tissues can be markedly different from those of tissues with more intimate access to the circulatory system.

The inflammatory response is held under strict control by many regulatory proteins. A critical protein complex in the cytoplasm is called the "inflammasome"—a structure of more than 700 kDa. The inflammasome complex has properties of a cytoplasmic pattern recognition receptor, as well as a signaling initiator. When a microbial molecule is bound, the inflammasome activates the proinflammatory caspases 1 and 5. These two caspases then lead to the

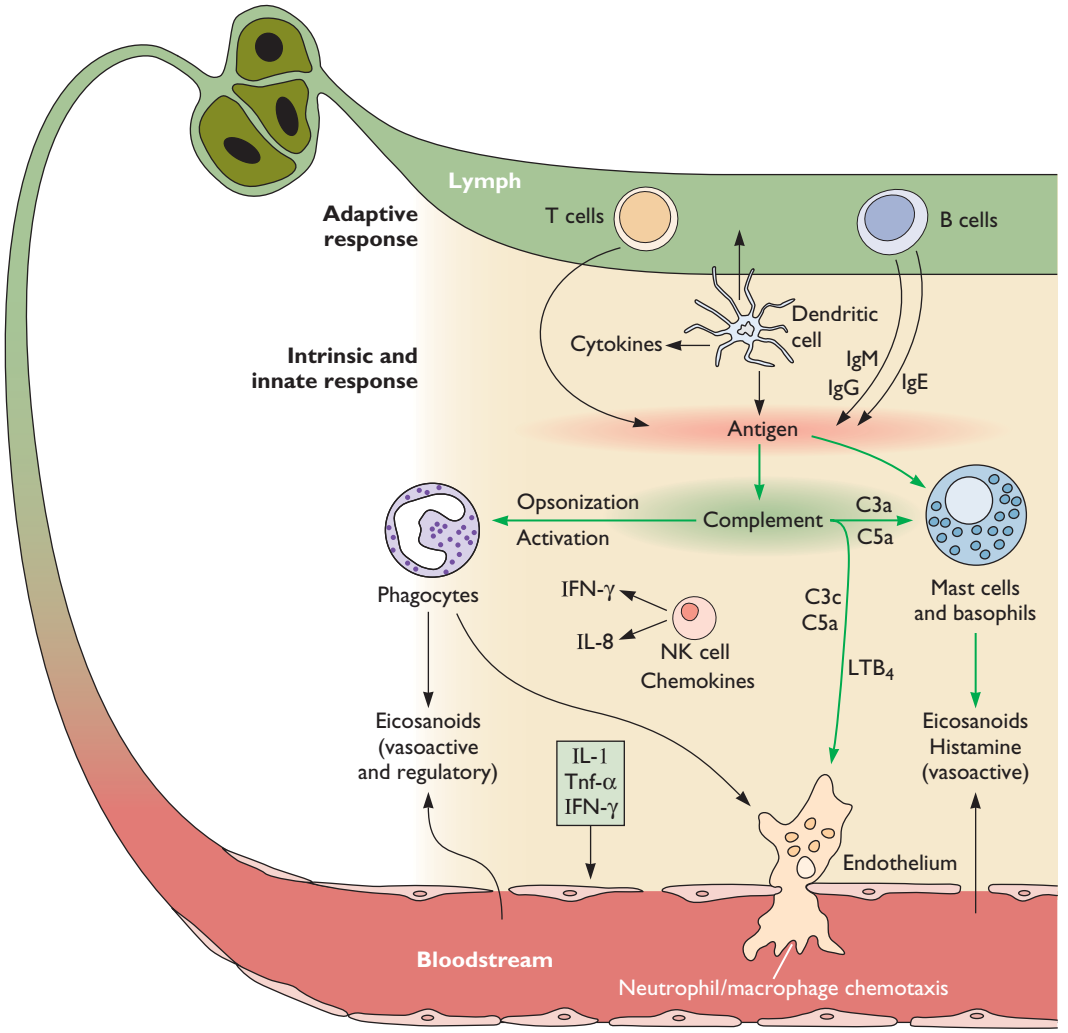


Figure 4.5 Inflammation provides integration and synergy with the main components of the immune system. Viral infections at entry sites in the body often trigger an inflammatory response. A stylized section of infected tissue served by the lympoid system (top, green) and the circulatory system (bottom, red) is shown. Inflammation reactions can be initiated in several ways, for example by cytokines such as IFN released by immature dendritic cells as they detect infection, by the classical or alternative pathway of complement activation, or by mast cells that migrate to sites of cell damage responding to cytokine release, where they can be activated by IgE antibody and antigen. C3a, C3c, and C5a are protease digestion products of the complement cascade that stimulate the inflammatory response. C3a increases vascular permeability and activates mast cells and basophils, C3c stimulates neutrophil release, and C5a increases vascular permeability and chemotaxis of basophils, eosinophils, neutrophils, and monocytes and stimulates neutrophils. The cytokines IL-1, IFN-γ, and Tnf-α act on the local capillary endothelium to enhance leukocyte adhesion and migration. IL-8 and other chemokines promote lymphocyte and monocyte chemotaxis. IL-1 and Tnf-α bind to receptors on epithelial and mesenchymal cells to cause division and collagen synthesis and stimulate prostaglandin and leukotriene synthesis (eicosanoid compounds). LTB_4 is a particularly active leukotriene that is vasoactive and chemotactic. The activities of cells that enter an infected site where inflammation reactions are occurring are controlled by locally produced cytokines, particularly Tnf-α, IL-1, and IFN-γ. Adapted from D. Male et al., *Advanced Immunology*, 3rd ed. (Mosby, St. Louis, MO, 1996).

processing and secretion of the proinflammatory cytokines IL-1β and IL-18. The role of the inflammasome in bacterial infections is well established, and it is likely to function similarly in viral infections.

Viral gene products known to modulate the inflammatory response include soluble cytokine receptors of poxviruses, the complement component-binding proteins of herpesviruses, and various cytokines and growth factors of beta- and gammaherpesviruses. Mutants that do not synthesize these proteins have markedly reduced virulence (virulence is discussed in Chapter 1). Such modulation can be critical for both host and virus. For example, if the normal inflammatory response induced by adenovirus infection is not suppressed by viral proteins encoded in the E3 gene complex, severe damage and even death of the host can result.

The Adaptive Immune Response

General Features

The adaptive response comprises two complex actions, the **humoral response** (antibody) and the **cell-mediated response** (helper and effector cells) (Fig. 4.6 and Table 4.3). As we discuss the cells and processes that characterize each response, it is important to understand that **both** are essential in antiviral defense and function in concert. However, the relative contribution of each in any given infection varies with the nature of both the infecting virus and the host. In general, antibodies bind to virus particles in the bloodstream and at mucosal surfaces, decreasing the number of cells that could have been infected, whereas T cells recognize and kill infected cells.

Like the innate response, the adaptive response to viral infection must distinguish infected from uninfected cells. This feat is accomplished in a markedly different fashion than occurs in the innate immune system. Highly specific molecular recognition is mediated by two antigen receptors: membrane-bound antibody on B cells or the T-cell receptor, and one of two membrane glycoprotein oligomers that display fragments of internal cellular proteins on the cell surface. These latter proteins are members of the MHC protein family. MHC class I proteins display protein fragments on the surface of almost all cells, whereas MHC class II proteins generally are found only on the surfaces of mature dendritic cells, macrophages, and B cells (the professional antigen-presenting cells).

While B- and T-cell receptors both bind foreign antigens, they do so in very different ways. The B-cell receptor is a membrane-bound antibody and binds discrete **epitopes** (contiguous sequences or unique conformations) in intact proteins. In contrast, the T-cell receptor binds short, linear **peptides** derived from proteolytically processed proteins. The act of binding to an epitope or peptide by either receptor has profound effects on the cell bearing that receptor: it responds by producing cytokines, by replicating and dividing, by killing the cell that bears the foreign protein or peptide, or by synthesizing antibodies. The entire sequence of events initiated by the binding of foreign peptides or epitopes comprises the adaptive immune response.

As in all defense mechanisms, an uncontrolled or inappropriate adaptive response can be damaging. Therefore, precise initiation and rapid cessation of the response are as important as its amplification. Regulation is achieved largely by multiple interactions among the surfaces of lymphocytes and infected cells, the short life spans of the activated cells, and the short half-lives of the chemical mediators. For example, complement proteins of the innate immune system play important roles in initiating and regulating the T-cell response (Fig. 4.7). When such regulation is disrupted actively or passively by viral infection, or when the cells of the immune system themselves are infected by viruses, severe consequences ensue. Many pathological effects associated with infection are manifestations of inappropriate reactions of the host immune defense system (see "Injury induced by viruses" below). On the other hand, all successful viral genomes encode proteins that modulate these host defenses. In some instances, viral infections can be long-lived, persistent, or latent in the face of an active adaptive immune system (these situations are discussed in Chapter 5).

Unlike the innate response, the adaptive response is tailored specifically to a particular invading organism or substance and requires more time (days to weeks) after exposure to become fully active. However, once a specific response has been established and the viral infection is subdued, the individual is immune to subsequent infection by the same invader (Fig. 4.8). Such memory of previous infections is one of the most powerful features of the adaptive immune response, and makes vaccines possible. While the primary response takes many days to reach its optimum, a subsequent encounter with an invader by an immune individual engenders a ferocious response that occurs within hours of the infection. The innate defenses also are stimulated during this secondary response to infection. The cellular and molecular mechanisms for maintenance of memory are the focus of considerable research and debate, but a subset of B and T lymphocytes called **memory cells** is maintained after each encounter with a foreign antigen. These cells survive for years in the body and are ready to respond immediately to any subsequent encounter by rapid proliferation and efficient production of their protective products. Because such a secondary response is usually stronger than the primary one, childhood infection

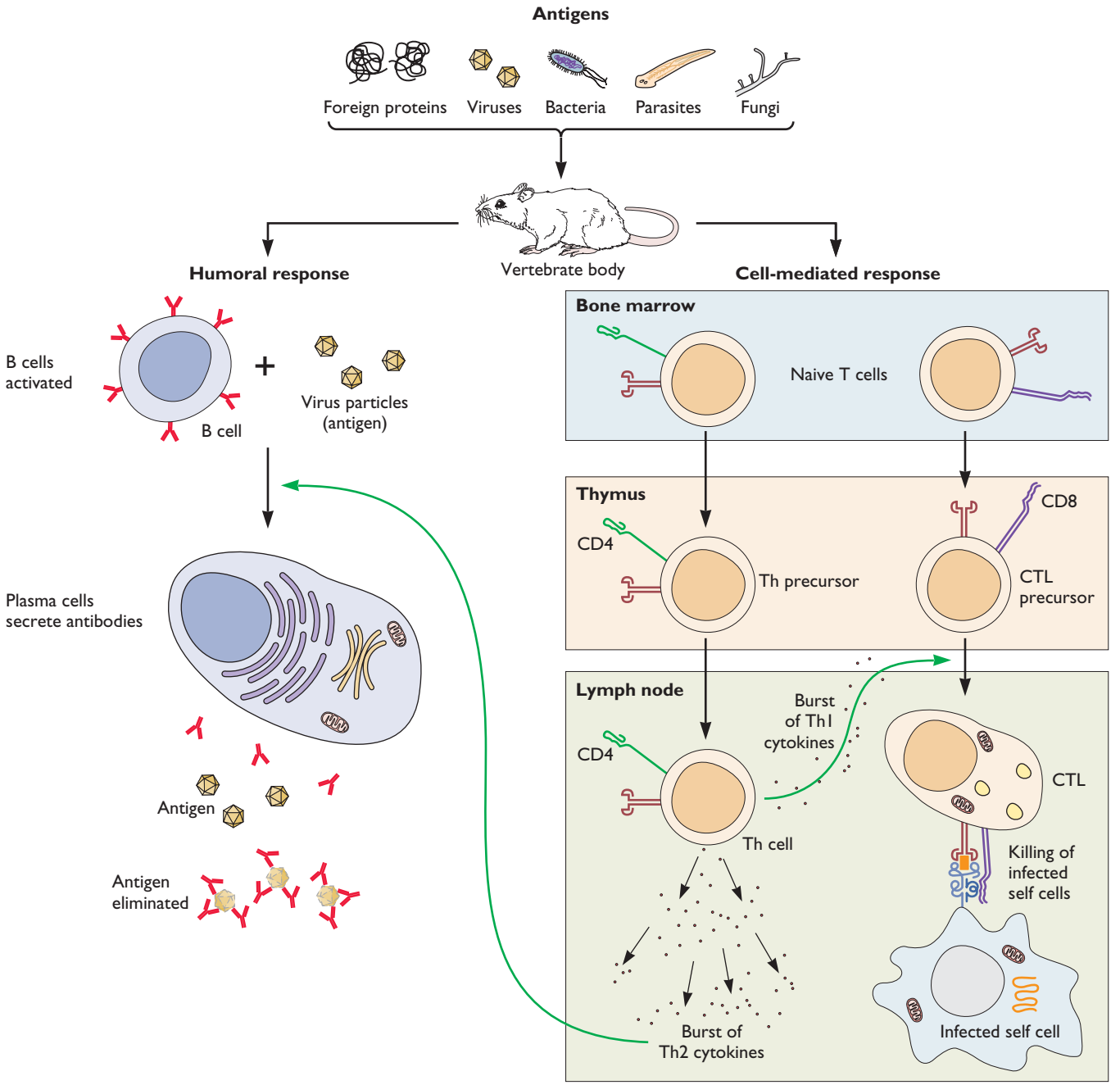


Figure 4.6 The humoral and cell-mediated branches of the adaptive immune system. A variety of foreign proteins and particles (antigens) may stimulate adapative immune responses after recognition by intrinsic and innate defense systems. **(Left)** The humoral branch comprises lymphocytes of the B-cell lineage. Antibodies are the important effector molecules produced by this response. The process begins with the interaction of a specific receptor on precursor B lymphocytes with antigens. Binding of antigen promotes differentiation into antibody-secreting cells (plasma cells). **(Right)** The cell-mediated branch comprises lymphocytes of the T-cell lineage that arise in the bone marrow and are selected in the thymus. The activation process is initiated in lymph nodes when the T-cell receptor on the surface of naive T lymphocytes bind viral peptides on dendritic cells complexed with the MHC class II protein. Two subpopulations of naive T cells are illustrated: the Th-cell precursor and the CTL precursor. The Th cell recognizes antigens bound to MHC class II molecules and produces powerful cytokines that "help" activated B cells to differentiate into antibody-producing plasma cells (Th2 cytokines) or CTL precursors (Th1 cytokines) to differentiate into CTLs capable of recognizing and killing virus-infected cells. The Th1 or Th2 cytokines are produced by different subsets of Th cells and promote or inhibit cell division and gene activity of B-cell or CTL precursors.

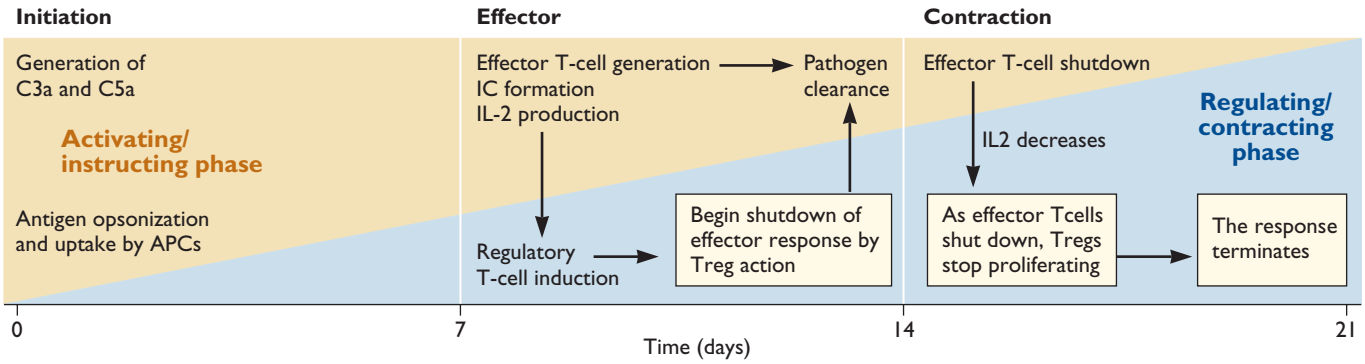


Figure 4.7 Regulation of the T-cell response by complement and regulatory T cells. The extent of the adaptive immune response is regulated in part by regulatory T cells (Treg cells). This model of an acute viral infection provides a view of how complement (part of the innate immune response) may regulate the three phases of a T-cell-mediated response. **(Initiation)** Soon after infection, antigen-presenting cells (APCs) take up viral proteins and make their way to local lymph nodes, where the T-cell response is initiated. The complement cascade stimulated at the site of infection produces a variety of effector proteins, including C3a, C5a, and C3b. The C3b opsonin facilities the uptake of C3b-coated antigens by antigen-presenting cells, while C3a and C5a stimulate their maturation. **(Effector)** Mature antigen-presenting cells then engage potential effector T cells in lymph nodes, resulting in production of IL-2 and activation of CD46-stimulated Treg cells. Ligands for CD46 include C3b-opsonized immune complexes (IC formation). Effector CTLs and Th1 cells are stimulated by the antigen-presenting cells and leave the lymph node to attack the site of infection and clear the infected cells and virions. The balance between activated CTLs and CD46-stimulated Treg cells determines the extent of CTL action as well as the degree of immunopathology promoted by CTL action. Too many CTL cells can cause damage, but too few cannot clear the viral infection; conversely, too many Treg cells shut down the effector response prematurely, while too few Treg cells promote continued CTL action and potential immunopathology. **(Contraction)** The dynamics of CTL and Treg cell proliferation promote the controlled contraction of the CTL response. Both CTLs and Treg cells rapidly decline in numbers at this stage. The contraction occurs in part because CD46-stimulated Treg cells divide more quickly than CTLs, and through the action of Treg cytokines, the CTL response shuts down. Because the activated CTLs and Th cells produce IL-2 necessary for Treg-cell replication, the pool of Treg cells then diminishes as the system returns to its unstimulated state. During this phase, memory CTL and memory Treg cells also are produced. Adapted from C. Kemper and J. Atkins, *Nat. Rev. Immunol*. **7:**9–18, 2007.

protects adults, and immunity conferred by vaccination can last for years.

Cells of the Adaptive Immune System

The adaptive response depends on two important cell groups: lymphocytes and antigen-presenting cells (Table 4.1). Lymphocytes are white blood cells produced by hematopoiesis in the bone marrow. They are migratory cells, leaving the bone marrow to circulate in the blood and lymphatic system, settling in various lymphoid organs throughout the body (Fig. 4.9). The small organs called lymph nodes are essential for initiation of the adaptive immune response: when lymph nodes are removed, viral infections do not stimulate adaptive immunity. Lymphoid tissues are the collection centers and sites of communication for cells in the circulatory system.

T cells and **B cells** represent two major classes of lymphocytes. T cells (lymphocytes that mature in the thymus) and particular cytokines are the primary components of the cell-mediated response. Immature T cells differentiate into two critical effector T cells, the T-helper (Th) cell and the cytotoxic T cell (cytotoxic T lymphocyte [CTL]). Plasma cells, whose immediate precursors are B cells (lymphocytes derived from the bone marrow, the mammalian equivalent of the avian bursa), make antibodies that bind to foreign molecules and define the antibody response (also known as the humoral response).

During the early maturation of T and B cells, cells that react against self tissues and proteins die. The remaining T and B cells have the capacity to recognize nonself molecules, but they remain in a dormant state in lymphoid tissue until they physically interact with particular lymphocytes. These lymphocytes (called professional antigen-presenting cells) move back and forth from peripheral tissues to lymphoid tissues. Their function is to expose on their surfaces peptides and proteins gathered from the peripheral tissues so that they can be bound by T- and B-cell receptors. When the peptides are recognized as nonself, the T or B cells are stimulated to divide and carry out their immune effector actions. Dendritic cells are crucial

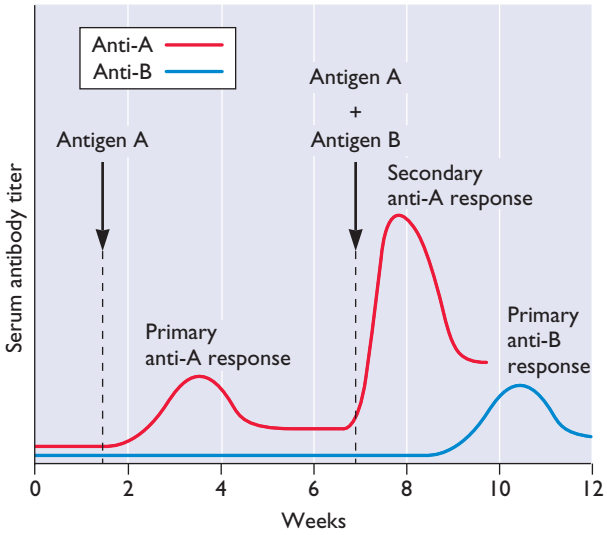


Figure 4.8 The specificity, self-limitation, and memory of the adaptive immune response. This general profile of a typical adaptive antibody response demonstrates the relative concentration of serum antibodies after time (weeks) of exposure to antigen A or a mixture of antigens A and B. The antibodies that recognize antigens A and B are indicated by the red and blue lines, respectively. The primary response to antigen A takes about 3 to 4 weeks to reach a maximum. When the animal is injected with a mixture of both antigens A and B at 7 weeks, the secondary response to antigen A is more rapid and more robust than the primary response. However, the primary response to antigen B again takes about 3 to 4 weeks. These properties demonstrate immunological memory. Antibody levels (also termed titers) decline with time after each immunization. This property is called self-limitation or resolution. From A. K. Abbas et al., *Cellular and Molecular Immunology* (The W. B. Saunders Co., Philadelphia, PA, 1994), with permission.

professional antigen-presenting cells, as discussed in Chapter 3. Immature B cells and cells of the monocyte lineage (e.g., macrophages) are also considered to be professional antigen-presenting cells.

The Mucosal and Cutaneous Arms of the Immune System

The adaptive immune system exhibits considerable decentralization of important cells and tissues. However, every major organ and body surface harbors components that are coordinated to mount a focused, adaptive immune response when signaled by the innate immune system. The **mucosal immune system** is usually the first adaptive defense to be engaged after infection. The lymphoid tissues below the mucosa of the gastrointestinal and respiratory tracts (often called mucosa-associated lymphoid tissue) (Fig. 4.9B) are vital in antiviral defense. These clusters of lymphoid cells include the collection called Peyer's patches in the lamina propria of the small intestine, the tonsils in the pharynx, the submucosal follicles of the upper airways, and the appendix. A specialized epithelial cell of mucosal surfaces is the **M cell** (microfold or membranous epithelial cell), which samples and delivers antigens to the underlying lymphoid tissue by transcytosis. M cells have invaginations of their membranes (pockets) that harbor immature dendritic cells, B and $CD4^+$ T lymphocytes, and macrophages. The secreted antibody IgA (important in antiviral defense at mucosal surfaces [see below]) is made by B cells that accumulate at adhesion sites in these M cell membrane pockets. After viral proteins transit through M cells, they emerge to be in intimate contact with all the appropriate immune cells. This process represents an essential step for the development of mucosal immune responses.

The skin, the largest organ of the body, possesses its own complex community of organized immune cells. Lymphocytes and Langerhans cells comprise the **cutaneous immune system** (also called skin-associated lymphoid tissue) (Fig. 4.9C). These cells are important in the initial response and resolution of viral infections of the skin. In particular, Langerhans cells, the predominant scavenger antigen-presenting cells of the epidermis, function as the sentinels or outposts of early warning and reaction. These abundant, mobile cells sample antigens and migrate to regional lymph nodes to transfer information to T cells, and to activate B lymphocytes directly. Certain T cells in the circulation have tropism for the skin and, after binding to the vascular endothelium, can enter the epidermis to interact with Langerhans cells and keratinocytes. These skin-tropic T cells play important roles in production of the virus-specific skin rashes and pox characteristic of measles virus and varicella-zoster virus infections.

Virus particles can interact with lymphoid cells associated with mucosal and cutaneous immune systems at the primary site of infection. Such events can suppress immune responses by killing or misregulation of immune cells. These interactions can govern the outcome of the primary infection and often establish the pattern of infection characteristic of a given virus. The M cells in the mucosal epithelium have been implicated in the spread from the pharynx and the gut to the lymphoid system of a variety of viruses, including poliovirus, enteric adenoviruses, human immunodeficiency virus type 1, and reovirus. These cells also have been suggested to be sites of persistent or latent infection for a number of other viruses, including herpes simplex virus.

Adaptive Immunity: the Action of Lymphocytes That Carry Distinct Antigen Receptors

Unlike the germ line-encoded pattern recognition receptors of the intrinsic defenses and innate immune system,

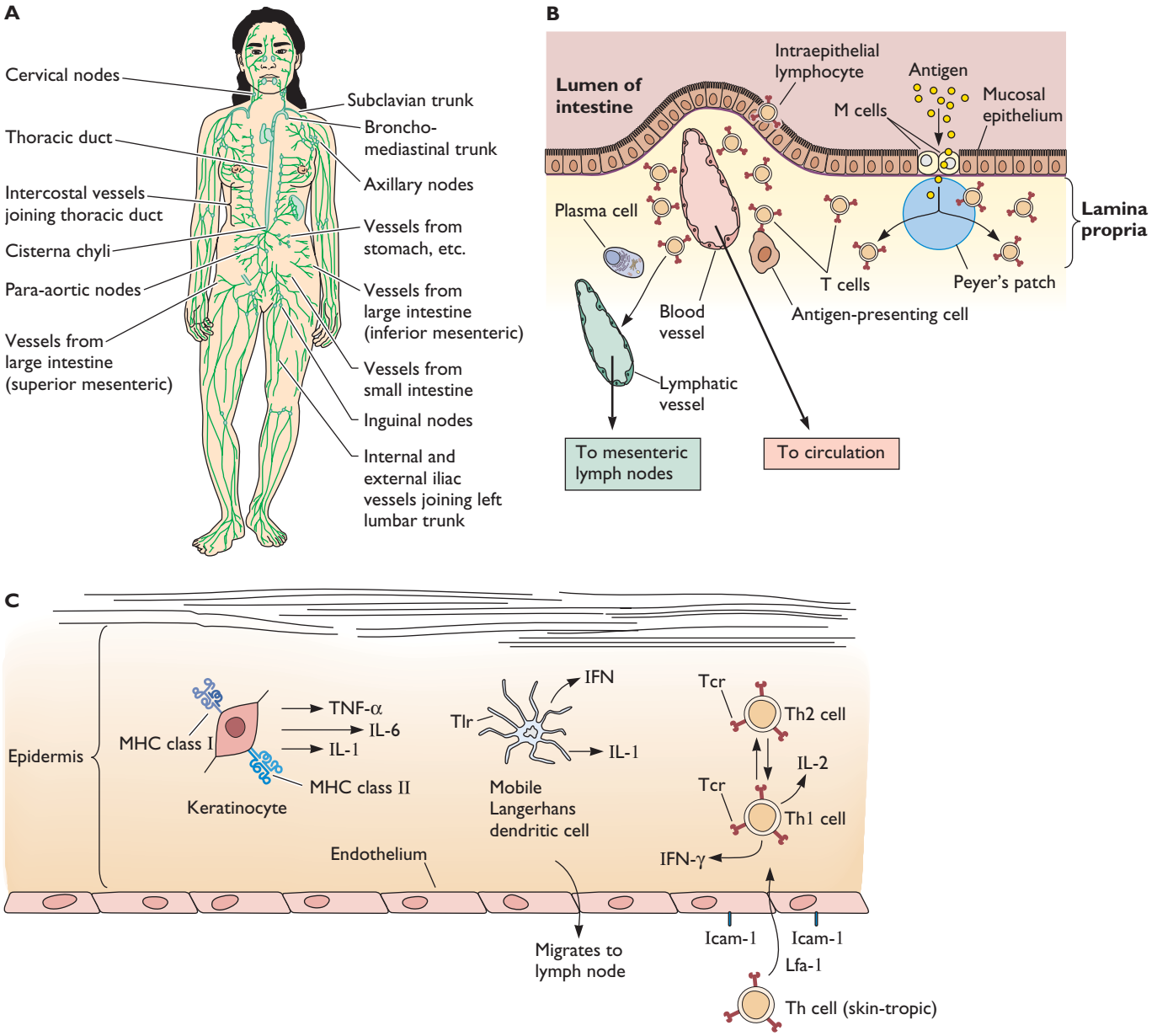


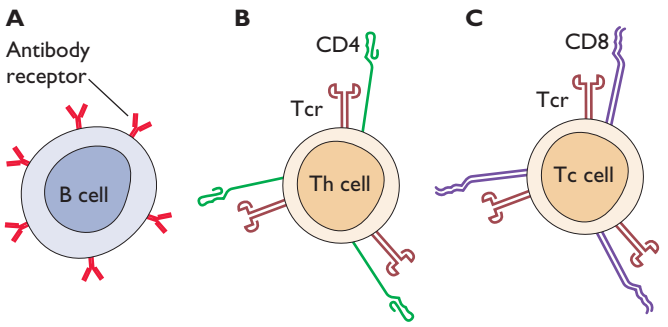
Figure 4.9 Components of the human lymphatic and mucosal immune systems. (A) The primary lymphatic system is illustrated in green. Many clusters of lymphatic tissue, called nodes, are found throughout the lymphatic system. Lymph nodes provide the interactive environment where mobile dendritic cells patrolling the local area exchange information with lymphocytes in the circulation. **(B)** Cellular components of the mucosal immune system in the gut (mucosa-associated lymphoid tissue). The lumen of the small intestine is at the top of the figure. The mucosal epithelial cells are shown with their basal surface oriented toward the lamina propria. Cross sections of a lymphatic vessel and a capillary are shown, illustrating their juxtaposition to cells of the mucosal immune system. M cells have large intraepithelial pockets filled with B and $CD4^+$ T lymphocytes, macrophages, and dendritic cells. M cells and intraepithelial lymphocytes are important in the transfer of antigen from the intestinal lumen to the lymphoid tissue in Peyer's patches, where an immune response can be initiated. **(C)** The cutaneous immune system (skin-associated lymphoid tissue) comprises three cell types: keratinocytes, Langerhans cells, and T cells. Keratinocytes actively secrete various cytokines, including Tnf-α, IL-1, and IL-6, and have phagocytic activity. They also synthesize both MHC class I and MHC class II proteins and can present antigens to T and B cells if stimulated by IFN-γ. Langerhans cells are migratory dendritic cells and are the major antigen-presenting cells in the epidermis. When products of viral infections in the skin are detected, Langerhans cells secrete IFN and undergo maturation. Mature dendritic cells migrate to the local draining lymph node, where they present viral peptides on both MHC class I and MHC class II proteins to antigen-specific T cells. Special skin-tropic T cells can cross the endothelium to enter the epidermis, where they can mature into Th1 or Th2 cells depending on the antigen and cytokine milieu. Activated T cells synthesize cytokines, including IFN-γ, that activate MHC expression from keratinocytes and Langerhans cells. Tcr, T-cell receptor. Adapted from A. K. Abbas et al., *Cellular and Molecular Immunology* (The W. B. Saunders Co., Philadelphia, PA, 1994), with permission.

the T- and B-cell antigen receptors are formed by somatic gene rearrangement during development of the organism. Their specificities are built without regard to any one target. Subsequently, cells that can bind a particular antigen are killed if they recognize self antigens, or stimulated to divide and prosper if they recognize a foreign invader or infected cell. Lymphocytes are responsible for specificity, memory, and self-nonself discrimination.

As noted above, each T or B cell carries on its surface a specific receptor that binds and responds to a particular peptide or epitope (Fig. 4.10). Antiviral defense is possible because T and B cells bearing these specific receptors have survived the normal selective process that eliminates those that respond to self peptides and self epitopes. The cells emerging from such selections enter the circulation and pass through the lymphatic system, or are retained in various tissues in the body (Fig. 4.6). They are said to be **naive,** because they are not completely differentiated and are not armed to produce their ultimate immune effector response. However, they are able to react to foreign signals with remarkable diversity and specificity. After such recognition, they rapidly acquire their specific immune effector activity.

The initial encounter with any foreign epitope, whether in lymphoid tissues or elsewhere in the body, involves only a few cells. For example, the frequency of B or T lymphocytes that recognize infected cells on first exposure is as few as 1 in 10,000 to 1 in 100,000. So few cells are certainly not sufficient for protection against an infection that is spreading in the host. What makes the adaptive response so powerful is that the initial response is amplified substantially during the ensuing 1 or 2 weeks: the number of virus-specific lymphocytes increases more than 1,000-fold. The original encounter stimulates these uncommitted, naive lymphocytes to differentiate, divide, and produce antibodies or cytokines. The antigen-stimulated cell is said to be **activated.** It then becomes fully differentiated and undergoes numerous rounds of cell division such that each daughter cell has the same specific immune reactivity as the original parent (often called a clonal response).

Figure 4.10 The antigen receptors on the surfaces of B and T cells. (A) Each B cell has about 100,000 molecules of a unique membrane-bound receptor antibody. Every receptor antibody on a given B cell has identical bivalent specificity for one antigen epitope. **(B and C)** Each T lymphocyte has about 100,000 T-cell receptors (Tcr), each with identical specificity. **(B)** T cells bearing the surface membrane protein CD4 always recognize peptide antigens bound to MHC class II proteins and generally function as Th cells. **(C)** T cells bearing the surface membrane protein CD8 always recognize peptide antigens bound to MHC class I proteins and generally function as cytotoxic T (Tc) cells.



B Cells

B cells are produced in the bone marrow. As they mature, each synthesizes an antigen receptor, which is a membrane-bound antibody (Fig. 4.6 and 4.10). When an antigen binds specifically to a membrane-bound antibody, a signal transduction cascade is initiated. As a consequence, new gene products are made and the cell begins to divide rapidly. The daughter cells produced by each division differentiate into effector plasma cells and a small number of memory B cells. As their name implies, memory B cells, or their clonal progeny, are long-lived and continue to produce the parental, membrane-bound antibody receptor. In contrast, plasma cells live for only a few days and no longer make membrane-bound antibody, but instead synthesize the same antibody in secreted form. A single plasma cell can secrete more than 2,000 antibody molecules per second.

T Cells

T-cell precursors are also produced in the bone marrow, but in contrast to a B-cell precursor, a T-cell precursor must migrate to the thymus gland to mature (Fig. 4.6). The notation "T" in "T cell" reminds us that the thymus is required for their development. T cells can be distinguished from other lymphocytes by a special receptor on their surface, called the T-cell receptor. Subsets of T cells have distinct functions.

The maturation process comprises two types of selection: positive selection for T cells that can bind appropriate surface molecules via the T-cell receptor and negative selection that efficiently kills T cells that recognize target cells displaying self peptides on their surfaces. As a result, only 1 to 2% of all immature T cells entering the thymus emerge potentially able to defend the host against viral infections. These naive T cells are now able to respond to nonself antigens in lymphoid tissues. When they encounter such antigens presented to them by mature dendritic cells, they radically change their complement of cell surface proteins. Some differentiate into effector cells that can kill target cells when they leave the lymph node (CTLs),

and some differentiate into helper cells that can stimulate B cells to make antibody (Th cells). Yet others become memory cells that retain the capacity to differentiate into effector cells when they reencounter the stimulating antigen, and some (Treg cells) function to shut down the T-cell-mediated response at the end of an immune response (Fig. 4.7).

As noted above, T cells synthesize the T-cell receptor, which is capable of binding peptides. The T-cell receptor is a disulfide-linked heterodimer composed of either alpha and beta or gamma and delta protein chains. The peptide-binding site of the T-cell receptor and the epitope-binding site of the B-cell receptor are very similar structures, formed by the folding of three regions in the amino-terminal domains of the proteins that participate in antigen recognition (the so-called hypervariable regions). However, unlike the B-cell receptor, which can recognize the epitope as part of an intact folded protein, the T-cell receptor can recognize **only** a peptide fragment produced by proteolysis. Furthermore, the peptide must be bound to MHC cell surface proteins (see below). When the T-cell receptor engages an MHC molecule carrying the appropriate antigenic peptide, a signal transduction cascade that leads to gene expression is initiated. As a result, the stimulated T cell is capable of differentiating to form memory and various effector T cells.

Th Cells and CTLs Are Distinguished by Unique Cell Surface Proteins

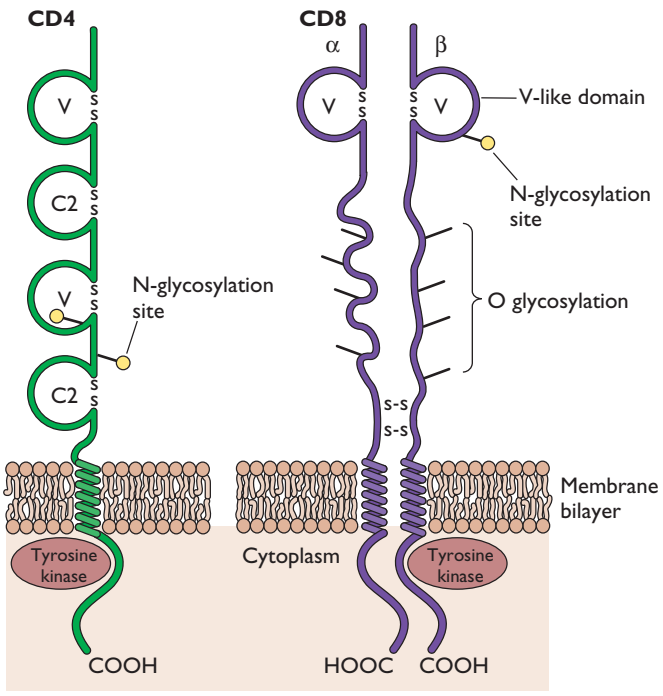
In general, lymphocytes can be distinguished by the presence on their surfaces of specific proteins called **cluster-of-differentiation (CD) markers** (e.g., CD3, CD4, and CD8). The presence of these proteins can be detected with antibodies raised against them in heterologous organisms; they are often referred to as "CD antigens." The over 247 individual CD markers known are invaluable in identifying lymphocytes of a particular lineage or differentiation stage. Two well-known subpopulations of T cells are defined by the presence of either the CD4 or the CD8 surface proteins (Fig. 4.11), which are coreceptors for MHC class II and MHC class I, respectively. When immature T cells leave the bone marrow, they do not synthesize CD4 or CD8 proteins (they are said to be "double-negative"). They differentiate sequentially in the thymus, initially producing both CD4 and CD8 proteins ("double-positive") and then either CD8 or CD4 ("single-positive"). These single-positive cells are the naive T cells that migrate to peripheral sites.

CD4⁺ T cells are generally Th cells capable of interacting with B cells and antigen-presenting cells that have MHC class II proteins on their surfaces. After such interactions, CD4⁺ Th cells mature into Th1 or Th2 cells (see below). Th cells synthesize cytokines and growth factors that stimulate (help) the specific classes of lymphocytes with which they interact. **CD8⁺ T cells** differentiate into CTLs that can interact with almost all cells of the body expressing the more ubiquitous MHC class I proteins. Cytotoxic T cells recognize foreign peptides complexed with MHC class I proteins and, when productively engaged, actively destroy the cell presenting the peptides. Mature cytotoxic T cells play important roles in eliminating virus-infected cells from the body by cell lysis and by production of IFN-γ and Tnf-α.

Th1 and Th2 Cells

When naive Th cells engage mature dendritic cells in lymphoid tissue, cytokines and receptor ligand interactions

Figure 4.11 Simplified representations of CD4 and CD8 coreceptor molecules. These two molecules associate with the T-cell receptor on the surface of T cells. The CD4 molecule is a glycosylated type 1 membrane protein and exists as a monomer in membranes of T cells. It has four characteristic immunoglobulin-like domains labeled here as V and C2. The V domains are similar to the variable domain of immunoglobulin in the tertiary structure. The first two domains form a binding site for MHC class II proteins. The cytoplasmic domain interacts with specific tyrosine kinases, endowing CD4 with signal transduction properties. The CD8 molecule is a type 1 membrane protein with both N and O glycosylation. It is a heterodimer of an α chain and a β chain covalently linked by disulfide bonds that interacts with MHC class I proteins. The two polypeptides are quite similar in sequence, each having an immunoglobulin-like V domain thought to exist in an extended conformation. Tyrosine kinases also associate with the CD8 cytoplasmic domain and participate in signal transduction reactions.



stimulate the T cell to differentiate into one of two Th cell types called Th1 and Th2 (Fig. 4.6; Fig. 4.12). These two cell types can be distinguished by the cytokines they produce and the processes they invoke. Th1 cells are important for controlling most, but not all, viral infections. They promote the cell-mediated response by stimulating the maturation of cytotoxic T-cell precursors. They accomplish this, in part, by producing IL-2 and IFN-γ, cytokines that stimulate inflammation (the proinflammatory response). In addition, Th1 cells provide stimulating cytokines to the antigen-presenting dendritic cell so that it can communicate with naive $CD8^+$ T cells. We know that if IL-12 is present at the time of antigen recognition, immature Th cells differentiate into Th1 cells. IL-12 also stimulates NK and Th1 cells to secrete IFN-γ, a cytokine important in increasing the activity of inflammatory cells such as macrophages.

In the presence of IL-4, immature Th cells differentiate into Th2 cells that stimulate the antibody response rather than the cell-mediated, proinflammatory response. The initial source of IL-4 may be the natural killer T cells (NKT cells) discussed below. Th2 cells promote the antibody response by inducing maturation of immature B cells and resting macrophages. They also reduce the inflammatory response by producing IL-4, IL-6, and IL-10, but not IL-2 or IFN-γ. Th2 cells are more active after invasion by extracellular bacteria or multicellular parasites. Nevertheless, the Th2 response is critical for controlling infections that produce large quantities of virus particles in the blood.

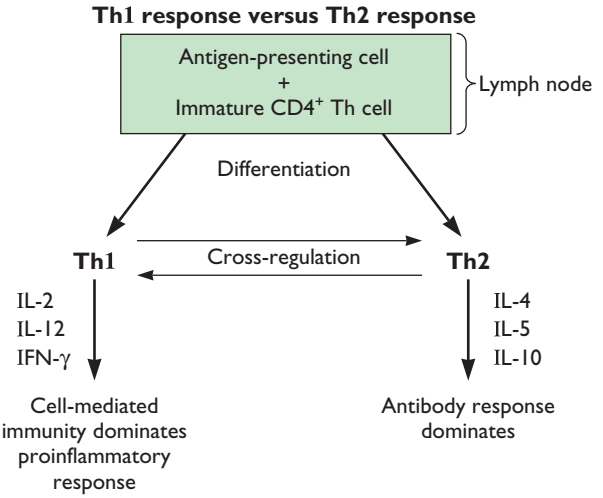
In general, Th1 and Th2 responses have a yin-yang relationship: as one increases, the other decreases (Fig. 4.12). While IFN-γ turns up the Th1 response, it also inhibits the synthesis of IL-4 and IL-5 by Th2 cells, effectively dampening the latter response. On the other hand, production of Th2 cytokines is an important mechanism to shut off the proinflammatory and potentially dangerous Th1 response.

Viral infection induces the production of proinflammatory Th1 cytokines such as IL-12. In contrast, bacterial infection induces synthesis of cytokines, such as IL-4, that promote the Th2 response. The mechanisms that provide such precise regulation are under study. One idea is that mature dendritic cells automatically produce proinflammatory cytokines as their default pathway, and always activate a Th1 response unless appropriate Th2 signals are provided. A similar idea gaining credence is that when dendritic cells detect CpG sequences, single-stranded RNA ssRNA, or double-stranded RNA dsRNA sequences via their Toll-like receptors, Nf-κb is activated and Th1 cytokine genes are transcribed.

We know that many viral proteins modulate the Th1-Th2 balance in interesting ways. For example, infection of B cells by Epstein-Barr virus and equine herpesvirus type 2 should stimulate an active Th1 response. However, both viral genomes encode proteins homologous to IL-10, a regulatory cytokine that represses the Th1 response. Viral IL-10 foils the Th1 antiviral defense that would kill infected B cells, while promoting differentiation into memory B cells that are important for long-term survival of the viral genome.

For most viral infections, a given Th response represents a spectrum of some Th1 and some Th2 cells, and consequently a mixture of cytokines. Establishment of the proper repertoire of Th cells therefore is an important early event in host defense; an inappropriate response has far-reaching consequences. For example, synthesis of the Th2 cytokine IL-4 by an attenuated mousepox virus recombinant resulted in lethal, uncontained spread of virus in an immune animal (see Box 2.8). How the fundamental Th1-Th2 decision is made is under much scrutiny in laboratories around the world. A practical reason for such interest

Figure 4.12 Th cells: the Th1 versus the Th2 response. Immature $CD4^+$ Th cells differentiate into two general subtypes called Th1 and Th2, defined functionally according to the cytokines they secrete. Th1 cells produce cytokines that promote the inflammatory response and activity of cytotoxic T cells, and Th2 cells synthesize cytokines that stimulate the antibody response. The cytokines made by one class of Th cell tend to suppress production of those of the other class.



Immune cross-regulation by cytokines

Th1 response		Th2 response	
Enhance	Suppress	Enhance	Suppress
IL-2 IL-12 IFN-γ	IL-4 IL-10	IL-4 IL-5 IL-6 IL-10	IFN-γ IL-12

is that the design of potent and effective vaccines depends on stimulating the appropriate spectrum of response.

Th17 Cells

This new class of $CD4^+$ helper cells plays central roles in control of the inflammatory response. These cells are found in the skin and the lining of the gastrointestinal tract and at other interfaces between the external and internal environments. When dendritic cells present antigens to them in the presence of Tgf-β and IL-6, Th17 cells secrete IL-17 and IL-21. In addition, the stimulated Th17 cells now express the receptor for IL-23, which leads to massive proliferation of the activated Th17 cells. The activated cells stimulate a strong inflammatory response, secrete defensins, and recruit neutrophils to the site of activation. Th17 cells have been implicated in autoimmune diseases involving chronic inflammation and in the control of bacterial infections. Their importance in controlling viral infections is only now being understood. For example, individuals with large numbers of Th17 cells in their gut mucosa appear to be able to control lentivirus infections much better than individuals with reduced numbers of these helper cells.

Memory T Cells

Memory T cells are long-lived, mature T cells. Each of their T-cell receptors binds to a specific nonself peptide. When the specific nonself peptide is bound, the cells divide rapidly, producing active effector T cells. Memory T cells can carry either CD8 or CD4 surface proteins. Whether memory cells are sequestered in various depots in the body for future use, or are constantly produced, is controversial. One idea of how memory is maintained is that follicular dendritic cells in lymph nodes bind antibody-antigen complexes and keep such complexes on their surfaces for long periods. When the circulating antibody concentration drops, as happens after an infection is cleared, antigen is released gradually from the immune complexes. Such slow release of antigen would provide continual stimulation of the immune response.

If memory cells are infected directly by viruses, as is the case for human immunodeficiency virus type 1, the host's secondary immune response to a wide variety of previous infectious agents can be compromised.

Regulatory T Cells

The regulatory T-cell (Treg) subset of T cells has been recognized for some time (initially they were called suppressor T cells). However, their function was controversial until recently. Now it is clear that Treg cells are pivotal players in the end-stage immune response to most if not all infectious agents. Their primary function is to terminate the immune response and bring the immune system back to ground state (immune homeostasis; Fig. 4.7). These cells are also important for immune suppression, self-tolerance, and control of the inflammatory response. Treg cells serve to maintain a balance between protection and immune pathology. Ironically, Treg action may limit the effectiveness of vaccines because they shut down the immune response.

NKT Cells

NKT cells have a T-cell receptor, a property that distinguishes them from natural killer (NK) cells described previously. NKT cells do have some surface molecules in common with NK cells, hence their name. NKT cells play critical roles in early innate and adaptive responses. Unlike conventional T cells that recognize peptides bound to MHC class I molecules on target cells, NKT cells recognize glycolipid molecules bound to CD1d, a distant cousin of the MHC class I proteins. If a foreign glycolipid is recognized, the NKT cell can act as a Th cell or a cytotoxic cell. NKT cells account for 20 to 30% of lymphocytes present in the liver, and are capable of releasing IFN-γ after viral infection. Their role in antiviral defenses is only now being appreciated.

γδ T Cells

γδ T cells, a small subset of T cells, develop in the thymus as do all T cells, but after that, the similarity ends. These cells reside at the crucial interface between the outside world and tissues. Their name derives from their unique cell surface γδ T-cell receptor. They are abundant in epithelial cell layers, including the gut mucosa, skin, and lining of the vagina. Curiously, the antigens recognized by γδ T cells are not bound to classical cell surface MHC proteins and include not only peptides but also intact proteins and organic molecules that contain phosphorus. These cells do not interact with professional antigen-presenting cells (dendritic cells or macrophages), nor do they express CD8 or CD4 proteins on their cell surface. Long ignored because they were difficult to identify, purify, and study, the γδ T cells now are seen as crucial players in front-line immune surveillance and antiviral action.

Antigen Presentation and Activation of Immune Cells

Naive T cells engage the professional antigen-presenting cells by binding to MHC class II proteins (Fig. 4.6). This encounter results in differentiation of T cells into effector cells, the **CTLs**. These cells enter the circulation and are able to distinguish infected cells from uninfected cells by specific interactions of the T-cell receptor with MHC class I proteins (Box 4.4). MHC class I proteins are found on the surfaces of nearly all nucleated cells. The MHC class I protein

BOX 4.4 EXPERIMENTS

Virology provides Nobel Prize-winning insight: MHC restriction

In 1974 at the John Curtin School of Medical Research in Canberra, Australia, Rolf Zinkernagel and Peter Doherty performed a classic experiment that provided insight into how CTLs can recognize virus-infected cells. Initially, they teamed up to determine the mechanism of the lethal brain destruction observed when mice are infected with lymphocytic choriomeningitis virus, an arenavirus that does not directly kill the cells it infects. They anticipated that the brain damage was due to CTLs responding to replication of the noncytopathic virus in the brain.

When they infected mice of a particular MHC type with the virus and then isolated T cells, these cells lysed virus-infected target cells in vitro **only** when the target cells and the T cells were of identical MHC haplotype. Uninfected target cells were not lysed, even when they shared identical MHC alleles. This requirement for MHC matching was called **MHC restriction**.

Their Nobel Prize-winning insight was that a CTL must recognize two determinants present on a virus-infected cell: one specific for the virus and one specific for the MHC of the host. We now know that CTLs recognize a short peptide derived from viral proteins and only engage the peptide when it is bound to MHC class I proteins present on the surface of target cells.

Zinkernagel, R. M., and P. C. Doherty. 1974. Restriction of in vitro T-cell mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogenetic system. *Nature* **248:**701–702.

comprises two subunits called the α chain (often called the heavy chain) and β_2-microglobulin (the light chain). Lymphocytes possess the highest concentration of MHC class I protein, with about 5×10^5 molecules per cell. In contrast, fibroblasts, muscle cells, and liver hepatocytes carry much smaller quantities, sometimes 100 or fewer molecules per cell. There are three MHC class I loci in humans (*A, B,* and *C*) and two in mice (*K* and *D*). Because there are many allelic forms of these genes in outbred populations, MHC class I genes are said to be **polymorphic**. For example, at the human MHC class I locus *hla-B,* more than 149 alleles with pairwise differences ranging from 1 to 49 amino acids have been identified. When cells bind cytokines such as IFN and IFN-γ, transcription of the MHC class I α chains, β_2-microglobulin, and the linked proteasome and peptide transporter genes (see below), is markedly increased.

Synthesis of MHC class II proteins occurs primarily in the professional antigen-presenting cells (dendritic cells, macrophages, and B cells). Other cell types, including fibroblasts, pancreatic β cells, endothelial cells, and astrocytes, can produce MHC class II molecules, but only on exposure to IFN-γ. As with MHC class I, there are many alleles of MHC class II genes.

The defining property of both MHC class I and class II proteins is that they specialize in binding short peptides produced inside the cell and presenting them for recognition by lymphocytes with T-cell receptors on their surfaces. Both classes of MHC protein have a peptide-binding cleft that is sufficiently flexible to accommodate the binding of many peptides (Fig. 4.13 and 4.14). Even so, not all possible peptides are bound. The ability of MHC molecules to bind and display peptides on the cell surface varies from individual to individual as a result of the many MHC alleles. Such diversity of MHC alleles plays an important role in an individual's capacity to respond to various infections. The more diversity, the more robust the immune response. This fact has dramatic consequences for the spread of viral diseases in a given population. For example, individuals in inbred populations lose MHC diversity over time and also have a reduced capacity to respond to infections. Protective immunity is difficult to establish, and epidemics are likely.

T Cells Recognize Infected Cells by Engaging the MHC Class I Receptors

The immune system must destroy virus-infected cells while ignoring uninfected cells. As viruses can infect many cell types, the recognition/destruction system certainly operates on all cells with high fidelity. Virus-infected cells are identified, in part, because they display small viral peptides as well as cell peptides complexed to MHC class I proteins on their surfaces. The viral and cellular peptides are produced by **endogenous antigen presentation** (Fig. 4.13).

In uninfected and infected cells, a fraction of most newly synthesized proteins is broken down in a controlled manner by the proteasome. The targeted protein is marked for destruction by the covalent attachment of multiple copies of a small protein called ubiquitin and, following adenosine 5′-triphosphate (ATP)-dependent unfolding, the protein is degraded in the inner chamber of the proteasome. The peptide products are released and transported into the endoplasmic reticulum (ER) by a specific peptide transporter system. Within the ER, peptides bind to newly synthesized MHC class I proteins, an interaction that allows MHC class I molecules to adopt their native conformation for transport to the cell surface via the secretory pathway. Hence, the MHC class I pathway displays an "inside-out" picture of the cell to the T cell. Patrolling CTLs move over the surface of potential target cells, engaging

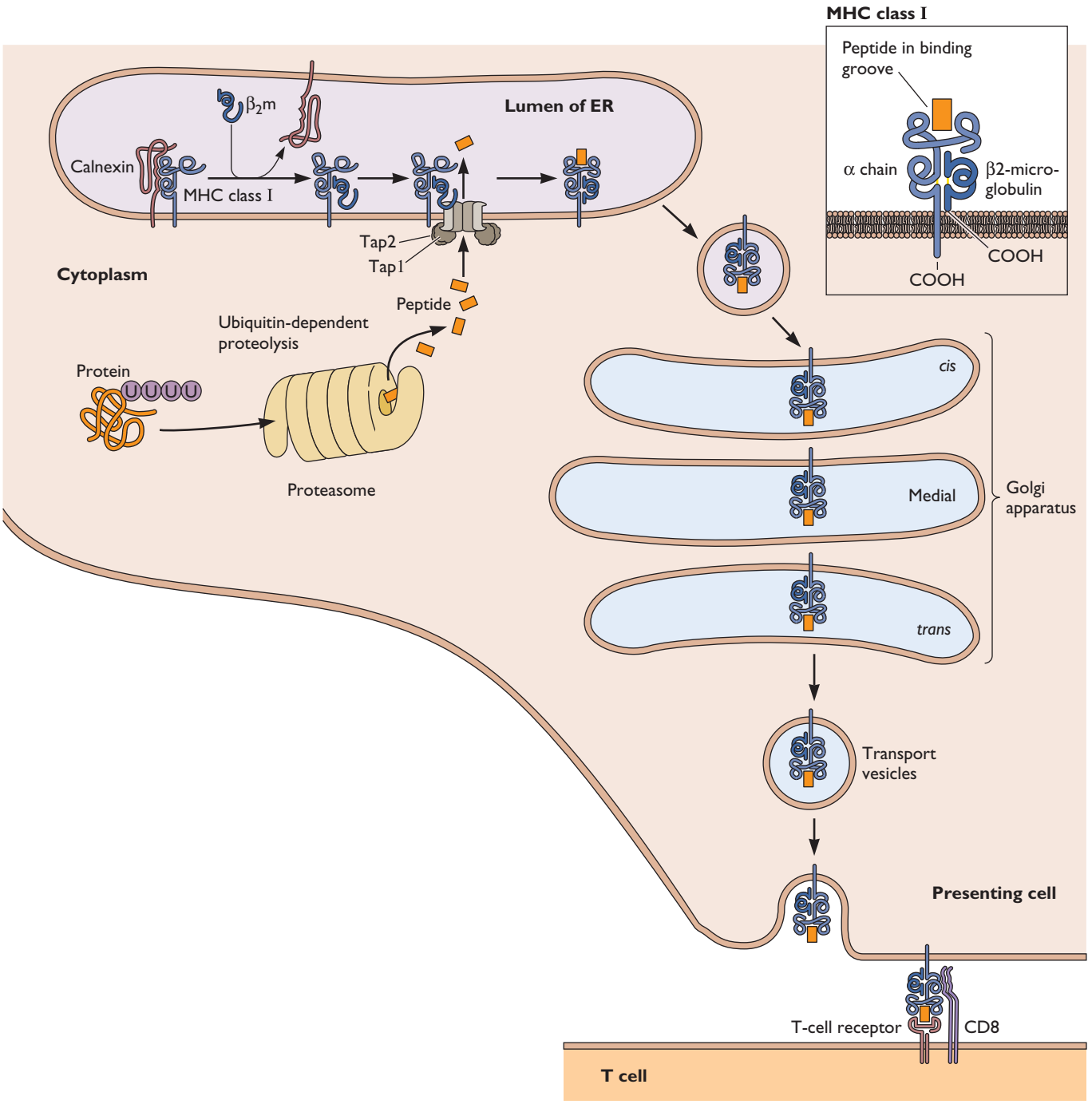


Figure 4.13 Endogenous antigen processing: the pathway for MHC class I peptide presentation. Intracellular proteins of both host cell and virus are degraded in the cytoplasm in a ubiquitin-dependent process. Proteins are marked for destruction by polyubiquitinylation. These modified proteins are then taken up and degraded by the proteasome. The resulting short peptides are transported into the ER lumen by the Tap1-Tap2 heterodimeric transporter in a reaction requiring ATP. Once in the ER lumen, the peptides associate with newly synthesized MHC class I molecules that bind weakly to the Tap complex. Assembly of the α chain and β_2-microglobulin of the MHC class I molecule is facilitated by the ER chaperone calnexin, but formation of the final native structure requires peptide loading. The MHC class I complex loaded with peptide is released from the ER to be transported via the Golgi compartments to the cell surface, where it is available for interaction with the T-cell receptor of a cytotoxic T cell carrying the CD8 coreceptor. **(Inset)** The MHC class I molecule is a heterodimer of the membrane-spanning type I glycoprotein α chain (43 kDa) and β_2-microglobulin (12 kDa) that does not span the membrane. The α chain folds into three domains, 1, 2, and 3. Domains 2 and 3 fold together to form the groove where peptide binds, and domain 1 folds into an immunoglobulinlike structure. Adapted from D. Male et al., *Advanced Immunology*, 3rd ed. (Mosby, St. Louis, MO, 1996).

MHC class I peptide complexes by their T-cell receptors. Binding of a viral peptide-MHC class I complex by the T-cell receptor triggers a series of reactions that activate the CTL for killing of the infected cell (see below). Surprisingly, the T-cell receptor has a low affinity, 1 μM or less, for its peptide-MHC class I ligand. How high-fidelity recognition is obtained from low-affinity binding has been a subject of intense research.

T Cells Recognize Professional Antigen-Presenting Cells by Engaging the MHC Class II Receptors

Both antibody and CTL responses are controlled precisely by the cytokines produced by Th cells. Such precision is achieved by specific antigen recognition by the MHC class II proteins. A Th cell is activated only when the peptide antigen is presented on the surfaces of professional antigen-presenting cells, such as dendritic cells. As dendritic cells mature, MHC class II glycoproteins loaded with peptides produced from their stores of endocytosed antigens appear on their surfaces. The mature antigen-presenting cells also carry high surface concentrations of costimulatory T-cell adhesion molecules that bind receptors on Th cells in the lymphoid tissue.

The process by which viral proteins are taken up from the outside of the cell and digested, and by which resulting peptides are loaded onto MHC class II molecules, is called **exogenous antigen presentation** (Fig. 4.14). In this case, the viral proteins are not produced inside the cell, as it is not infected, and their digestion takes place in endosomes rather than the proteasome. Furthermore, the peptides and MHC class II molecules are brought together by vesicular fusion. As with MHC class I, the complex is then transported to the surface of the antigen-presenting cell, where it is available to interact with appropriate T cells in the lymph node. Interaction of T-cell receptors on the naive $CD4^+$ Th cell with the MHC class II-peptide complex induces concerted changes in the Th cell, leading to its activation and differentiation (Fig. 4.6). Full activation requires the interactions of other surface proteins and costimulatory molecules (Fig. 4.15).

Th cells activated in this fashion produce IL-2, as well as a high-affinity receptor for this cytokine. The secreted IL-2 binds to the newly synthesized receptors to induce autostimulation and proliferation of the Th cell. Such clonal expansion of specific Th1 or Th2 cells then promotes the activation of CTLs and B lymphocytes (Fig. 4.6).

While MHC class II proteins are definitive components of the professional antigen-presenting cell, some MHC class I molecules also can be loaded with peptides produced via the exogenous route in dendritic cells (a process called **cross-presentation**). This pathway may be a significant mechanism for activating the adaptive immune response.

The Cell-Mediated Adaptive Response

In general, the cell-mediated response facilitates recovery from a viral infection, because it eliminates virus-infected cells without damaging uninfected cells. While the Th2-promoted antibody response is important for some infections in which virus particles spread in the blood, antibody alone is often unable to contain and clear an infection. Indeed, antibodies have little or no effect in many natural infections that spread by cell-to-cell contact (e.g., infections by neurotropic viruses such as alphaherpesviruses) or in infections by viruses that infect circulating immune cells (e.g., infections by lentiviruses and paramyxoviruses). These infections can be stopped only by CTL-produced antiviral cytokines and overt killing of infected cells.

CTLs

CTLs are superbly equipped to kill virus-infected cells, and once they complete one killing, they can detach and kill again. These lethal effector cells mature by a multistep pathway that fully arms them for killing. At least two reactions are required for realization of their full killer potential. These reactions are the interaction of their T-cell receptor with foreign antigens presented by MHC class I molecules, and the binding of additional surface proteins (the coreceptors) on the precursor CTL to their ligands on the infected cell.

Signaling from the T-cell receptor when it engages the peptide antigen-MHC complex requires clustering (aggregation) of a number of T-cell receptors and reorganization of the T-cell cytoskeleton in a particular structure called the **immunological synapse** (Fig. 4.16). Only after these reactions have taken place can the CTL lyse an infected cell.

The term "immunological synapse" was coined because the proteins that mediate target and T-cell recognition show an unexpected degree of spatial organization at the site of T-cell–target cell contact. This focal collection of membrane proteins and their respective binding partners has functional analogy to the neuronal synapse, a site of informational transfer between neurons. The synapse structure contributes to stabilizing signal transduction by the T-cell receptor for the prolonged periods required for gene activation. In addition, membrane proteins in the structure engage the underlying cytoskeleton and polarize the secretion apparatus so that a high local concentration of effector molecules is attained at the site of contact. Small numbers of peptide ligands complexed to MHC class I molecules apparently can stimulate a T cell because they serially engage a large total number of T-cell receptors on the opposing cell surface in the immunological synapse. Unengaged T-cell receptors

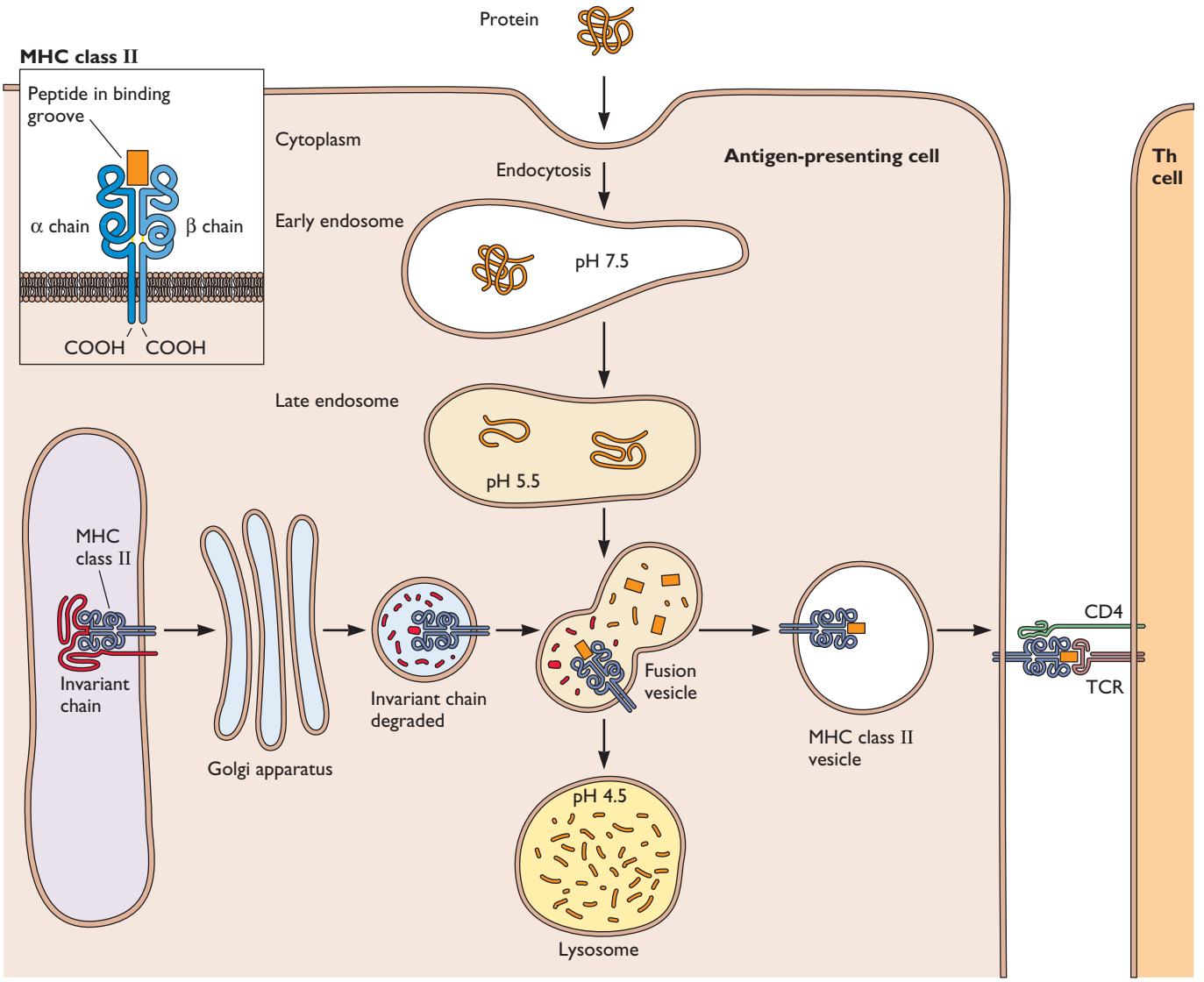


Figure 4.14 Exogenous antigen processing in the antigen-presenting cell: the pathway for MHC class II peptide presentation. Peptides in the ER lumen of the antigen-presenting cell are prevented from binding to the MHC class II peptide groove by association of a protein called the invariant chain with MHC class II molecules. The complex is transported through the Golgi compartments to a post-Golgi vesicle, where the invariant chain is removed by proteolysis. This reaction activates MHC class II molecules to accept peptides. The peptides are derived, not from endogenous proteins, but from extracellular proteins that enter the antigen-presenting cell. In some antigen-presenting cells, the proteins enter by endocytosis (top) and are internalized to early endosomes with neutral luminal pH. Endocytotic vesicles traveling to the lysosome via this pathway are characterized by a decrease in pH as they "mature" into late endosomes. The lower pH activates vesicle proteases that degrade the exogenous protein into peptides. Internalized endosomes with their peptides fuse at some point with the vesicles containing activated MHC class II. The newly formed peptide-MHC class II complex then becomes competent for transport to the cell surface, where it is available for interaction with the T-cell receptor (Tcr) of a Th cell carrying the CD4 coreceptor. **(Inset)** The MHC class II molecule is a heterodimer of the membrane-spanning type I α-chain (34-kDa) and β-chain (29-kDa) glycoproteins. Each chain folds into two domains, 1 and 2, and together the α and β chains fold into a structure similar to that of MHC class I. The two amino-terminal domains from α and β chains form the groove in which peptide binds. Unlike the closed MHC class I peptide groove, the MHC class II peptide-binding groove is open at both ends. The second domain of each chain folds into an immunoglobulinlike structure. Human genomes contain three MHC class II loci (*DR*, *DP*, and *DQ*), and mouse genomes have two (*IA* and *IE*). MHC class II molecules are dimers comprising α and β subunits. The three sets of human genes give rise to four types of MHC class II molecules. Adapted from D. Male et al., *Advanced Immunology*, 3rd ed. (Mosby, St. Louis, MO, 1996).

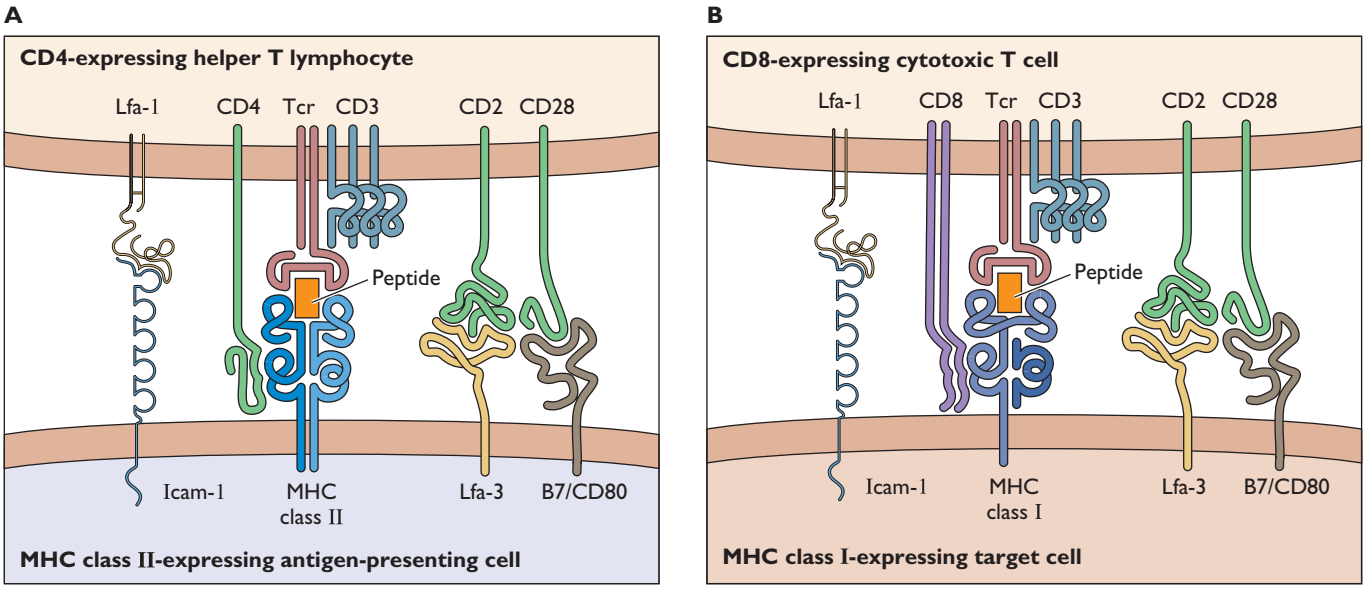


Figure 4.15 T-cell surface molecules and ligands. The interactions of these receptors and ligands are important for antigen recognition and initiation of signal transduction and other T-cell responses. **(A)** Interaction of a Th cell producing the CD4 coreceptor with an antigen-presenting cell. This cell exhibits an MHC class II-peptide complex in addition to Icam-1, Lfa-3, and CD80 (B7) membrane proteins. These complexes all are capable of binding cognate receptors on the Th cell as illustrated. **(B)** Interaction of a CTL producing the CD8 coreceptor with its target cell. The target cell exhibits an MHC class I-peptide complex in addition to Icam-1, Lfa-3, and CD80 (B7) membrane proteins. These complexes all can be recognized and bound by receptors on the CTL as illustrated.

subsequently entering this zone have an increased likelihood of binding specific ligand and signaling.

The primary interaction of the MHC-peptide complex with the T-cell receptor is often called **signal 1**. This interaction is not sufficient to activate the T cell. A second essential signal, often called **signal 2**, is produced by clustering and binding of accessory or costimulatory molecules on the CTL to appropriate ligands that form the immunological synapse with the infected cell (Fig. 4.16). One interaction required to produce signal 2 is the binding of CTL CD28 protein with the target cell CD80 protein. Other interactions include those that facilitate adhesion of T cell and target cell to one another, such as binding of CD2 or leukocyte function antigen 1 (Lfa-1) on the CTL with Lfa-3 or Icam-1, respectively, on the infected cell.

Given the central role of the T-cell receptor and formation of the immunological synapse in adaptive immune defense, it should come as no surprise that viral gene products can affect the structure, function, and localization of the T-cell receptor and the various coreceptors. Indeed, infection by several members of the *Retroviridae* and *Herpesviridae* families leads to reduction of T-cell receptor function. Viral infection also can affect the abundance of various accessory molecules on cell surfaces and therefore alter CTL recognition and subsequent effector function.

CTLs kill by two primary mechanisms: transfer of cytoplasmic granules from the CTL to the target cell, and induction of apoptosis. These killing systems are formed during the differentiation process. The maturing CTL fills with cytoplasmic granules that contain macromolecules required for lysis of target cells, such as **perforin**, a membrane pore-forming protein, and **granzymes**, members of a family of serine proteases. Granules are released by CTLs in a directed fashion when in direct membrane contact with the target cell, and are taken up by that cell via receptor-mediated endocytosis. Perforin, as its name implies, makes holes in the endosomal membrane, allowing the release of granzymes that induce apoptosis of the infected cell. CTL killing by the perforin pathway is rapid, occurring within minutes after contact and recognition. Activated CTLs also can induce apoptotic cell death via binding of the Fas ligand on their surfaces to the Fas receptor on target cells. Fas pathway killing is much slower than perforin-mediated killing. Many activated CTLs also secrete IFN-γ, which, as we have seen, is a potent inducer of both the antiviral state in neighboring cells, and synthesis of MHC class I and II proteins. Activated CTLs also secrete powerful

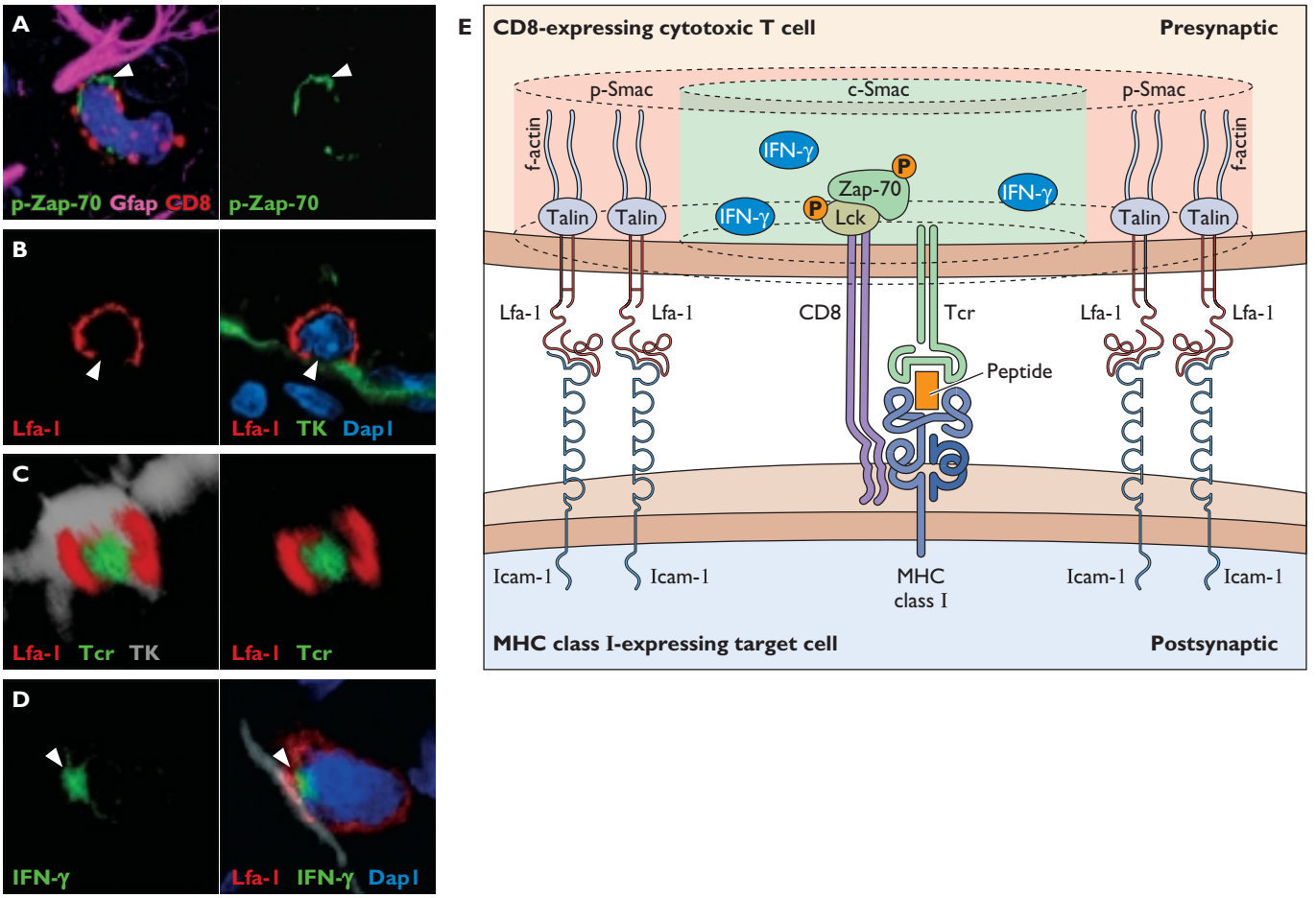


Figure 4.16 The immunological synapse. The morphological characteristics of an *in vivo* immunological synapse between CD8⁺ CTLs and adenovirus-infected astrocytes is illustrated. Colors are specific antibody reactions to identify proteins. The striatum of rats was injected with a recombinant adenovirus vector expressing the herpes simplex virus thymidine kinase (TK) gene. **(A)** Interaction between a CD8⁺ CTL (red) and an infected astrocyte (Gfap, magenta [marks astrocytes]) stimulates T-cell receptor (Tcr) signaling, resulting in phosphorylation and polarization of tyrosine kinases such as Zap70 (green) toward the interface with the infected cell. The white arrow indicates polarized pZap70. **(B)** Adhesion molecules such as Lfa1(red) aggregate to form a **peripheral** ring (p-SMAC: peripheral supramolecular activation cluster) at the junction formed by the immunological synapse. The postsynaptic astrocyte process can be identified by staining with antibody to TK, a marker of adenovirus infection (green). Note the characteristic absence of Lfa1 at the central portion of the immunological synapse between the T cell and the infected astrocye (white arrow). **(C)** A rotated image from a three-dimensional (3-D) reconstruction demonstrates the typical **central** polarization (c-SMAC) of Tcr molecules (green) toward the infected astrocyte (TK, white), and the peripheral distribution of Lfa1 in the p-SMAC (red). **(D)** The effector molecule IFN-γ (green) within a Tcr⁺ (red) CTL is directed toward the site of close contact with an infected target cell (TK, white); the white arrow indicates the T-target cell contact zone. The diameter of a CTL is ~10 μm. **(E)** Schematic cross section of an immunological synapse showing the characteristic polarized arrangement of the cytoskeleton (actin and talin proteins indicated) and organization of the adhesion molecule Lfa1 toward the peripheral supramolecular activation cluster (p-SMAC). The Tcr molecules are directed toward the central-supramolecular activation cluster (c-SMAC), The phosphorylated TKs (Zap70 and Lck) and effector IFN-γ molecules are in the center of the immunological synapse. Figure kindly provided by Pedro Lowenstein, Kurt Kroeger, and Maria Castro. See C. Barcia et al., *J. Exp. Med.* **203:**2095–2107, 2006.

cytokines, such as IL-16, and chemokines such as Rantes (regulated on activation, normal T-cell expressed and secreted) protein. Their release by virus-specific CTLs following recognition of an infected target cell may assist in coordination of the antiviral response.

Typically, for a cytopathic virus infection, CTL activity appears within 3 to 5 days after infection, peaks in about a week, and declines thereafter. The magnitude of the CTL response depends on such variables as titer of infecting virions, route of infection, and age of the host. The critical contribution of CTLs to antiviral defense is demonstrated by **adoptive-transfer** experiments in which virus-specific CTLs from an infected animal can be shown to confer protection to nonimmunized recipients (see also Box 4.5). However, CTLs can also cause direct harm by large-scale cell killing. Such immunopathology often follows infection by noncytopathic viruses, when cells can be infected yet still function. For example, the liver damage caused by hepatitis viruses is actually due to CTL killing of persistently infected liver cells. Other examples of immunopathology are discussed later in this chapter.

Control of CTL Proliferation

By using assays described in Box 4.6, several investigators discovered a hitherto unknown, massive CTL precursor expansion after acute primary infections by viruses such as lymphocytic choriomeningitis virus and Epstein-Barr virus. For example, more than 50% of CTLs from the spleen of a lymphocytic choriomeningitis virus-infected mouse were specific for a **single** viral peptide. The response reached a maximum 8 days after infection, but up to 10% of virus-specific T cells were still detectable after a year. Such results are in contrast to those for hepatitis B virus or human immunodeficiency virus infection: less than 1% of the CTLs from spleens of infected patients are specific for a single viral peptide.

These findings are stimulating a variety of new avenues of study. We must understand why there is a large primary expansion of particular CTL precursors for some infections and not others. It may be that this expansion is necessary to thwart the infection, or it may simply reflect an overreaction or lack of control in these controlled laboratory infections. The mechanisms that control this rapid expansion remain to be discovered. Other studies to determine the relationship of T-cell function (e.g., cell killing) to peptide specificity are under way.

As discussed in Chapter 5, viral proteins can blunt the deadly CTL response with far-reaching effects ranging from rapid death of the host to long-lived, persistent infections. Many such proteins confound CTL recognition by disguising or reducing antigen presentation by MHC class I molecules (see Fig. 5.5). In the case of human immunodeficiency virus type 1, the viral genome encodes three proteins that interfere with CTL action: Nef and Tat induce Fas ligand production and subsequent Fas-mediated apoptosis of CTLs, while Env engages the CXCr4 chemokine receptor, triggering the death of the CTL. Human cytomegalovirus-infected cells contain at least six viral proteins that interfere with the MHC class I pathway and also evoke apoptosis of virus-specific CTLs by increasing synthesis of Fas ligand (see Table 5.3).

Noncytolytic Control of Infection by T Cells

Complete clearance of intracellular viruses by the adaptive immune system does not depend solely on the destruction of infected cells by CTLs. The production of cytokines, such as IFN-γ and Tnf-α, by CTLs can lead to purging of viruses from infected cells without cell lysis. Such a mechanism requires that the infected cell retain the ability to activate antiviral pathways induced by binding of these cytokines to their receptors, and that viral replication be sensitive to the resulting antiviral response.

In certain circumstances, such as infection of the liver by hepatitis B and C viruses, there are orders of magnitude more infected cells than there are virus-specific CTLs. Furthermore, if vital organs such as the brain or liver are

BOX 4.5 DISCUSSION

An adoptive-transfer assay for T-cell-mediated delayed-type hypersensitivity

The delayed-type hypersensitivity response in a previously infected mouse typically is measured by an ear-swelling assay. Viral antigen is injected intradermally on the dorsal pinna of a mouse ear, while the other ear is injected with control antigen. Twenty-four hours later, the antigen-injected ear becomes inflamed, and swells as a result of delayed-type hypersensitivity, but the control-injected ear does not.

The same swelling reaction is observed in an animal that has not been infected when it is injected with purified, activated T cells from an infected animal. This adoptive-transfer experiment shows that delayed-type hypersensitivity is due to T cells.

BOX 4.6 DISCUSSION

Measuring the antiviral cellular immune response

The Classic Assay: Limiting Dilution and Chromium Release

For the past 45 years, virologists have determined the presence of CTLs in blood, spleen, or lymphoid tissues of immune animals by using the limiting-dilution assay and chromium release from lysed target cells. This assay measures $CD8^+$ CTL precursors, or memory CTLs, based on two attributes: (i) the action of foreign proteins and peptides to stimulate CTL proliferation and (ii) the ability of activated CTLs to lyse target cells.

Lymphocytes are obtained from an animal that has survived virus infection and are cultured for 1 to 2 weeks in the presence of whole inactivated virus, its proteins, or synthetic viral peptides. Under these conditions, virus-specific CTL precursors begin to replicate and divide (clonal expansion).

The expanded CTL population is then tested for its ability to destroy target cells that display viral peptides on MHC class I molecules. The target cells loaded with the viral antigen in question are then exposed to chromium-51, a radioactive isotope that binds to most intracellular proteins. After being washed to remove external isotope, cultured CTLs are incubated with the target cells, and lysis is measured by the release of chromium-51 into the supernatant.

Serial dilution before assay provides an estimate of the number of CD8+ CTL precursors in the original cell suspension (providing the name of the assay: "limiting dilution"). This assay does provide a quantitative measure of cellular immunity, but it is time-consuming, technically demanding, and expensive.

Identifying and Counting Virus-Specific T Cells

The limiting-dilution assay defines T cells by function, but not by their peptide specificity. Until recently, scientists have tried with little success to identify individual T cells based on their peptide recognition properties. This failure has been attributed to the low affinity and high "off" rates of the MHC-peptide complex and T-cell receptor. Without this information, it was difficult, if not impossible, to measure antigen-specific T-cell responses.

An important advance is the use of an **artificial MHC tetramer** as an antigen-specific, T-cell-staining reagent. The extracellular domains of MHC class I proteins are produced in *Escherichia coli*. These engineered MHC class I molecules have an unusual C-terminal 13-amino-acid sequence that enables them to be biotinylated. The truncated MHC class I proteins are folded in vitro with a synthetic peptide that will be recognized by a specific T cell. Biotinylated tetrameric complexes are purified and mixed with isolated T-cell pools from virus-infected animals. Individual T cells that bind the biotinylated MHC class I-peptide complex are detected by a variety of immunohistochemical techniques. Cell-sorting techniques can be used, and stained cells are viable.

Another assay for counting single T cells is the **enzyme-linked immunospot assay (ELISPot)**. In this assay, the cytokines are used as a surrogate for the T cell of interest. Fresh lymphoid cells are put into culture medium on plates that have been coated with antibody specific for cytokines under study. These cells are stimulated with virus-specific peptides. CTLs that recognize the peptide secrete cytokines locally where they are bound by the antibody on the plate. After the plate is washed, the foci of bound cytokine are stained and enumerated.

The **intracellular cytokine assay** is a relatively rapid method to count specific CTLs. Fresh lymphoid cells are treated with brefeldin A. This fungal metabolite blocks the secretory pathway and prevents the secretion of cytokines. The cells are then fixed with a mild cross-linking chemical that preserves protein, such as glutaraldehyde. Treated cells are permeabilized so that a specific antibody for a given cytokine can react with cytokines retained. Cells that react with the antibody can be quantified in a fluorescence-activated cell sorter. With appropriate software and calibration, the staining intensity corresponds to the level of cytokine expression, and the number of cells responding to a particular epitope and MHC class I molecule can be determined.

Measuring the Antiviral Antibody Response

Antibodies are the primary effector molecules of the humoral response. There are many methods to detect antibodies. However, a standard method in virology is the **neutralization assay**. Here, viral infectivity is determined in the presence and absence of antibody. Two variations on this general theme include the **plaque reduction assay** and the **neutralization index**. In the plaque assay, a known number of plaque-forming units (PFU) are exposed to serial dilutions of the antibody or serum in question. The highest dilution that will reduce the plaque count by 50% is taken as the plaque reduction titer of the serum or antibody. To compute a neutralization index, the titer of a virus stock is compared in the presence and absence of test antibody or serum. The index is calculated as the difference in viral titers. Obvious requirements for these assays is that the virus in question can be propagated in cultured cells, and that a measure of virus growth such as plaque formation is available.

Other important assays to determine binding of antibody to viral proteins are immunoprecipitation, Western blot, enzyme-linked immunosorbent assay, and hemagglutination inhibition.

infected, CTL killing can do more harm than good. When hepatitis B virus-specific CTLs are transferred to another animal (adoptive transfer), the IFN-γ and Tnf-α produced appear to clear the infection from thousands of cells without their destruction.

Noncytolytic clearing of infection by IFN-γ and Tnf-α produced by CTLs has now been documented for many viral infections, including those caused by primate lentiviruses, arenaviruses, adenoviruses, coronaviruses, hepadnaviruses, and picornaviruses. Additional cytokines produced

by a variety of immune system cells are likely to participate in viral clearance. In addition, the defensins (described previously) secreted by CTLs are known to promote the noncytolytic clearing of human immunodeficiency virus. $CD4^+$ T cells can also accomplish noncytolytic clearing of some infections with little involvement of CTLs. Such cases include infections by vaccinia virus, vesicular stomatitis virus, and Semliki Forest virus. CTL production of powerful, secreted, antiviral cytokines provides a simple explanation for how CTLs are able to control massive numbers of infected cells. However, how cytokines effect nonlethal purging of infection is not well understood.

Rashes and Poxes: Examples of T-Cell-Mediated, Delayed-Type Hypersensitivity

Many infections, including those of measles virus, smallpox virus, and varicella-zoster virus, produce a characteristic rash or lesion over extensive areas of the body, even though the primary infection began at a distant mucosal surface. This phenomenon results when the primary infection escapes the local defenses and virions or infected cells spread in the circulation to initiate many foci of infected cells in the skin. Th1 cells and macrophages that were activated by the initial infection home in on these secondary sites and respond by aggressive synthesis of cytokines, including IL-2 and IFN-γ. Such cytokines then act locally to increase capillary permeability, a reaction partially responsible for a characteristic local reaction referred to as **delayed-type hypersensitivity** (Box 4.5). This reaction is responsible for many virus-promoted rashes and lesions with fluid-filled vesicles.

The Antibody Response

Specific Antibodies Are Made by Activated B Cells Called Plasma Cells

When the B cell emerges from the bone marrow into the circulation and travels to lymph and lymphoid organs, it differentiates and synthesizes antibody **only** when its surface antibody receptor is bound to the cognate antigen. The activating signal requires clustering of receptors complexed with antigen. Such receptor clustering activates signaling via Src family tyrosine kinases that associate with the cytoplasmic domains of the closely opposed receptors. B-cell coreceptors, such as CD19, CD21, and CD8, enhance signaling by recruiting tyrosine kinases to clustered antigen receptors and coreceptors. Like dendritic cells, the B cell is an antigen-presenting cell that uses the MHC class II system and exogenous antigen processing (Fig. 4.14).

Binding of antigen to the B-cell receptor is only part of the activation process. Cytokines from Th cells also are required. When the T-cell receptor of Th2 cells recognizes MHC class II-peptide complexes present on the B-cell surface, these Th2 cells produce a locally high concentration of stimulatory cytokines, as well as CD40 ligand (a protein homologous to Tnf). The engagement of CD40 ligand with its B-cell receptor facilitates a local exchange of cytokines that further stimulates proliferation of the activated B cell and promotes its differentiation. Fully differentiated plasma cells produce prodigious amounts of specific antibodies: the rate of synthesis of IgG can be as high as 30 mg/kg of body weight/day.

Antibodies

Antibodies (immunoglobulins) have the common structural features illustrated in Fig. 4.17. Five classes of immunoglobulin—IgA, IgD, IgE, IgG, and IgM—are defined by their distinctive heavy chains—α, δ, ε, γ, and μ, respectively. Their properties are summarized in Table 4.4. IgG, IgA, and IgM are commonly produced after viral infection. During B-cell differentiation, "switching" of the constant region of heavy-chain genes occurs by somatic recombination and is regulated in part by specific cytokines. Consequently, each cell produces a specific type of antibody after such switching.

During the **primary antibody response**, which follows initial contact with antigen or viral infection, the production of antibodies follows a characteristic course. The IgM antibody appears first, followed by IgA on mucosal surfaces or IgG in the serum. The IgG antibody is the major antibody of the response and is remarkably stable, with a half-life of 7 to 21 days. Specific IgG molecules remain detectable for years, because of the presence of memory B cells. A subsequent challenge with the same antigen or viral infection promotes a rapid antibody response, the **secondary antibody response** (Fig. 4.8).

Virus Neutralization by Antibodies

While the cell-mediated response clearly plays an important role in eliminating virus-infected cells, the antibody response is crucial for preventing many viral infections and may also contribute to resolution of infection. Virions that infect mucosal surfaces will be exposed to secretory IgA antibodies. Similarly, virions that spread in the blood will be exposed to circulating IgG and IgM antibody molecules. Antibodies in the blood and lymph circulation are an important defense against infections caused by rabies virus, vesicular stomatitis virus, and enteroviruses. We know that immunodeficient animals can be protected from some lethal viral infections by injection with virus-specific antiserum or purified monoclonal antibodies (also called passive immunization). In studies of humans infected with eastern and western equine encephalitis viruses (alphaviruses in the family *Togaviridae*), patients with high antibody titers recovered, while those with low antibody titers were more likely to die of viral encephalitis.

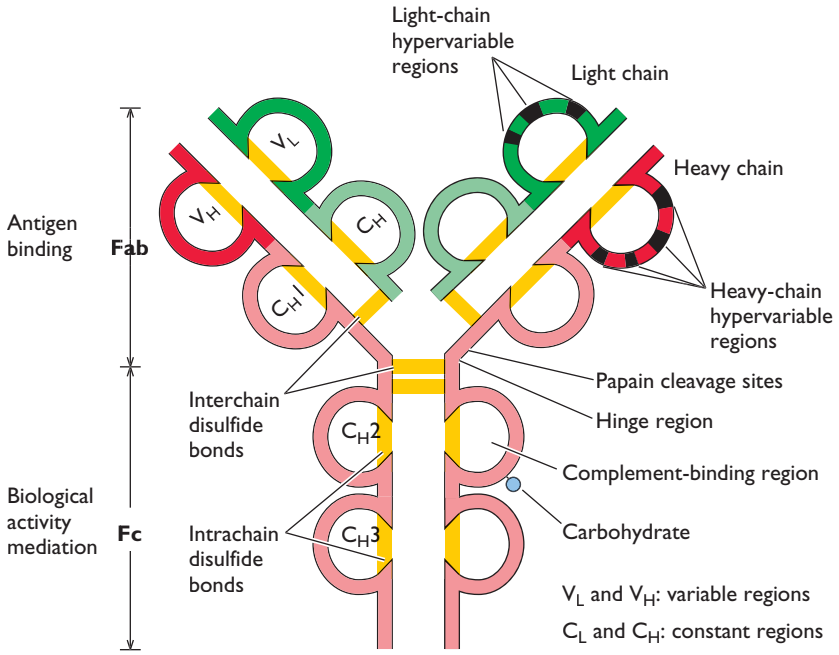


Figure 4.17 The structure of an antibody molecule. This is a schematic representation of an IgG molecule delineating the subunit and domain structures. The light and heavy chains are held together by disulfide bonds (yellow bars). The variable regions of the heavy (V_H) and light (V_L) chains, as well as the constant regions of the heavy (C_H) and light (C_L) chains, are indicated on the left part of the molecule. The hypervariable regions and invariable regions of the antigen-binding domain (Fab) are emphasized. The constant region (Fc) performs many important functions, including complement binding (activation of the classical pathway) and binding to Fc receptors found on macrophages and other cells. Clusters of papain protease cleavage sites are indicated, as this enzyme is used to define the Fab and Fc domains.

Perhaps the best example of the importance of antibodies in antiviral defense is the success of the poliovirus vaccine in preventing polio. We have learned that the type of antibody produced can influence the outcome of a viral infection significantly. Poliovirus infection stimulates strong IgM and IgG responses in the blood, but it is mucosal IgA that is vital in defense. This isotype can neutralize poliovirus directly in the gut, the site of primary infection. The live attenuated Sabin poliovirus vaccine is effective because it elicits a strong mucosal IgA response. IgA is synthesized by plasma cells that underlie the mucosal epithelium. This antibody is secreted from these cells as dimers of two conventional immunoglobulin subunits. The dimers then bind the polymeric immunoglobulin receptor on the basolateral surface of epithelial cells (Fig. 4.18). This complex is then internalized by endocytosis,

Table 4.4 The five classes of immunoglobulins

Property	IgA	IgD	IgE	IgG	IgM
Function	Mucosal; secretory	Surface of B cell	Allergy; anaphylaxis; epithelial surfaces	Major systemic immunity; memory responses	Major systemic immunity; primary response; agglutination
Subclasses	2	1	1	4	1
Light chain	κ, λ	κ, λ	κ, λ	κ, λ	κ, λ
Heavy chain	α	δ	ε	γ	μ
Concn in serum (mg/ml)	3.5	0.03	0.00005	13	1.5
Half-life (days)	6	2.8	2	25	5
Complement activation					
Classical	–	–	–	+	++
Alternative	–/+	–	+	–	–

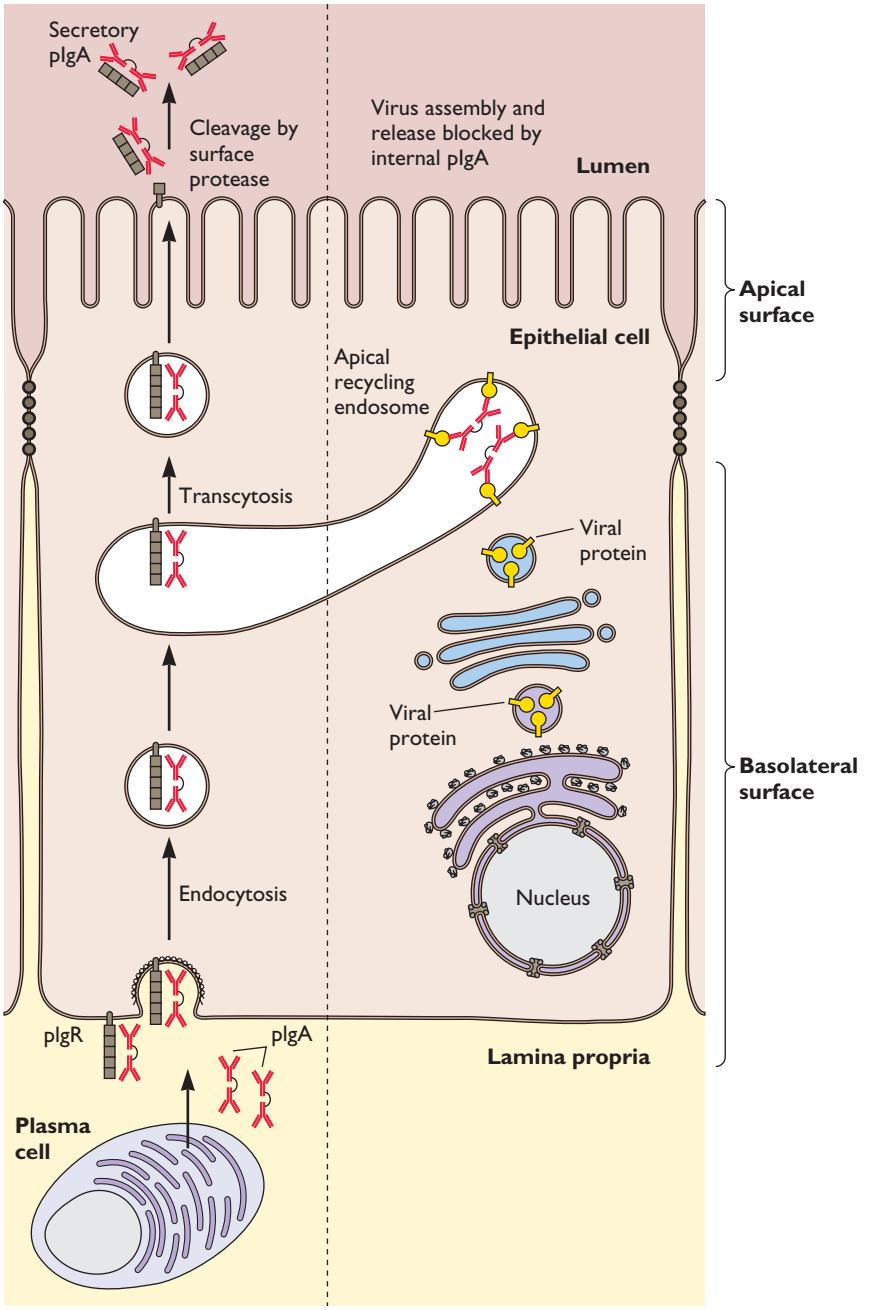


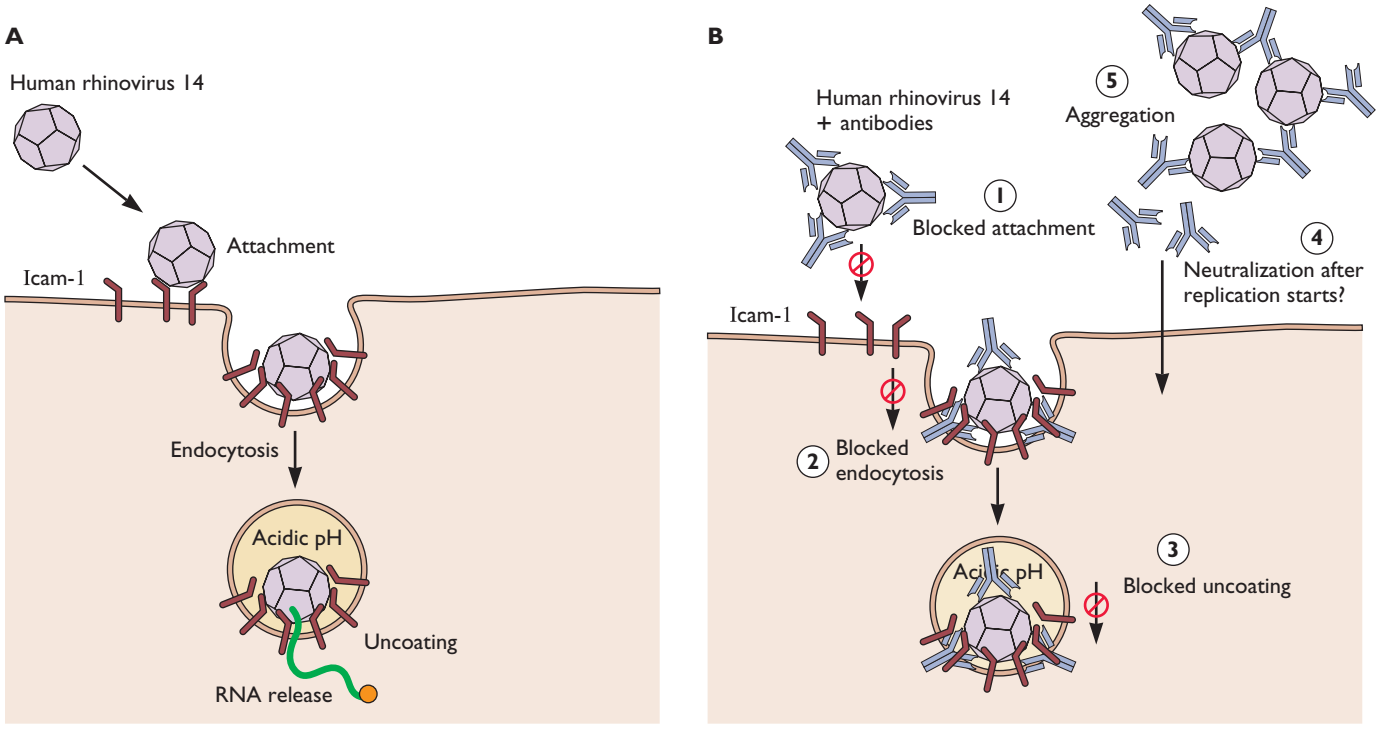
Figure 4.18 Secretory antibody, IgA, is critical for antiviral defense at mucosal surfaces. A single polarized epithelial cell is illustrated. The apical surface is shown at the top, and the basal surface is shown at the bottom. **(Left)** Antibody-producing B cells (plasma cells) in the lamina propria of a mucous membrane secrete the IgA antibody (also called polymeric IgA [pIgA]). pIgA is a dimer, joined at its Fc ends. For IgA to be effective in defense, it must be moved to the surface of the epithelial cells that line body cavities. This process is called transcytosis. **(Right)** Interestingly, a virus particle infecting an epithelial cell potentially can be bound by internal IgA if virus components intersect with the IgA in the lumen of vesicles during transcytosis. This process is likely to occur for enveloped viruses, as their membrane proteins are processed in many of the same compartments as those mediating transcytosis. Adapted from M. E. Lamm, *Annu. Rev. Microbiol*. **51**:311–340, 1997, with permission.

and moved across the cell (**transcytosis**) to the apical surface. Protease cleavage of the receptor releases dimeric IgA into mucosal secretions, where it can interact with incoming virions.

IgA may also block viral replication inside infected mucosal epithelial cells (Fig. 4.18). Because IgA must pass through such a cell en route to secretion from the apical surface, it is available during transit for interaction with viral proteins produced within the cell. The antigen-binding domain of intracellular IgA lies in the lumen of the ER, the Golgi compartment, and any transport vesicles of the secretory pathway. It is therefore available to bind to the external domain of any type I viral membrane protein that has the cognate epitope of that IgA molecule. Such interactions have been demonstrated with Sendai virus and influenza virus proteins during infection of cultured cells. In these experiments, antibodies colocalized with viral antigen only when the IgA could bind to the particular viral envelope protein. These studies suggest that clearing viral infection of mucosal surfaces need not be limited to the lymphoid cells of the adaptive immune system.

It is widely assumed that the primary mechanism of antibody-mediated viral neutralization is via steric blocking of virion-receptor interaction (Fig. 4.19). While some antibodies do prevent virions from attaching to cell receptors, the vast majority of virus-specific antibodies are likely to interfere with the concerted structural changes that are required for entry. Antibodies also can promote aggregation of virions, and thereby reduce the effective concentration of infectious particles. Many enveloped viruses can be destroyed in a test tube when antiviral antibodies and serum complement disrupt membranes (the classical

Figure 4.19 Interactions of neutralizing antibodies with human rhinovirus 14. (A) The normal route of infection. The virus attaches to the Icam-1 receptor and enters by endocytosis. As the internal pH of the endosome decreases, the particle uncoats and releases its RNA genome into the cytoplasm. **(B)** Possible mechanisms of neutralization of human rhinovirus 14 by antibodies. With well-characterized monoclonal antibodies, at least five modes of neutralization have been proposed and are illustrated: (1) blocked attachment—binding of antibody molecules to virus results in steric interference with virus-receptor binding; (2) blocked endocytosis—antibody molecules binding to the capsid can alter the capsid structure, affecting the process of endocytosis; (3) blocked uncoating—antibodies bound to the particle fix the capsid in a stable conformation so that pH-dependent uncoating is not possible; (4) blocked uncoating, inside cell—antibodies themselves may be taken up by endocytosis and interact with virions inside the cell after infection starts; (5) aggregation—because all antibodies are divalent, they can aggregate virus particles affecting concentration of virus particles and facilitating their destruction by phagocytes. (A) Adapted from T. J. Smith et al., *Semin. Virol.* **6**:233–242, 1995, with permission.



complement activation pathway). Nonneutralizing antibodies are also prevalent after infection; they bind specifically to virus particles, but do not interfere with infectivity. In some cases, such antibodies can even enhance infectivity: antibody bound to virions is recognized by Fc receptors on macrophages, and the entire complex is brought into the cell by endocytosis.

Much of what we know about antibody neutralization comes from the isolation and characterization of "antibody escape" mutants or **monoclonal antibody-resistant mutants**. These mutants are selected after propagating virus in the presence of neutralizing antibody. The analysis of the mutant viruses allows a precise molecular definition, not only of antibody-binding sites but also of parts of viral proteins important for entry. Antigenic drift (see Chapters 5 and 10) is a consequence of selection and establishment of antibody escape mutants in viral populations.

Antibodies can provoke other remarkable responses in virus-infected cells. For example, in a process analogous to CTL purging, antibodies that bind to the surface proteins of many enveloped viruses (e.g., alphaviruses, paramyxoviruses, and arenaviruses) can clear these viruses from persistently infected cells. This process is noncytolytic and complement independent. In this case, antibodies act synergistically with IFN and other cytokines. Virus-specific antibodies bound to surfaces of infected cells can inhibit virion budding at the plasma membrane and can also reduce surface expression of viral membrane proteins by inducing endocytosis. Antiviral antibodies injected into the peritoneal cavities of mice can block the neurotropic transmission of reovirus, poliovirus, and herpesvirus to the brain, even when the virions are injected at sites far removed from the peritoneum. The molecular bases for these intriguing effects remain elusive.

Antibody-Dependent Cell-Mediated Cytotoxicity: Specific Killing by Nonspecific Cells

The Th1 response results in production of a particular isotype of IgG that can bind to antibody receptors on macrophages and some NK cells. These receptors are specific for the carboxy-terminal, more conserved region of an antibody molecule, the Fc region (Fig. 4.16). If an antiviral antibody is bound in this manner, the amino-terminal antigen-binding site is still free to bind viral antigen on the surface of the infected cell. In this way, the antiviral antibody targets the infected cell for elimination by macrophages or NK cells. This process is called **antibody-dependent cell-mediated cytotoxicity** (often referred to as ADCC). The antibody provides the specificity for killing by a less discriminating NK cell. While this mechanism is well documented in cultured cells, its importance in controlling viral infections in animals is unknown.

The Immune System and the Brain

Cells of the central nervous system (brain and spinal cord) can initiate a robust and transient innate defense, but, surprisingly, they are unable to mount an adaptive response. A primary reason for this deficit is that the central nervous system is devoid of lymphoid tissue and dendritic cells. In addition, the central nervous system of vertebrates is separated from many cells and proteins of the bloodstream by tight endothelial cell junctions that comprise the so-called **blood-brain barrier**. As a consequence of these features, viral infections of the central nervous system can have unexpected outcomes. For example, if virus particles are injected directly into the ventricles or membranes covering the brain that are in contact with the bloodstream, the innate immune system is activated, a strong inflammatory response occurs, and the adaptive response ensues. In contrast, if virus particles are injected directly into brain tissue, avoiding the blood vessels and ventricles, only a transient inflammatory response is found. The adaptive response is not activated; antibodies and antigen-activated T cells are not made.

Although the central nervous system is unable to initiate an adaptive immune response, it is not isolated from the immune system. Indeed, the blood-brain barrier is open to entry of activated immune cells circulating in the periphery. Antigen-specific T cells regularly enter and travel through the brain, performing immune surveillance. Moreover, at least two glial cell types, astrocytes and microglia, have a variety of cell surface receptors that can engage these T cells. Astrocytes, the most numerous

Table 4.5 Cells and mechanisms associated with immunopathology

Proposed mechanism	Virus
$CD8^+$ T-cell mediated	Coxsackievirus B
	Lymphocytic choriomeningitis virus
	Sin Nombre virus
	Human immunodeficiency virus type 1
	Hepatitis B virus
$CD4^+$ T-cell mediated	Theiler's virus
Th1	Mouse coronavirus
	Semliki Forest virus
	Measles virus
	Visna virus
	Herpes simplex virus
Th2	Respiratory syncytial virus
B-cell mediated (antibody)	Dengue virus
	Feline infectious peritonitis virus

cell type in the central nervous system, respond to a variety of cytokines made by cells of the immune system. All natural brain infections begin in peripheral tissue, and any infection that begins outside the central nervous system activates the adaptive immune response. However, if the infection spreads to the brain, the resulting immune attack on this organ can be devastating. A careful scientist can inject virus particles experimentally into the brain without infecting peripheral tissue, thereby avoiding an adaptive response. On the other hand, if the animal is first immunized by injecting virus particles into a peripheral tissue, the adaptive response is activated as expected. Subsequent injection of an identical virion preparation into the brain of the immunized animal elicits massive immune attack on that organ: any virus-infected target in the brain is recognized and destroyed by the peripherally activated T cells. In both natural and experimental infections, the inflammatory response is not transient but sustained, resulting in capillary leakage, swelling, and cell death. Swelling of the brain in the closed confinement of the skull has many deleterious consequences, and, when this is coupled with bleeding and cell death, the results are disastrous. It is clear that although the brain is "immunoprivileged" in some sense, it is not completely isolated and, when infected, is vulnerable to attack by T cells produced and stimulated by the immune system.

Immunopathology: Too Much of a Good Thing

The clinical symptoms of viral disease in the host (e.g., fever, tissue damage, aches, pains, and nausea) result primarily from the host response to infection (Table 4.5). Damage caused by the immune system is called **immunopathology**, and it may be the price paid by the host to eliminate a viral infection. For noncytolytic viruses it is likely that the immune response is the sole cause of disease. In fact, most viral infections with an immunopathological component are noncytolytic and persistent (see Chapter 5). Most immunopathology is induced by activated T cells, but there are examples of disease caused by antibodies or an excessive innate response (Box 4.7).

Immunopathological Lesions

Lesions Caused by CTLs

The best-characterized example of CTL-mediated immunopathology occurs during the infection of mice with lymphocytic choriomeningitis virus. Infection is not cytopathic and induces tissue damage only in immunocompetent animals. Experiments using adoptive transfer of T-cell subtypes, depletion of cells, and gene knockout and transgenic mice clearly show that tissue damage requires CTLs (Box 4.8). The mechanism by which these cells cause damage is not clear, but may be a result of

BOX 4.7 EXPERIMENTS

Defective viral vectors and lethal immunopathology

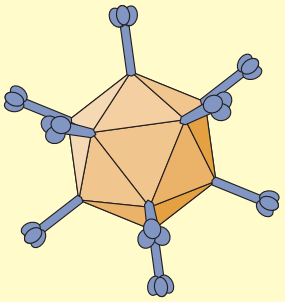
In September 1999, an 18-year-old man participated in a clinical trial to test the safety of a defective adenovirus designed as a gene delivery vector. It seemed like a routine procedure: normally, even replication-competent adenoviruses cause only mild respiratory disease. Most humans harbor adenoviruses as persistent colonizers of the respiratory tract and, indeed, produce antibodies against the virus. The young man was injected with a large dose of the viral vector. Four days after the injection, he died of multiple-organ failure. What caused this devastating response to such an apparently benign virus?

Some relevant facts:

- Natural adenovirus infection never occurs by direct introduction of virions into the circulation. Most infections occur at mucosal surfaces with rather small numbers of infecting virions.
- Most humans have antibodies to the adenoviral vectors used for gene therapy.
- A large dose of virus was injected directly into his bloodstream.

One compelling idea is that most of the infecting defective virions were bound by antibody present in the young man's blood. As a consequence, the innate immune system, primarily complement proteins, responded to the antibody-virus complex, resulting in massive activation of the complement cascade. The amplified complement cascade caused widespread inflammation in the vessel walls of the liver, lungs, and kidneys, resulting in multiple-organ failure.

Given the complexities of the immune response to infections, this idea is most certainly an oversimplification. Nevertheless, this fatal trial resulted in a large-scale reassessment of gene delivery methods and clinical protocols. Research is ongoing to find methods and tests to identify, reduce, or eliminate immunopathology so that the promise of gene therapy can be realized.



BOX 4.8 BACKGROUND

Transgenic mouse models prove useful in defining the antiviral contribution of CTLs

Studies of mice carrying mutations in genes that encode proteins required for CTL killing, B-cell function, and IFN responses have provided a wealth of information on the contributions of these gene products in immune defense. The only requirement is that the virus of interest be able to infect the transgenic mouse.

Genetic manipulation of the CTL response has been particularly informative. In the case of some noncytopathic viruses, perforin-mediated, but not granzyme-mediated, or Fas-mediated, killing is an important component of host CTL defense. When virus was not cleared in perforin-defective mice, the resulting persistent infection led to a lethal overproduction of cytokines, including Tnf-α and IFN-γ. In contrast, granzyme-deficient mice recover completely from viral infection. How the various CTL killing proteins are coordinated to control other viral infections is an important area of research.

Transgenic models also demonstrate an unexpectedly limited contribution of the CTL killing functions in defense against certain cytopathic viruses. Vaccinia virus and vesicular stomatitis virus pathogenesis was not affected by lack of CTL killing systems: rather, recovery from primary infection depended largely on production of neutralizing IgG antibodies. In fact, cytokines secreted by $CD4^+$ Th cells and CTLs are required for **early** immune defense, but not for recovery from infection by many different cytopathic viruses. One way to think about these observations is that recognition and lysis of infected cells by CTLs may be too slow to be effective as a primary defense against acute cytopathic infections: the rapid innate defenses and early cytokine responses of lymphocytes are the critical frontline systems.

Rall, G., D. Lawrence, and C. Patterson. 2000. The application of transgenic and knockout mouse technology for the study of viral pathogenesis. *Virology* **271:**220–226.

Yeung, R., J. Penninger, and T. Mak. 1994. T-cell development and function in gene knockout mice. *Curr. Opin. Immunol.* **6:**298–307.

their cytotoxicity. For example, knockout mice lacking perforin (the major cytolytic protein of CTLs) develop less severe disease after infection. CTLs may also release proteins that recruit inflammatory cells to the site of infection, which in turn elaborate proinflammatory cytokines.

Liver damage caused by hepatitis B virus also appears to depend on the action of CTLs. Production of the hepatitis B virus envelope proteins in transgenic mice has no effect on the animals. When the mice are injected with hepatitis B virus-specific CTLs, liver lesions that resemble those observed in acute human viral hepatitis develop. First, CTLs attach to hepatocytes and induce apoptotic cell death. Next, cytokines released by these lymphocytes recruit neutrophils and monocytes, which cause even more extensive cell damage. Death from hepatitis can be prevented by the administration of antibody to IFN-γ or by depletion of macrophages. CTLs are required for immunopathology, but tissue damage is mediated largely by nonspecific cytokines and cells recruited to the site of infection.

Myocarditis (inflammation of the heart muscle) caused by coxsackievirus B infection of mice also requires the presence of CTLs. In particular, perforin is a major determinant of myocarditis. Mice lacking the perforin gene develop a mild form of heart disease yet are still able to clear the infection. Chemokines contribute to disease by controlling directional migration of lymphocytes into infected tissues. For example, mice lacking the chemokine macrophage inflammatory protein 1α do not develop myocarditis. These observations indicate that inflammation of heart muscle following infection is a result of the combined action of immune-mediated tissue damage and virus-induced cytopathology.

Lesions Caused by $CD4^+$ T Cells

$CD4^+$ T lymphocytes elaborate far more cytokines than do CTLs and recruit and activate many nonspecific effector cells. Such inflammatory reactions are usually called delayed-type hypersensitivity responses. Most of the recruited cells are neutrophils and mononuclear cells, which are protective but can cause tissue damage. Immunopathology is the result of release of proteolytic enzymes, reactive free radicals such as peroxide and nitric oxide (see below), and cytokines such as Tnf-α. For noncytopathic persisting viruses, the $CD4^+$-mediated inflammatory reaction is largely immunopathological. This response is protective against cytopathic viruses, although there may be cases in which immunopathology occurs.

$CD4^+$ Th1 cells. The cytokines produced by $CD4^+$ Th1 cells cells facilitate the inflammatory response but not the antibody response. These cells are necessary for demyelination caused by viral infection of the nervous system and provoke central nervous system disease of rodents infected with several different viruses. When mice are infected with Theiler's murine encephalomyelitis virus (a picornavirus), proinflammatory cytokines produced by $CD4^+$ Th1 cells activate macrophages and microglial cells that mediate demyelination of neurons. It is not known

how demyelination occurs, but it has been proposed that the activated phagocytic cells release superoxide and nitric oxide free radicals in addition to Th1 cell proinflammatory cytokines, and the combination destroys oligodendrocytes, which are the source of myelin. That a similar demyelinating pathology is caused by so many different viral infections is consistent with the hypothesis that an underlying immunopathology is at work.

Herpes stromal keratitis is one of the most common causes of vision impairment in developed countries of the world. The eye damage is caused almost entirely by immunopathology. In humans, herpes simplex virus infection of the eye induces lesions on the corneal epithelium, and repeated infections result in opacity and reduced vision. Studies of a mouse model for this disease have demonstrated the importance of $CD4^+$ Th1 cells in an interesting example of immunopathology. The surprise was that while viral replication occurs in the corneal epithelium, $CD4^+$ T-cell-mediated inflammation was restricted to the underlying uninfected stromal cells. An important observation was that viral replication in the cornea had ceased by the time that $CD4^+$ T cells attacked the stromal cells. Herpes stromal keratitis is now thought to result from a damaging inflammatory reaction directed to uninfected cells in the stroma that is stimulated by secreted cytokines produced by infected cells in the corneal epithelium (bystander cell activation).

$CD4^+$ Th2 cells. The cytokines produced by $CD4^+$ Th2 cells facilitate the humoral response. Respiratory syncytial virus disease is an important cause of lower respiratory tract disease in infants and the elderly. Models for this particular disease have been difficult to produce, but some success has come with immunosuppressed mice. When these animals are infected, lesions of the respiratory tract are minor, but they become severe after adoptive transfer of viral antigen-specific, $CD4^+$ Th2 cells. The respiratory tract lesions contain many eosinophils, which may be responsible for pathology. One possibility is that the cytokines produced by $CD4^+$ Th2 cells recruit and stimulate proliferation of these eosinophils.

The Balance of Th1 and Th2 Cells

An inappropriate Th1 or Th2 response can have pathogenic effects. For example, respiratory syncytial virus causes respiratory infections which can be prevented by vaccination that induces a Th1 response in young children. These children were protected and suffered no ill effects. However, when children were vaccinated with a formalin-inactivated whole-virus vaccine that elicited a Th2 response, they not only remained susceptible to infection but also developed an atypically severe disease characterized by increased infiltration of eosinophils into the lungs. This particular pathology had been predicted by adoptive transfer of $CD4^+$ Th2 cells in mice (discussed above). Current vaccination efforts are based on preparations that induce only the Th1 response, which does not cause immunopathological disease.

Immunopathological Lesions Caused by B Cells

Virus-antibody complexes accumulate to high concentrations when extensive viral replication occurs at sites inaccessible to the immune system or continues in the presence of an inadequate immune response. Such complexes are not cleared efficiently by the reticuloendothelial system and continue to circulate in the blood. They become deposited in the smallest capillaries and cause lesions that are exacerbated when the complement system is activated (Fig. 4.20). Deposition of such immune complexes in blood vessels, kidneys, and brain may result in vasculitis, glomerulonephritis, and mental confusion, respectively. This type of immunopathology was first described in mice infected with lymphocytic choriomeningitis virus. Although immune complexes have been demonstrated in humans, viral antigens have been found in the complexes only in hepatitis B virus infections.

Antibodies may also cause an enhancement of viral infection. This mechanism probably accounts for the pathogenesis of dengue hemorrhagic fever. This disease is transmitted by mosquitoes and is endemic in the Caribbean, Central and South America, Africa, and Southeast Asia, where billions of people are at risk. The primary infection is usually asymptomatic, but may result in an acute febrile illness with severe headache, back and limb pain, and a rash. It is normally self-limiting, and patients recover in 7 to 10 days. There are four viral serotypes, and antibodies to any one serotype do not protect against infection by another. After infection by another serotype of dengue virus, nonprotective antibodies bind virus particles and facilitate their uptake into normally nonsusceptible peripheral blood monocytes carrying Fc receptors. Consequently, the infected monocytes produce proinflammatory cytokines, which in turn stimulate T cells to produce more cytokines. This vicious cycle results in high concentrations of cytokines and other chemical mediators that are thought to trigger the plasma leakage and hemorrhage characteristic of dengue hemorrhagic fever. There may be so much internal bleeding that the often fatal dengue shock syndrome results. Dengue hemorrhagic fever occurs in approximately 1 in 14,000 primary infections. However, after infection with a dengue virus of another serotype,

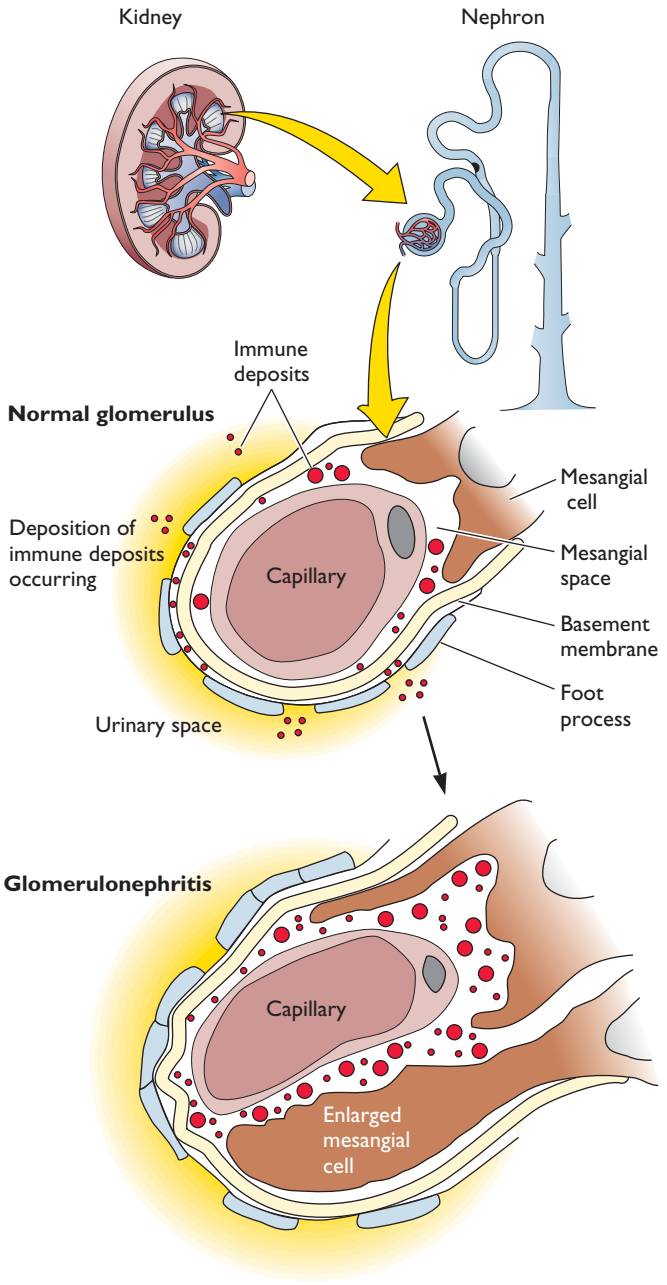


Figure 4.20 Deposition of immune complexes in the kidneys, leading to glomerulonephritis. (Top) Normal glomerulus and its location in the nephron and kidney. **(Middle)** Normal glomerulus. Red dots are immune complexes. The smaller complexes pass to the urine, and the larger ones are retained at the basement membrane. **(Bottom)** Glomerulonephritis. Complexes have been deposited in the mesangial space and around the endothelial cell. The function of the mesangial cell is to remove complexes from the kidney. In glomerulonephritis, the mesangial cells enlarge into the subepithelial space. This results in constriction of the glomerular capillary, and foot processes of the endothelial cells fuse. The basement membrane becomes leaky, filtering is blocked, and glomerular function becomes impaired, resulting in failure to produce urine. Adapted from C. A. Mims et al., *Mims' Pathogenesis of Infectious Disease* (Academic Press, Orlando, FL, 1995), with permission.

the incidence of hemorrhagic fever increases dramatically to 1 in 90, and the shock syndrome is seen in as many as 1 in 50.

Viral Infection-Induced Immunosuppression

Modulation of the immune defenses by viral gene products can range from a mild and rather specific attenuation to a marked global inhibition of the response (Table 4.6). The molecular mechanisms of immune modulation are discussed above. Immunosuppression by viral infection first was observed over 100 years ago because patients were unable to respond to a skin test for tuberculosis during and after measles infection. However, progress in understanding the phenomenon was slow until the human immunodeficiency virus epidemic was under way and the well-known, devastating immunodeficiency syndrome stimulated unprecedented efforts. Immunosuppression by common human viruses such as rubella and measles viruses is also a serious public health concern. For example, the vast majority of the million children who die from measles virus each year in Third World countries succumb to other infections that arise during this transient immunosuppression.

Systemic Inflammatory Response Syndrome

An important tenet of immune defense is that when viral replication exceeds a certain threshold, immune defenses are mobilized and amplified, resulting in a global response. How this threshold is determined is not known. Normally, the global response is well tolerated, and it quickly contains the infection. However, if the threshold is breached too rapidly, or if the immune response is not proportional to the infection, the large-scale production and systemic release of inflammatory cytokines and stress mediators can overwhelm and kill an infected host. Such a disastrous outcome often results if the host is naive and has not coevolved with the invading virus (zoonotic infections; see Chapter 10), or if the host is very young, malnourished, or otherwise compromised. This type of pathogenesis is called the **systemic inflammatory response syndrome** and is sometimes referred to as a "cytokine storm." The lethal effects of the 1918 influenza virus have been attributed to this response. Similar syndromes (e.g., toxic shock syndrome and toxic sepsis) are triggered by other microbial pathogens.

Autoimmune Diseases

Autoimmune disease is caused by an immune response directed against host tissues (often described as "breaking immune tolerance"). In laboratory animals, viral infection

Table 4.6 Some mechanisms of immunosuppression by viruses

Mechanism	Representative virus	Immunosuppression	Specific for infecting virus only
Infection of immune cells	Human immunodeficiency virus, canine distemper virus, lymphocytic choriomeningitis virus	Marked	No
Tolerance after infection of fetus	Rubella virus	Moderate	Yes
Disruption of cytokine defense pathways	Measles virus	Moderate	No
Virokines and viroceptors	Poxviruses, herpesviruses	Mild	Yes

can trigger potent autoimmune responses, but it has been difficult to find evidence for any role for viral infections in human autoimmune disease. How can viral infections promote autoimmune disease in animals? One model posits that replication at an anatomically sequestered site leads to the release and subsequent recognition of self antigens. A modified version of this hypothesis proposes that infection leads to exposure of cellular self antigens normally hidden from the immune system. Cytokines, or even virus-antibody complexes that modulate the activity of proteases in antigen-presenting cells, might cause the unmasking of self antigens. Cytokines produced during infection may stimulate inappropriate surface expression of host membrane proteins that are recognized by host defenses. Another possibility is that during virion assembly, host proteins normally not exposed to the immune system are packaged in virions (Volume I, Chapter 4); these host proteins are delivered to cells during infection and are recognized by the immune system.

A popular hypothesis for virus-induced autoimmunity states that viral and host proteins share antigenic determinants. This idea is often called **molecular mimicry**. Infection leads to the elaboration of immune responses to such shared determinants, resulting in an autoreactive response. Although many peptide sequences are shared among viral and host proteins, direct evidence for this hypothesis has been difficult to obtain. One reason is the long lag period between events that trigger human autoimmune diseases and the onset of clinical symptoms. To circumvent this problem, transgenic mouse models in which the products of foreign genes are expressed as self antigens have been established. This approach permits a precise study of how the autoreactive immune response is initiated and what cell types and soluble mediators are involved (Box 4.9).

Heterologous T-Cell Immunity

The immune memory cells of the adaptive immune system enable a subsequent rapid and specific response to previously encountered infections. Surprisingly, these memory cells are not always as specific as once thought. This realization came from many studies, but one fact was particularly informative. It is well known that common infections can run surprisingly different courses in different individuals. Why this should be so is complicated, but from experiments with genetically identical mice, it become clear that the history of previous infections can dictate the outcome of a new infection. The phenomenon is called **heterologous T-cell immunity**: memory T cells specific for a particular virus epitope can be activated during infection with a completely unrelated virus. The presence of a cross-reactive memory T cell has the potential to influence the new immune response to be protective or pathological or even to change the balance between Th1 and Th2 responses. Cross-reacting T-cell epitopes are another type of molecular mimicry that plays a significant role in the outcome of an infection.

When the T-cell receptor on a memory T cell engages its cognate epitope, the cell begins to proliferate and to secrete cytokines. These cytokines almost instantly affect the subsequent immune response. If a heterologous memory T cell responds to a similar epitope, it can be activated by an unrelated infection. Consequently, the immediate immune response is not tailored to the new pathogen and instead produces a response appropriate for the unrelated previous infection. When mice are immunized against one of several viruses and then challenged with a panel of other viruses, the animals show partial, but not necessarily reciprocal protection to the heterologous infection. For example, infection with lymphocytic choriomeningitis virus, an areanavirus, provided substantial protection against vaccinia virus, a poxvirus, but not vice versa. For other virus pairs such as murine cytomegalovirus and vaccinia virus, the protection was partially reciprocal. The significance of these findings to human infections is only now emerging. For example, patients experiencing Epstein-Barr virus-induced mononucleosis may have a strong T-cell response to a particular influenza virus epitope rather than the typical response to an immunodominant

BOX 4.9 EXPERIMENTS

Viral infections promote or protect against autoimmune disease

Promotion

Transgenic mice that synthesize proteins of lymphocytic choriomeningitis virus in β cells of the pancreas or oligodendrocytes have been developed. Expression of these viral proteins has no consequence; the mice are healthy. The viral transgene products are present in the mouse throughout development, and therefore are self antigen.

Infection of these transgenic animals with lymphocytic choriomeningitis virus stimulates an immune response in which the self antigen is recognized, leading to insulin-dependent diabetes mellitus or central nervous system demyelinating disease, respectively.

The action of virus-specific CTLs leads to inflammation, insulitis, and diabetes. Curiously, in uninfected animals, virus-specific lymphocytes are present in the peripheries as determined from in vitro studies, but they do not respond to the viral transgene product. The mechanisms that maintain such unresponsiveness to self antigens and prevent autoimmunity can be elucidated with such transgenic models. It was determined that tolerance to self antigens is circumvented depending on the number of autoreactive T cells and their affinity for peptides presented by MHC class II proteins, the number of memory cells induced by infection, and the cytokine milieu.

Hypothesis: If an individual becomes infected in the first few months after birth with an organ-tropic virus that establishes a persistent infection, tolerance to the virus is established. Later in life, an infection with the same virus leads to an immune-mediated attack on virus-infected cells in the organ.

Protection

Both the nonobese diabetic mouse strain and a particular strain of rat spontaneously develop type 1 diabetes. Infection of either animal with lymphocytic choriomeningitis virus reduces the incidence of diabetes. IL-12 is known to exacerbate autoimmune disease in these experimental models by promoting expansion and activation of autoreactive $CD4^+$ T cells. Moreover, production of IFN results in reduction in IL-12 production by monocytes and dendritic cells.

Explanation: Lymphocytic choriomeningitis virus infection induces synthesis of IFN that, in turn, protects against autoimmune disease by decreasing IL-12 expression.

Fujinami, R. 2001. Viruses and autoimmune disease—two sides of the same coin? *Trends Microbiol.* **9:**377–381.

von Herrath, M., and M. Oldstone. 1996. Virus-induced autoimmune disease. *Curr. Opin. Immunol.* **8:**878–885.

Epstein-Barr virus epitope. It appears that the Epstein-Barr virus infection activated memory T cells established by previous exposure to influenza virus. These individuals had a different course of mononucleosis, often more severe, than did individuals with no previous exposure to influenza virus.

T-cell cross-reactivities among heterologous viruses are more frequent than commonly expected. These reactions modulate the course of disease in animal models and are likely to exert similar influences in human infections. The important principle is that prior infection affects the defense against pathogens that have not yet been encountered.

Superantigens "Short-Circuit" the Immune System

Some viral proteins are extremely powerful T-cell mitogens known as **superantigens**. These proteins bind to MHC class II molecules on antigen-presenting cells, and interact with the Vβ chain of the T-cell receptor. As approximately 2 to 20% of **all** T cells produce the particular Vβ chain that binds the superantigen, superantigens short-circuit the interaction of MHC class II-peptide complex and the T cell. Rather than activation of a small, specific subset of T cells (only 0.001 to 0.01% of T cells usually respond to a given antigen), **all subsets** of T cells producing the Vβ chain to which the superantigen binds are activated and proliferate. Superantigens clearly interfere with a coordinated immune response and divert the host's defenses.

All known superantigens are microbial products, and many are produced after infection by viruses. The best-understood viral superantigen is encoded in the U3 region of the mouse mammary tumor virus long terminal repeat. This retrovirus is transmitted efficiently from mother to offspring via milk. However, the virus replicates poorly in most somatic tissues, so the question is, how do virions get into the milk? When B cells in the neonatal small intestine epithelium are infected, the viral superantigen is produced and recognized by T cells carrying the appropriate T-cell receptor Vβ chain. Consequently, extraordinarily large numbers of T cells are activated, and produce growth factors and other molecules that stimulate the infected B cells. These cells then carry the virus to the mammary gland. Infection of mice with mutants harboring a deletion of the superantigen gene results in limited viral replication and minimal transmission to offspring via milk. Expression of the superantigen stimulates the immune system to

facilitate the spread of infection from the gut to the mammary gland.

Mechanisms Mediated by Free Radicals

Two free radicals, superoxide ($O_2^{\cdot}$) and nitric oxide (NO), are produced during the inflammatory response and may play important roles in virus-induced pathology. Superoxide is produced by the enzyme xanthine oxidase, present in phagocytes. The production of $O_2^{\cdot}$ is significantly increased in the lungs of mice infected with influenza virus or cytomegalovirus. Inhibition of xanthine oxidase protects mice from virus-induced death. However, $O_2^{\cdot}$ is not toxic for some cells and viruses, and its effects might be the result of formation of peroxynitrite ($ONOO^{\cdot}$) through interaction with nitric oxide.

Nitric oxide is produced in abundance in virus-infected tissues during inflammation as part of the innate immune response (Fig. 4.21). This gas has been shown to inhibit the replication of many viruses in cultured cells and in animal models. It probably acts intracellularly to inhibit viral replication, but the molecular sites of action are not well understood. Nitric oxide is produced by three different IFN-inducible isoforms of nitric oxide synthase. While low concentrations of NO have a protective effect, high concentrations or prolonged production have the potential to contribute to tissue damage. For example, treating infected animals with inhibitors of nitric oxide synthase prevents tissue damage. Although NO is relatively inert, it reacts rapidly with $O_2^{\cdot}$ to form peroxynitrite ($ONOO^{\cdot}$), which is much more reactive than either molecule and may be responsible for cytotoxic effects on cells (Fig. 4.21). In the central nervous system, NO is produced by activated astrocytes and microglia, and may be directly toxic to oligodendrocytes and neurons.

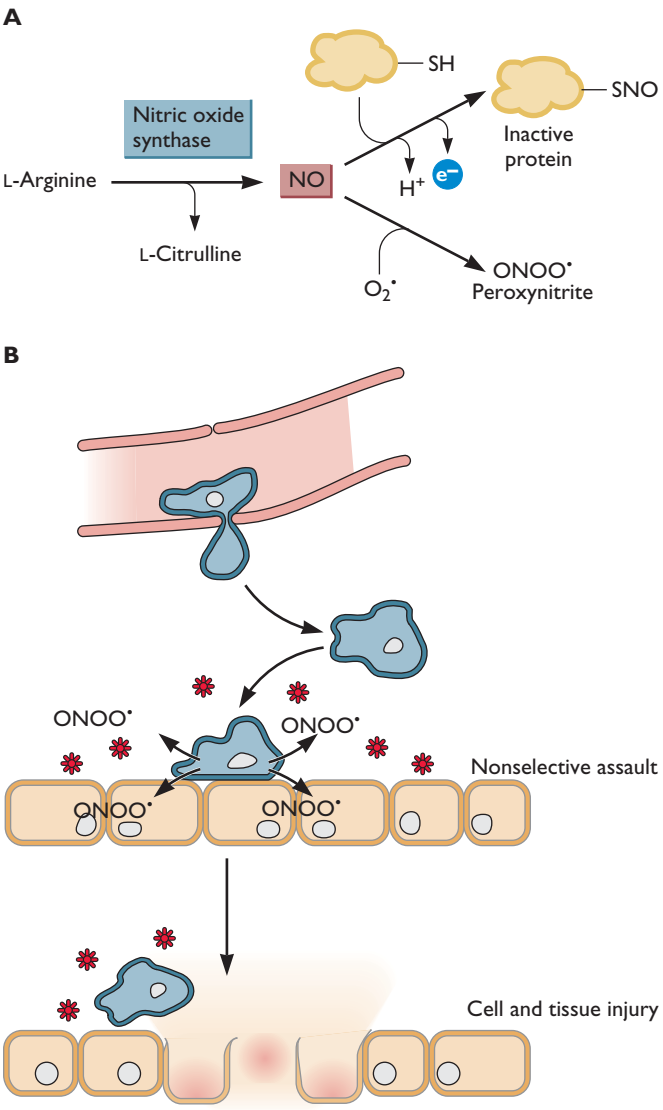


Figure 4.21 Consequences of nitric oxide production. **(A)** Formation of NO and $ONOO^{\cdot}$. Nitric oxide, which is produced from L-arginine by nitric oxide synthase, reacts with proteins, inactivating them, or with $O_2^{\cdot}$, forming peroxynitrite, $ONOO^{\cdot}$. Adapted from C. S. Reiss and T. Komatsu, *J. Virol.* **72:**4547–4551, 1998, with permission. **(B)** Biological effects of $NO/O_2^{\cdot}/ONOO^{\cdot}$. Phagocytes move into the area of active virus replication and produce $NO/O_2^{\cdot}/ONOO^{\cdot}$, which destroys cells, causing tissue injury but perhaps limiting virus yields. Adapted from T. Akaike et al., *Proc. Soc. Exp. Biol. Med.* **217:**64–73, 1998, with permission.

Perspectives

The breadth and complexity of the host response to infection can be initially confusing, if not overwhelming. To illuminate the important principles, it is useful to consider a hypothetical acute viral infection that is cleared by the host response (Fig. 4.22). Remember also this central fact: all successful viruses encode gene products that modulate their host's defenses (Table 4.7; Figure 4.23).

To initiate the primary infection, physical barriers are breached and virus particles enter permissive cells. Almost immediately, viral proteins and viral nucleic acids are bound by pathogen recognition receptors. Signal transduction cascades release latent transcription activation proteins from the cytoplasm. The infected cell now synthesizes cytokines, such as IFN. As new viral proteins are produced, the cell initiates other intrinsic defenses, such as apoptosis or autophagy. Local sentinel cells, the immature dendritic cells and macrophages, engage the locally released cytokines and internalize

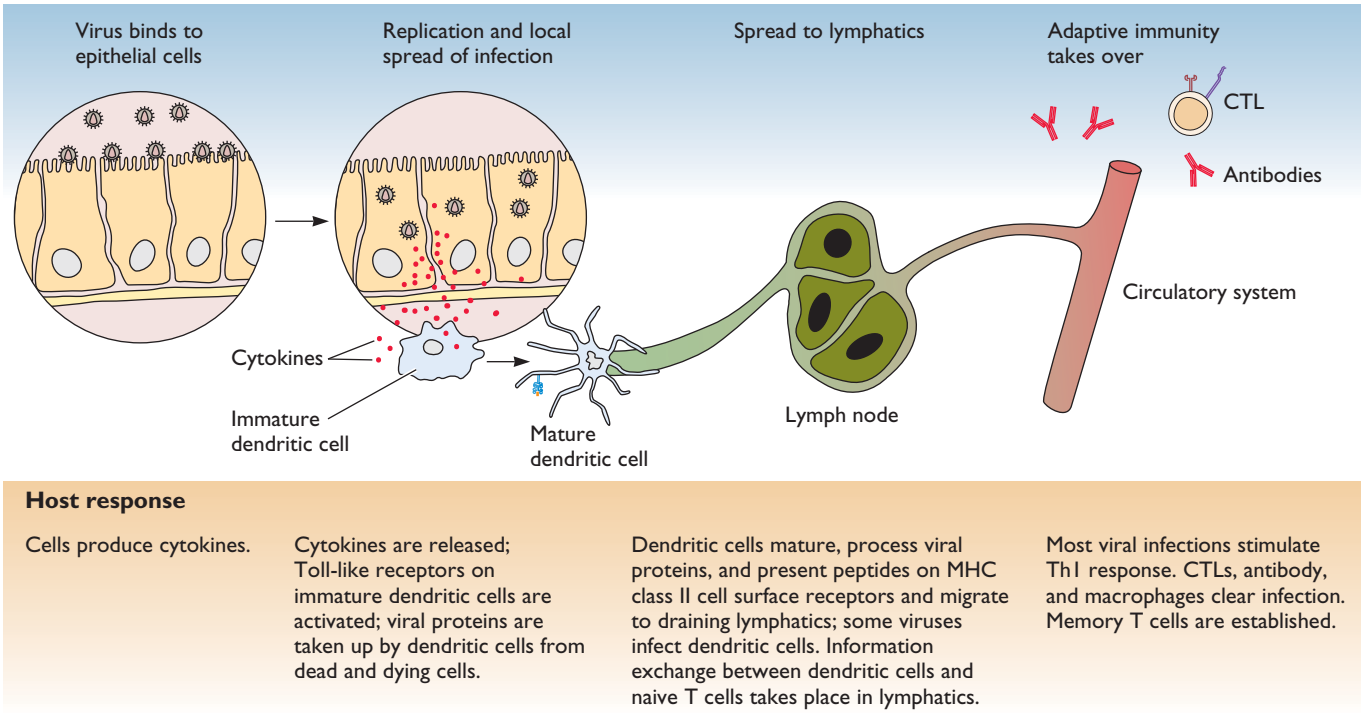


Figure 4.22 The stages of viral infection and responses of the host. Four stages are illustrated for a typical viral infection at an epithelial surface, a typical site of viral entry. The virus must attach to epithelial cells and then initiate replication. Infected cells produce cytokines that signal local dendritic cells, which respond accordingly. As a result, most often, local defenses contain the infection. However, if virus replication is not stopped, the adaptive immune system is activated by contact with mature dendritic cells and cytokines in local lymph nodes. The adaptive response leads to production of activated effector T and B cells that are released into the circulation to clear the infection and provide precise memory of the particular invader.

viral proteins produced by infected cells. The first response of the immature dendritic cell is to produce massive quantities of IFN and other cytokines. If viral anti-IFN or antiapoptotic gene products are made, progeny virions are released. If the newly infected cells have already bound IFN, protein synthesis is inhibited when viral nucleic acid is produced. Soon thereafter, NK cells can recognize the infected cells because of new surface antigens and a low or aberrant display of MHC class I proteins. The IFN produced by infected cells stimulates the NK cells to intensify their activities, which include target cell destruction and synthesis of IFN-γ (Box 4.10). In some cases, serum complement can be activated to destroy enveloped viruses and infected cells. In general, the intrinsic and innate defenses bring most viral infections to an uneventful close before the adaptive response is required.

If viral replication outpaces the innate defense, a critical threshold is reached: increased IFN production by circulating immature dendritic cells elicits a more global host response, and flulike symptoms are experienced by the infected individual. As viral replication continues, viral antigens are delivered by mature dendritic cells to the local lymph nodes or spleen to establish sites of information exchange with T cells. T-cell recirculation is shut down because of the massive recruitment of lymphocytes into lymphoid tissue. The swelling of lymph nodes so often characteristic of infection is a sign of this stage of immune action.

Within days, Th cells and CTLs appear; these cells are the first signs of activation of the adaptive immune response. Th cells produce cytokines that begin to direct the amplification of this response. The synthesis of antibodies, first of IgM and then of other isotypes, quickly

Table 4.7 Immune modulation strategies deduced from virus-infected cells[a]

Strategy	Example(s)
Secreted modulators	Virokines (ligand mimics)
	Viroceptors (receptor mimics)
Modulators on the infected cell surface	Complement inhibitors
	Coagulation regulators
	Immune receptors
	Adhesion molecules
Stealth	Latency
	Infection of immunoprivileged tissue
Antigenic hypervariability	Error-prone replicase
	Antigenic drift by antibody selection
	Epitope drift by CTL selection
Bypassing or killing of lymphocytes	Direct cytopathic infection
	Blockage of NK cell recognition
	Interference with signal transduction, maturation, or effector functions
Blockage of adaptive immune response	Alteration of MHC-I or MHC-II production
	Blockage of antigen processing
	Blockage of transcription of immune response genes
Inhibition of complement	Soluble receptor mimics
	Viral Fc receptors
Inhibition of cytokine action	Blockage of signal transduction cascade
Modulation of apoptosis	Blockage of cell death signaling pathways
	Scavenging of free radicals
	Blockage of death receptors or ligands
Interference with pattern recognition receptors	Alteration of ligands
	Production of decoy ligands
	Blockage of downstream signaling

[a]Adapted from G. Finlay and G. McFadden, *Cell* **124:**767–782, 2006.

follows. The relative concentrations of these isotypes are governed by the route of infection and the pattern of cytokines produced by the Th cells. As the immune response is amplified, CTLs kill or purge infected cells, and antibodies bind to virus particles and infected cells. Specific antibody-virus complexes can be recognized by macrophages and NK cells to induce antibody-dependent cell-mediated cytotoxicity, and can also activate the classical complement pathway. Both of these processes lead to the directed killing of infected cells and enveloped viruses by macrophages and NK cells.

An inflammatory response often occurs as infected cells die, and innate and adaptive responses develop. Cytokines, chemotactic proteins, and vasodilators are released at the site of infection. These proteins, invading white blood cells, and various complement components all contribute to the swelling, redness, heat, and pain characteristic of the inflammatory response. Many viral proteins modulate this response and the subsequent activation of immune cells.

If infection spreads from the primary site, second and third rounds of replication can occur in other organs. T cells that were activated at the initial site of infection can cause delayed-type hypersensitivity (usually evident as a characteristic rash or lesion) at the sites of later replication. Immunopathology, particularly after infection by noncytopathic viruses, is caused by the cytokines, antibodies, and cells in response to the infection and can contribute to the severity of the disease. Finally, the combination of innate and adaptive responses clears the infection, and the host is immune because of the presence of memory T and B cells and long-lived antibodies. The high concentrations of immune lymphocytes drop dramatically as these cells

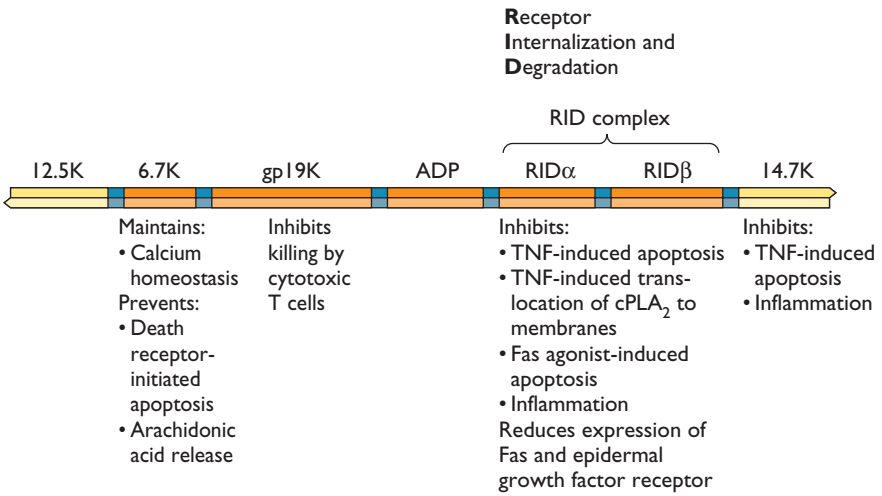


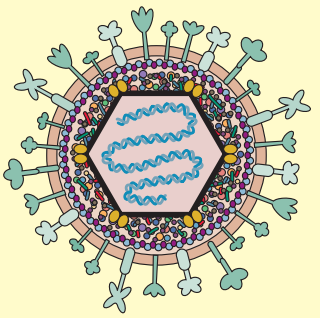
Figure 4.23 The adenovirus type 2 E3 region, a cluster of five genes encoding proteins that mediate host defense modulation. The proteins were named initially according to their apparent molecular masses (e.g., 14.7K for 14.7 kDa). Recently, some of the proteins have been given names that reflect their known functions. RID is an acronym for "receptor internalization and degradation." The RID protein complex is composed of RIDα and RIDβ. RID proteins have multiple functions, as indicated. These functions may or may not represent the same molecular mechanism. Integral membrane proteins are indicated by reddish shading. gp19K is a glycoprotein that reduces MHC class I protein synthesis, and inhibits killing of infected cells by CTLs. The 14.7K protein inhibits Tnf-induced apoptosis. The 6.7K protein maintains calcium homeostasis and prevents death-receptor-initiated apoptosis and arachidonic acid release. $cPLA_2$, cytoplasmic phospholipase A2. Adapted from W. S. M. Wold and A. E. Tollefson, *Semin. Virol.* **8:**515–523, 1998, with permission.

die by apoptosis, and the system returns to its normal, preinfection state. The adaptive response can be avoided completely, or in part, when organs or tissues that have poor immune responses or none are infected, when new viral variants are produced rapidly because of high mutation rates, or when progeny virions spread directly from cell to cell. Consequently, there can be dramatic differences in patterns of infection ranging from short-lived (acute) to lifelong (persistent), the topic of Chapter 5.

BOX 4.10 EXPERIMENTS

Setting the threshold for activation of innate immunity

The threshold that will trigger an innate immune response when crossed is not the same for every individual. However, the molecular mechanisms that set this threshold are poorly understood. Some insight into the problem came recently from an unexpected source: studies of γHV68 latent infections in mice. γHV68 is a gammaherpesvirus that establishes a latent infection in mouse B cells. The conventional view had been that latent infections are quiescent to the point of being inapparent to the host. Unexpectedly, latent γHV68 herpesvirus infections confer prolonged cross-protection against a variety of bacterial pathogens, including *Listeria* (listeriosis) and *Yersinia* (plague). The latent infection activates macrophages because immune recognition of the latently infected B cells leads to production of IFN-γ. The authors conclude that the latent virus infection sets the level of innate immunity. Moreover, not only is the latent infection an active immunological state, but also infected animals enjoy a benefit from this curious symbiotic relationship.



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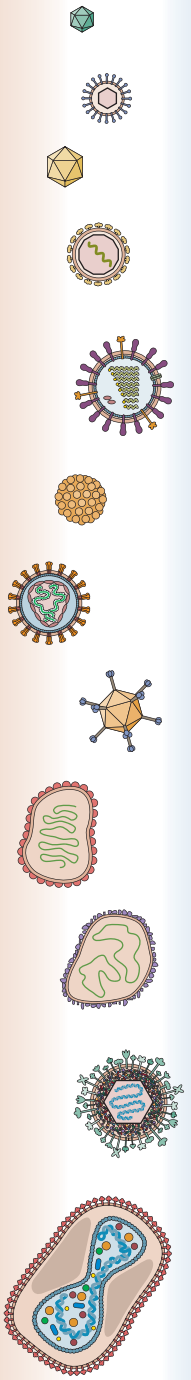

5

Patterns of Infection

You know something's happening, but you don't know what it is, do you, Mr. Jones?

Bob Dylan
Ballad of a Thin Man

How can you expect one species to follow the logic of another?

Philip K. Dick
The Crack in Space

Introduction

Viral infections of individuals in populations differ from infections of cultured cells in the laboratory. In the former, initiation of the infection and its eventual outcome rest upon complex variables such as host defenses, composition of the host population, and the environment. Despite such complexity and the plethora of viruses and hosts, common patterns of infection do appear. In general, natural infections can be rapid and self-limiting (**acute infections**) or long-term (**persistent infections**). These patterns can be surprisingly stable over time and characteristic for many virus families. Variations and combinations of these two modes abound (Fig. 5.1). It can be argued that all patterns begin with an acute infection, and differences in the subsequent management of that infection engender the many variations. For example, most **latent infections** begin as an acute infection of one cell type, but then when a different cell type is infected, no infectious particles are produced at all. Nevertheless, the genome persists to be reactivated in the future. Intermediate patterns that lie between rapid viral growth and latent infection can be thought of as "**smoldering infections**" in which low-level viral replication occurs in the face of a strong immune response. Similarly, **slow infections**, **abortive infections**, and **transforming infections** are more complicated variants of persistent infections.

While we can provide detailed descriptions of individual patterns of infection, we are in the early days of understanding the molecular mechanisms required to initiate or maintain any specific one. In this chapter, we discuss the principles that are the foundations of the observed patterns of infection.

Life Cycles and Host Defenses

A cursory examination of the animal viruses that grow in cultured cells identifies many distinctive life cycles with common features. Some infections rapidly kill the cell while producing a burst of new particles (**cytopathic viruses**). Others yield virions without causing immediate host cell death (**noncytopathic viruses**). Alternatively, some infections neither kill the cell nor produce any

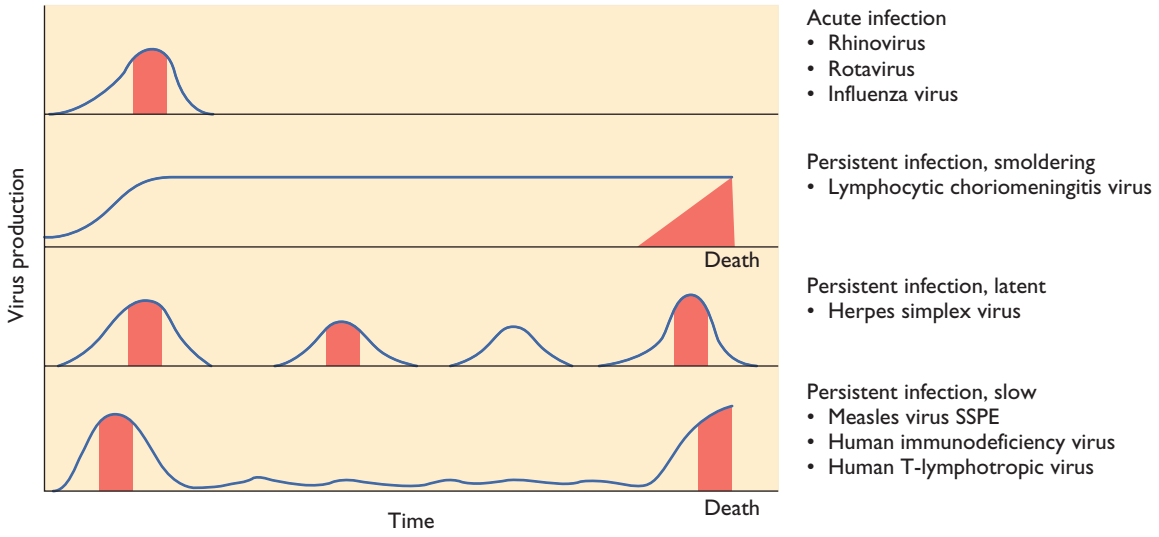

Figure 5.1 General patterns of infection. Relative virion production is plotted as a function of time after infection (blue line). The time when symptoms appear is indicated by the red shaded area. The top panel is the typical profile of an acute infection, in which virions are produced, symptoms appear, and the infection is cleared within 7 to 10 days after infection. The second panel is the typical profile of a persistent smoldering infection, in which virion production continues for the life of the host. Symptoms may or may not appear just before death, depending upon the virus. Virions are usually produced throughout the infection. The bottom two panels are variations of the profile of a persistent infection. The third panel depicts a latent infection in which an initial acute infection is followed by a quiescent phase and repeated bouts of reactivation. Reactivation may or may not be accompanied by symptoms, but generally results in the production of virions. The fourth panel depicts a slow virus infection, in which a period of years intervenes between a typical primary acute infection and the usually fatal appearance of symptoms. The production of infectious particles during the long period between primary infection and fatal outcome may be continuous (e.g., human immunodeficiency virus) or absent (e.g., measles virus SSPE). Adapted from F. J. Fenner et al., *Veterinary Virology* (Academic Press, Inc., Orlando, FL, 1993), with permission.

progeny. These life cycle characteristics are only part of the processes that produce stable patterns. Host responses also play central roles in the evolution of patterns of infection. As we discuss in Chapters 3 and 4, the interplay between the molecular biology of viral life cycles and host defense systems is of paramount importance to the outcome of any infection. These interactions are dynamic and, despite appearing rather chaotic at the molecular and cellular level, can be remarkably stable and predictable when averaged over many individuals infected over long periods.

Mathematics of Growth Correlate with Patterns of Infection

The changes in size of a viral population can be described by one simple concept: the rate of increase is the difference between the rate of replication and the rate of elimination. We can write this statement as

$$dN/dt = (b - d)N$$

where dN/dt is the rate of change of the population (N) with respect to the change in time. The terms b and d are the average rates of birth and death, respectively. The term $(b - d)$ is usually written as a constant r, the intrinsic rate of increase. Therefore, we obtain equation 5.1:

$$dN/dt = rN$$
$$\text{and } \ln N = rt \qquad \textbf{(5.1)}$$

This is the equation for exponential population growth. Plotting ln N versus t yields a straight line with slope r (Fig. 5.2A).

If b far exceeds d (as is the case for infections in cultured cells), progeny accumulate. When b equals d, the population maintains a stable size. If we assume a linear relationship for increase and decrease of the population, then the slope of the increase of replication rate is equal to k_b and the slope of death or removal rate is equal to k_d. The stability of the population N then can be written as follows:

$$b_0 - k_b N = d_0 + k_d N$$

or

$$N = (b_0 - d_0)/(k_d + k_b)$$

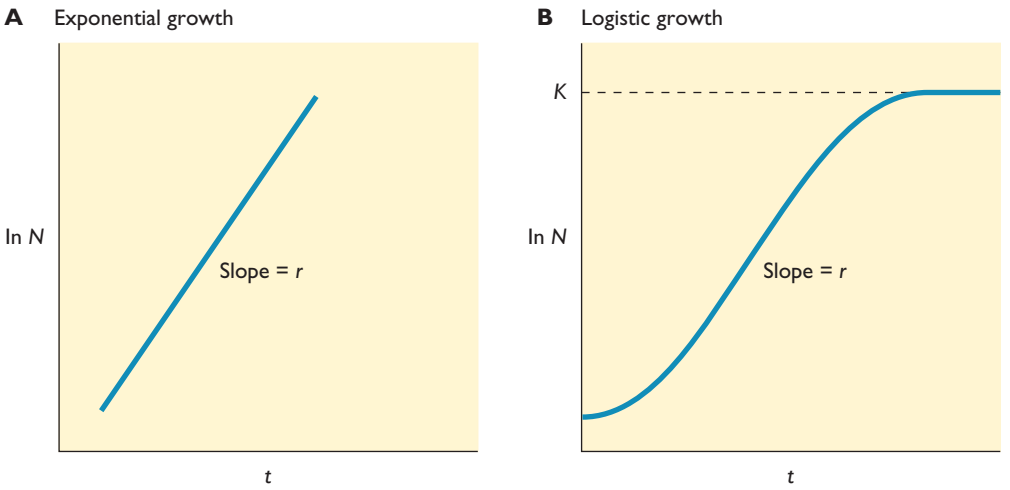

Figure 5.2 Two plots of standard growth equations. (A) A graph of simple exponential growth. **(B)** A graph of the pattern termed logistic growth illustrating *K*, the limit to growth. *r* is the slope in both types of plot.

This description of *N* is called the **carrying capacity (*K*)** of the environment. The term "environment" can define a single cell, an individual, or the entire host population. For any value of *N* greater than *K*, the viral population will decrease, and for any value of *N* less than *K*, the population will increase. The carrying capacity *K* is of particular interest in virology, as it defines the upper boundary of the growing population and, as we will note later, influences patterns of infection.

Therefore, by knowing that $r = (b_0 - d_0)$ and $K = (b_0 - d_0)/(k_b + k_d)$, we can substitute these values in equation 5.1 to obtain the basic equation for growth and regulation of a population, sometimes called the logistic growth equation (equation 5.2).

$$dN/dt = rN(K - N/K) \qquad \textbf{(5.2)}$$

Plotting ln N versus *t* yields the curve illustrated in Fig 5.2B. Here, *K* is easily seen to be the limit to growth, and the rate of increase is *r*.

Two fundamental viral growth strategies are apparent in nature, and these are strongly correlated with distinctive patterns of viral infection. The first is the ***r*-replication strategy**, in which large numbers of progeny are produced and growth is maintained by a steady, unbroken lineage of serial infections and never reaches a limit as long as there are susceptible hosts (equation 5.1; Fig. 5.2A). The second is the ***K*-replication strategy**, in which the host population is at or close to its saturation density (e.g., new susceptible hosts are rare or nonexistent [equation 5.2; Fig. 5.2B]). In addition, rates of viral propagation may be slow or vanishingly small.

r-replication strategies often manifest as **acute infections**: virulent, short reproductive cycles with production of many progeny. Pathogenesis may or may not be obvious, but high rates of shedding and efficient transmission are the rule. Acute infections following an *r*-replication strategy will "burn out" in the absence of susceptible hosts. One can mimic an *r*-selection environment in cell culture by low-multiplicity-of-infection (MOI) infections: susceptible cells abound to sustain multiple rounds of replication, but transmission stops abruptly when all the cells are infected.

K-replication strategies often appear as persistent or **latent infections**. In this pattern, infected hosts survive for extended times (resources for both host and viral reproduction are maximized). In cell culture, this selection environment can be mimicked by high-MOI infections: most cells are infected and faster replication confers no selective advantage. For some bacteriophages, this environment leads to efficient integration of viral DNA and formation of lysogens (formation of prophage).

Other examples of persistent infection appear as a mixture of *r* and *K* strategies: virion production occurs continuously over the life of most of the individuals in a population, but is balanced by immune elimination (a smoldering infection). In other situations (e.g., latent infection), the viral genome survives as long as the host prospers. Often infectious virions are produced only sporadically during the host's lifetime, when the host is in danger or stressed. Under these conditions, the mechanisms for viral shedding and transmission are so efficient that even a small number of virions have a high probability of surviving and infecting others in the population.

Viruses and their hosts exist along a continuum of values for *r* and *K*. Hosts can have generation times of minutes to years, which influence the selection of viruses that infect

them. These host growth properties also play pivotal roles in evolution of viral growth patterns. Hosts that grow rapidly with generation times of minutes or hours (e.g., bacteria), or have rapid growth as part of their life cycles (cycling cells), tend to be infected by rapidly replicating viruses. Hosts with generation times of years (e.g., mammals, fish, and plants), tend to be infected by viruses that can establish persistent infections of some type. Even in these slow-growing hosts, rapidly growing viruses can be selected. However, in these cases, rapid growth is quickly met by host defenses that limit pathogenesis and spread.

The growth equations, as written in their simplest form above, can be used to model replication in single, identical cells in culture. However, to describe how a viral infection is propagated and maintained in a large population of hosts, more terms must be included. These terms would define the rate of shedding from infected individuals, the rate of transmission to other individuals, the probability that one infected individual will infect more than one person, and the number and density of susceptible individuals (Box 5.1). Some of these parameters are discussed in Chapter 10.

Acute Infections

Definition and Requirements

Acute infections are common and well studied. The term "acute" refers to rapid onset of disease with a short but occasionally severe course. Hallmarks of an acute viral infection are rapid production of infectious virions, followed by resolution and elimination of the infection by the host ("clearing" the infection). These infections occur only when intrinsic and innate defenses are transiently bypassed. Acute infections are the typical, expected course for agents such as influenza virus and rhinovirus (Fig. 5.3). The disease symptoms tend to be relatively brief and resolve over a period of days. In a healthy host, virions and virus-infected cells are destroyed (cleared) by the adaptive immune system within days. Nevertheless, during the rapid replication phase, some progeny invariably are shed and spread to other hosts **before** the infection is resolved (Boxes 5.2 and 5.3). If the initial infection escapes local defenses and spreads via hematogenous or neural routes to other parts of the body, several distinct rounds of replication may occur in the same animal, with new and distinctive symptoms. The pattern of multiple infections is characteristic of varicella-zoster virus, an alphaherpesvirus that causes the childhood disease chickenpox (Fig. 5.4).

Occasionally, an acute infection results in limited or even no obvious symptoms. Indeed, **inapparent** (asymptomatic) acute infections are quite common, and can be major sources of infections in populations (Box 5.3). Such infections are recognized by the presence of virus-specific antibodies with no reported history of disease. For example, over 95% of the unvaccinated population of the United States has antibody to varicella-zoster virus, but less than half report that they have had chickenpox.

Acute Infections Tend To Be Efficiently Contained and Cleared

Once immediate host defenses have been breached and an infection is established, a cascade of new defensive reactions occurs in the host (see Chapters 3 and 4). Symptoms and disease may or may not be obvious, depending upon the virus, the infected tissue, and the host defenses. The initial period before the characteristic symptoms of a disease are obvious is called the **incubation period**. During this time,

BOX 5.1 BACKGROUND

Viral spread in space and time: modeling epidemic spread

In the simple growth equations discussed in the text, susceptible cells or animals were assumed to be well mixed. In addition, viral growth was considered only with respect to time. In reality, of course, populations are not well mixed, population centers are separated geographically, and individuals are free to move around the country and the world. When spatial dimensions that describe these conditions are added to the basic r and K expressions, extremely rich patterns of infection emerge. In epidemics, the patterns often appear as waves of infection propagating through the nonhomogeneous population. For example, more people live in cities than in the country so cities form nodes of high propagation. As populations become immune and infected individuals move to different locations where susceptible hosts abound, more complex patterns result. For example, the spread of measles across the United Kingdom and sub-Saharan Africa exhibits complicated waveforms. These studies provide important data for epidemiologists and those who wish to implement effective control strategies.

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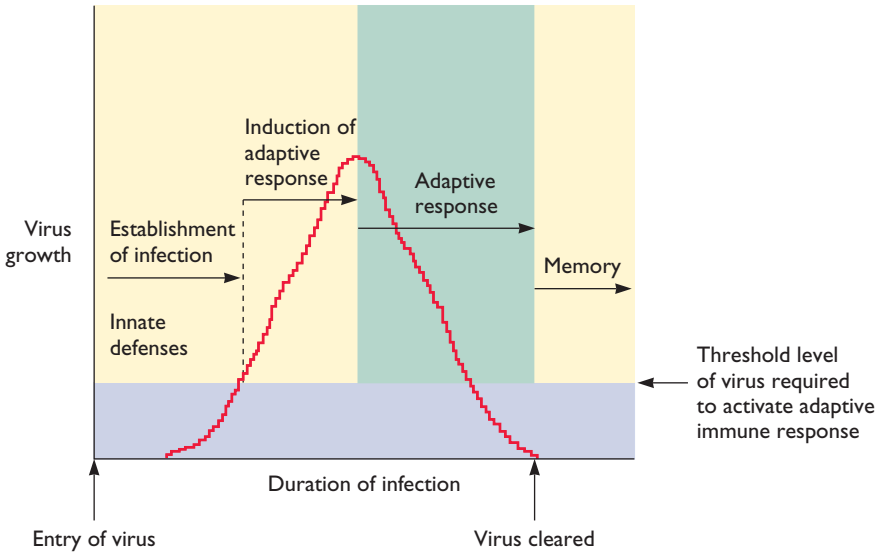


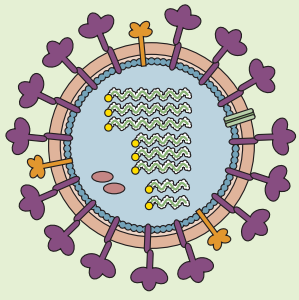
Figure 5.3 The course of a typical acute infection. Relative virus growth plotted as a function of time after infection. The concentration of virions increases with time, as indicated by the jagged red line. During the establishment of infection, only the innate defenses are at work. If the infection reaches a certain threshold level characteristic of the virus and host (purple), the adaptive responses initiate. After 4 to 5 days, effector cells and molecules of the adaptive response begin to clear infected tissues and virions (green). After this action, memory cells are produced, and the adaptive response ceases. Antibodies, residual effector cells, and memory cells provide lasting protection should the host be reinfected at a later date. Redrawn from C. A. Janeway, Jr., and P. Travers, *Immunobiology: the Immune System in Health and Disease* (Current Biology Ltd. and Garland Publishing, New York, NY, 1996), with permission.

viral genomes are replicating and the host is responding, producing cytokines such as interferon (IFN) that can have global effects that manifest as the classical symptoms of an acute infection (e.g., fever, malaise, aches, pains, and nausea). Remarkably, incubation periods can vary from 1 or 2 days to years (Table 5.1). Short incubation times usually indicate that actions at the primary site produce the characteristic symptoms of the disease. Long incubation times indicate that the host response, or the tissue damage required to reveal the symptoms of infection, result from actions other than those at the primary site of infection. The events that occur during the incubation period certainly influence the observed pattern of infection, but identification and characterization of these processes remain major challenges for those studying pathogenesis. Meeting these challenges is of paramount importance if we are to design

BOX 5.2 BACKGROUND

Uncomplicated acute infection by influenza virus

An influenza virus infection begins in the upper respiratory tract by inhalation of droplets produced when an infected individual sneezes or coughs. Viral replication occurs in ciliated columnar epithelial cells of the respiratory epithelium, and progeny virions spread to nearby cells. Infectious particles can be isolated from nasal secretions or throat swabs for 1 to 7 days, with the peak occurring on the fourth or fifth day after infection. About 48 h after the initial infection, symptoms appear abruptly, such that infected individuals can almost pinpoint the hour that they noticed they had the flu. Symptoms last for about 3 days and then begin to abate.

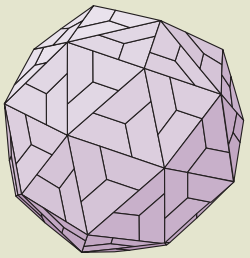


The infection typically resolves within a week through action of the innate and acquired immune systems, but it may take several weeks before the individual feels completely well because of the lingering effects of the host responses.

BOX 5.3 DISCUSSION

Inapparent acute infections

It is important to distinguish an inapparent acute infection from an unsuccessful one. Inapparent infections are successful acute infections that produce no symptoms or disease. Sufficient virions are made to maintain the infection in the host population, but the quantity is below the threshold required to induce symptoms in infected individuals. The usual way an inapparent infection is detected is by a rise in antiviral antibody concentrations in an otherwise healthy individual. Well-adapted pathogens often follow this pattern, as demonstrated by poliovirus, in which more than 90% of infections are inapparent.



and implement useful diagnostic tools and treatments. In Chapter 3, we present more discussion of the adage that "whatever happens early in a viral infection dictates what happens later on."

The intrinsic and innate responses limit and contain most acute infections. When these defenses are lacking or compromised, acute infections can be disastrous, primarily because the infection becomes systemic and multiple organs can be damaged. In a naive host, the adaptive immune response (antibody and activated cytotoxic T lymphocytes [CTLs]) does not influence viral replication for several days, but is essential for final clearance of virions and infected cells. The adaptive response also provides **memory T and B cells** for defense against subsequent exposure to the same infectious agent.

Antigenic Variation Provides a Selective Advantage in Acute Infections

If an individual survives a typical acute infection, he or she often is immune to infections by the same virus. Nevertheless, some acute infections (e.g., infections with rhinovirus [the common-cold virus], influenza virus, and human immunodeficiency virus) occur repeatedly despite a robust immune response to them. These recurring infections are possible because selection pressures during the initial acute infections lead to shedding of virions that are resistant to immune clearance. In many cases, the structural properties of virions and the capacity of neutralizing antibodies to block infectivity are critical parameters.

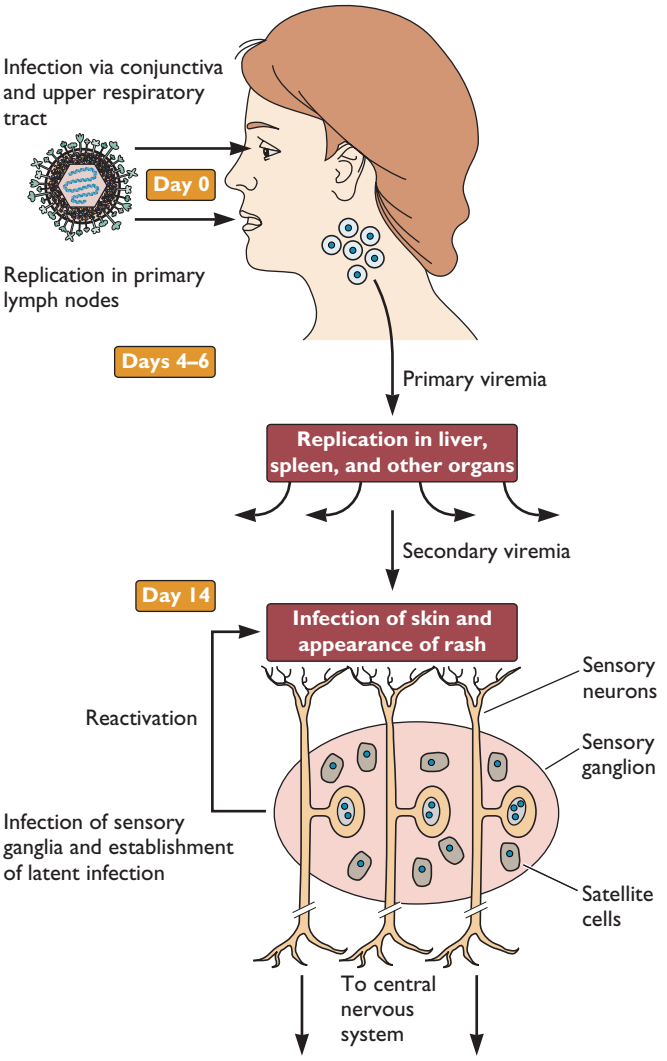


Figure 5.4 Model of varicella-zoster virus infection and spread. Infection initiated on the conjunctiva or mucosa of the upper respiratory tract spreads to regional lymph nodes. After 4 to 6 days, infected T cells enter the bloodstream, causing a **primary viremia**. These infected cells subsequently invade the liver, spleen, and other organs, initiating a second round of infection. Virions and infected cells are then released into the bloodstream in a **secondary viremia**. Infected skin-homing T cells efficiently invade the skin and initiate this third round of infection about 2 weeks from the initial infection. The characteristic vesicular rash of chicken pox appears as a result of immune defensive action. Next, virions produced in the skin infect sensory nerve terminals and spead to dorsal root ganglia of the peripheral nervous system, where a latent infection is established. The latent infection is maintained by active immune surveilance. Later in life, perhaps as the immune system wanes, reactivations are not contained and another infectious cycle is initiated. Virions leave the peripheral neurons to infect the skin. The characteristic recurrent disease called shingles is often accompanied by a long-lasting painful condition called postherpetic neuralgia. Normally, an infected individual experiences only one visible reactivation event, probably because reactivation stimulates the immune system. Such restimulation of the immune system is the rationale for administering the varicella-zoster live vaccine to adults to prevent reactivation and shingles.

Table 5.1 Incubation periods of some common viral infections

Disease	Incubation period (days)[a]
Influenza	1–2
Common cold	1–3
Bronchiolitis, croup	3–5
Acute respiratory disease (adenoviruses)	5–7
Dengue	5–8
Herpes simplex	5–8
Enterovirus disease	6–12
Poliomyelitis	5–20
Measles	9–12
Smallpox	12–14
Chickenpox	13–17
Mumps	16–20
Rubella	17–20
Mononucleosis	30–50
Hepatitis A	15–40
Hepatitis B and C	50–150
Rabies	30–100
Papilloma (warts)	50–150
AIDS	1–10 yr

[a]Until first appearance of prodromal symptoms.

Virions that can tolerate many amino acid substitutions in their structural proteins and remain infectious are said to have **structural plasticity** (e.g., influenza virus and human immunodeficiency virus). If viral replication occurs in the presence of antibody that neutralizes virions, antibody-resistant mutants are selected and shed. These mutant virions can reinfect individuals who were immune to the initiating virus. In contrast, there are other virions that cannot tolerate many amino acid changes in their structural proteins (e.g., those of poliovirus, measles virus, and yellow fever virus). For these viruses, even if the mutation rate is high, antibody-resistant virions have a low probability of being selected.

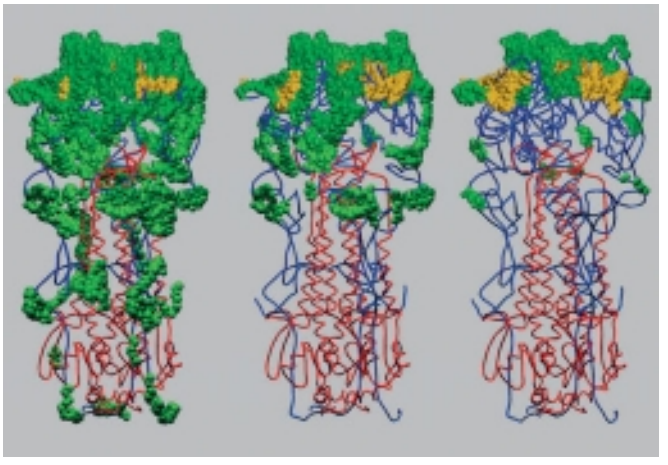
The principles underlying the selection and maintenance of antibody-resistant virions in natural infections are not well developed. Exceptions to rules such as structural plasticity are common. For example, the virions of rhinoviruses have remarkable structural plasticity, while those of poliovirus, a related picornavirus, do not. Over 100 different serotypes of rhinovirus are maintained in humans at all times, a property that accounts for the fact that individuals may contract more than one common cold each year. This fact also explains why it is difficult to produce a vaccine against the common cold. Why just three serotypes of poliovirus are circulating around the world is a mystery. Fortunately, this property ensures that the poliovirus vaccines that were effective in the 1950s are just as potent in the 21st century. Similarly, enveloped influenza virus particles more resistant to antibodies are readily selected, while the enveloped particles of measles virus and yellow fever virus exhibit little variation in membrane protein amino acid sequence, and antibody-resistant variants are rarely observed. Consequently, an influenza vaccine is required every year, while a single measles virus vaccination lasts a lifetime.

Antigenic variation is the change of virion proteins in response to antibody selection. In an immunocompetent host, antigenic variation comes about by two distinct processes. **Antigenic drift** is the appearance of virions with slightly altered surface proteins (antigen) following passage in the natural host (Fig. 5.5). In contrast, **antigenic shift** is a major change in the surface protein(s) of a virion as genes encoding completely new surface proteins are acquired. This dramatic change in virion composition results when a host is coinfected with two viral serotypes. Viruses with segmented genomes can exchange segments, or coreplicating genomes can produce recombinant genomes. The new reassortant and recombinant viruses have exchanged blocks of genetic information, and the resulting hybrid virions may temporarily avoid immune defenses (Box 5.4; see also Chapter 10).

Acute Infections Present Common Public Health Problems

An acute infection is most frequently associated with serious epidemics of disease affecting millions of individuals every year (e.g., influenza and measles). The nature of an

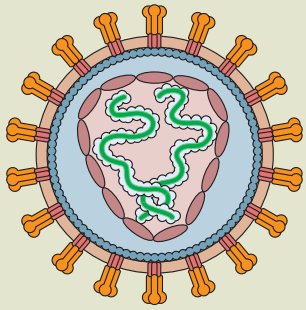
Figure 5.5 Antigenic drift: distribution of amino acid residue changes in hemagglutinins (HA) of influenza viruses isolated during the Hong Kong pandemic era (1968 to 1995). The space-filling models represent the virus-receptor binding site (yellow) and the substituted amino acids (green). **(Left)** All substitutions in HAs of virions isolated between 1968 and 1995; **(middle)** amino acid substitutions that were retained in subsequent years; **(right)** amino acid substitutions detected in monoclonal antibody-selected variants of A/Hong Kong/68 HA. The α-carbon tracings of the HA1 and HA2 chains are shown in blue and red, respectively. Adapted from T. Bizebard et al., *Curr. Top. Microbiol. Immunol*. **260:**55–64, 2001, with permission.



BOX 5.4 DISCUSSION

Recombination and antigenic shift during human immunodeficiency virus infections

While the contribution of antigenic shift to the dramatic influenza virus pandemics is well documented, the role of this process in human immunodeficiency virus pathogenesis is only now becoming appreciated. In this case, antigenic shift can occur only by recombination. The process requires packaging of two distinct genomes into a single particle for subsequent reverse transcription and copy-choice replication. At first glance, recombination appears to be an unlikely event. However, results of recent experiments indicate that recombination may be more frequent than expected. Splenocytes from human immunodeficiency virus-infected patients harbor as many as three or four distinct



proviral genomes per cell, and give rise to huge numbers of recombinants. Antigenic shift arising from recombination can be thought of as a double-edged sword for pathogen and host: virions can escape elimination by immune defenses with serious consequences to the host, but if the immune system is functional, CTLs and antibodies will recognize the new combinations, and broaden the immune repertoire against the infected cell.

Jung, A., R. Maier, J.-P. Vartanian, G. Bocharov, V. Jung, U. Fischer, E. Meese, S. Wain-Hobson, and A. Meyerhans. 2002. Multiply infected spleen cells in HIV patients. *Nature* **418:**144.

acute infection presents difficult problems for physicians, epidemiologists, drug companies, and public health officials. The main problem is that by the time people feel ill or mount a detectable immune response, most acute infections are essentially complete, and the infection has spread to the next host. Such infections can be difficult to diagnose retrospectively, or to control in large populations or crowded environments (e.g., day care centers, military camps, college dormitories, nursing homes, schools, and offices). Effective antiviral drug therapy requires treatment early in the infection, often before symptoms are manifested, because by the time the patient feels ill, the infection has been resolved. Antiviral drugs can be given in anticipation of an infection, but this strategy demands that the drugs be affordable, safe, and free of side effects. Moreover, as we discuss in Chapter 8, our arsenal of antiviral drugs is very small, and drugs effective for most common acute viral diseases simply do not exist.

Persistent Infections

Definition and Requirements

Persistent infections occur when the primary infection is not cleared efficiently by the adaptive immune response. Instead, virions, proteins, and genomes continue to be produced or persist for long periods, often for the life of the animal. Virions may be produced continuously or intermittently for months or years, even in the face of an active immune response (Fig. 5.1). In some instances, viral genomes remain after viral proteins are no longer detected. Distinctions are often made between persistent infections that are eventually cleared (**chronic infections**) and those that last the life of the host (**latent infections** or **slow infections**).

The persistent pattern is surprisingly common (Table 5.2), particularly for noncytopathic viruses. For example, some arenaviruses, such as lymphocytic choriomeningitis virus, are inherently noncytopathic in their natural hosts and maintain a persistent infection if the host cannot clear the infected cells. Other viral life cycles include a distinct noncytopathic phase in addition to a cytolytic phase. Epstein-Barr virus infections include alternative transcription and replication programs that maintain the viral genome in some cell types with no production of viral particles. In other cases, ubiquitous infections, such as those produced by adenoviruses, circoviruses, and human herpesvirus 7, persist uneventfully in most human populations. Adenoviruses can be isolated from lymphoid tissue, including adenoids and tonsils, in most respiratory infections, but cultured lymphoid cells do not support efficient viral replication. It is possible that delayed kinetics of infection and replication observed in these cells contribute to the long-term maintenance of adenovirus in a fraction of lymphoid cells. What is clear from these examples is that no single mechanism is responsible for establishing a persistent infection. However, one common theme does emerge: when viral cytopathic effects and host defenses are reduced, a persistent infection is likely.

Table 5.2 Some persistent viral infections of humans

Virus	Site(s) of persistence	Consequence(s)
Adenovirus	Adenoids, tonsils, lymphocytes	None known
Epstein-Barr virus	B cells, nasopharyngeal epithelia	Lymphoma, carcinoma
Human cytomegalovirus	Kidneys, salivary gland, lymphocytes,[a] macrophages,[a] stem cells,[a] stromal cells[a]	Pneumonia, retinitis
Hepatitis B virus	Liver, lymphocytes	Cirrhosis, hepatocellular carcinoma
Hepatitis C virus	Liver	Cirrhosis, hepatocellular carcinoma
Human immunodeficiency virus	$CD4^+$ T cells, macrophages, microglia	AIDS
Herpes simplex virus types 1 and 2	Sensory and autonomic ganglia	Cold sore, genital herpes
Human T-lymphotropic virus types 1 and 2	T cells	Leukemia, brain infections
Papillomavirus	Skin, epithelial cells	Papillomas, carcinomas
Polyomavirus BK	Kidneys	Hemorrhagic cystitis
Polyomavirus JC	Kidneys, central nervous system	Progressive multifocal leukoencephalopathy
Measles virus	Central nervous system	Subacute sclerosing panencephalitis, measles inclusion body encephalitis
Rubella virus	Central nervous system	Progressive rubella panencephalitis
Varicella-zoster virus	Sensory ganglia	Zoster (shingles), postherpetic neuralgia

[a]Proposed, but not certain.

An Ineffective Intrinsic or Innate Immune Response Can Promote a Persistent Infection

Primary infections are cleared by the adaptive immune response, but this action can occur only through close integration with intrinsic and innate defenses. If such early action and communication among defense systems does not occur, the host may die as the infection spreads out of control. Alternatively, the infection may persist. For example, apoptosis is a common intrinsic cellular defense that can limit or expand viral replication and spread. In some vertebrate cell lines, Sindbis virus infection is acute and cytopathic because apoptosis is induced. However, if the host Bcl2 protein blocks apoptosis, a persistent infection is established. Similarly, Sindbis virus causes a persistent infection of cultured postmitotic neurons because these cells are intrinsically resistant to virus-induced apoptosis. The *in vitro* studies are recapitulated in host animals: when Sindbis virions are injected into an adult mouse brain, a persistent noncytopathic infection is established. In contrast, when the same preparation is injected into neonatal mouse brains, the infection is cytopathic and lethal, because neonatal neurons lack the gene products to block virus-induced apoptosis.

The host IFN response plays unexpected roles in establishing patterns of infection. For example, bovine viral diarrhea virus, a pestivirus in the *Flaviviridae* family, establishes a lifelong persistent infection in the vast majority of cattle around the world. Remarkably, persistently infected animals have no detectable antibody or T-cell responses to viral antigens. Cytopathic and noncytopathic strains have been useful in understanding how a persistent infection occurs. Infection of pregnant cattle with the noncytopathic biotype during the first 120 days of pregnancy results in birth of persistently infected calves. In contrast, infection of the fetus by cytopathic virus is contained quickly and eliminated. We now understand that the phenotype depends on a rapid IFN response by the fetus that clears the infection. In contrast, noncytopathic infection of fetal tissue does not stimulate production of IFN. Consequently, the adaptive immune system is not activated, and because infection does not kill cells, a persistent infection is established.

Modulation of the Adaptive Immune Response Perpetuates a Persistent Infection

Interference with Production and Function of MHC Proteins

The CTL response is one of the most powerful adaptive host defenses against viral infection. This response depends, in part, upon the ability of host T cells to detect viral antigens present on the surfaces of infected cells, and to kill them. Recognition requires the presentation of viral peptides by major histocompatibility complex (MHC) class I proteins. The pathway by which endogenous peptide antigens are produced and transported to the cell surface is discussed in Chapter 4 (see Fig. 4.13). Obviously, any mechanism that prevents viral peptides from binding to MHC class I molecules, even transiently, provides a potential selective advantage. The production of MHC class I

Table 5.3 Viral regulation of MHC class I antigen synthesis

Virus	Observed effect or postulated mechanism
RNA	
Human immunodeficiency virus type 1	Tat protein-induced reduction in MHC class I gene transcription; Vpu interferes with an early step in synthesis of MHC class I; Nef promotes endocytosis of MHC class I from the cell surface
Mouse hepatitis virus	Decrease in transcription of specific MHC class I genes
Respiratory syncytial virus	Decrease in MHC class I gene transcription
Poliovirus	Protein 3A inhibits transport of vesicles containing MHC class I proteins
DNA	
Adenovirus	E3 gp19kDa retains MHC class I in endoplasmic reticulum (ER); reduced transcription of MHC class I genes
Epstein-Barr virus	EBNA-4 may block production of antigenic peptides or their transport from the cytosol to the ER; allele-specific decrease in MHC class I appearance on cell surface
Human cytomegalovirus	US3 retains MHC class I molecules in the ER; US6 inhibits peptide translocation by TAP (ER lumenal domain); US11 and US2 dislocate MHC class I molecule from the ER lumen to the cytosol by different mechanisms; UL10 decreases rate of MHC class I export from ER; UL83 blocks IE-1 peptide presentation
Herpes simplex virus	ICP47 binds to TAP transporter and blocks import of peptides into the ER
Vaccinia virus	Lower abundance of MHC class I on cell surface by unknown mechanisms

proteins is modulated after many acute infections (Table 5.3). Presumably such modulation prevents or delays elimination by early-acting CTLs so that sufficient progeny can be disseminated. Any process that reduces immune clearing of infected cells has the potential to promote a subsequent persistent infection.

The MHC class I pathway is vital to the immune response and is highly regulated. However, many of the MHC-processing or regulatory steps were not known until it became obvious that many viral infections interfere with this pathway. Identifying the mechanisms used by the viral proteins was revealing. For example, several viral proteins block MHC class I function at various points in the pathway (Fig. 5.6; Table 5.3). Peptide presentation by MHC class I proteins can be reduced by lowering the expression of the MHC genes, by blocking the production of peptides by the proteasome, or by interfering with subsequent assembly and transport of the MHC-peptide complex to the cell surface.

Human cytomegalovirus, a betaherpesvirus, causes a common childhood infection with inapparent to mild effects in healthy individuals (Appendix A). These infections are never cleared, and a persistent infection is established in salivary and mammary glands as well as the kidneys. Infectious virions are secreted in saliva, milk, and urine. In addition, a latent infection is established in early precursor cells of the monocyte/macrophage lineage; no virions are produced from these cells. When latently infected individuals become immunosuppressed by drugs during organ or bone marrow transplantation, or by human immunodeficiency virus infections, cytomegalovirus replication resumes, often causing a life-threatening disease.

Human cytomegalovirus deserves special mention, because MHC class I presentation of viral antigens is inhibited by multiple mechanisms during an acute infection. The viral US6 protein inhibits translocation of peptides into the endoplasmic reticulum lumen. The viral US3 protein binds to, and detains, MHC class I proteins in the endoplasmic reticulum, while the US11 and US2 proteins eject MHC class I molecules from that organelle into the cytoplasm, where they are degraded by the proteasome. Why human cytomegalovirus encodes so many proteins to block antigen presentation remains an open question. One possibility is that multiple gene products act additively or synergistically to delay immune clearance until macrophage/monocyte precursors are infected, and a latent infection is established.

Ubiquitinylation of proteins is an important regulatory mechanism that directs endocytosis, sorting, and degradation. The genomes of many gammaherpesviruses and poxviruses encode a class of zinc-binding RING finger proteins with E3 ubiquitin ligase activity, which disable the adaptive immune response, and also stimulate replication and inhibit apoptosis. The K3 and K5 genes of human herpesvirus 8 and the MK3 gene of murine gammaherpesvirus 68 encode such proteins. The K5 and MK3 proteins are related type III transmembrane proteins but, unexpectedly, act at different steps in the MHC class I pathway. The K5 protein reduces the concentrations of MHC class I

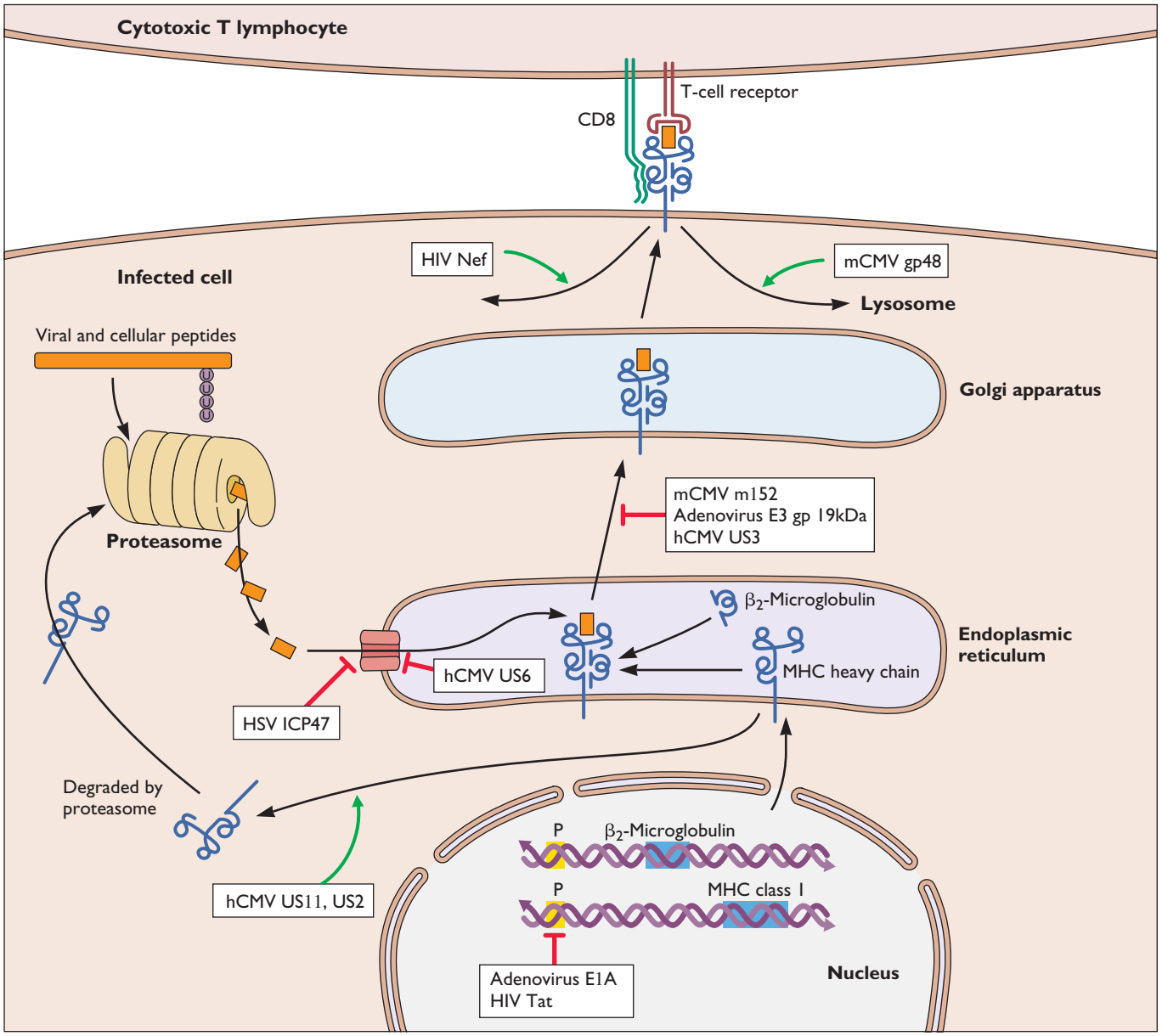


Figure 5.6 Viral proteins block cell surface antigen presentation by the MHC class I system. In almost every cell, a fraction of newly synthesized proteins translated in the cytoplasm is targeted to the proteasome, where the molecules are digested into peptide fragments (orange). These peptides are transported to the lumen of the endoplasmic reticulum by the Tap transporters in the endoplasmic reticulum membrane (pink channel). The peptides then bind to a cleft in newly synthesized MHC class I proteins (blue) that comprise a MHC class I heavy chain and a β_2-microglobulin chain. The complete peptide-MHC class I complex moves into the Golgi apparatus and then to the cell surface, where it can be recognized by T-cell receptors present on $CD8^+$ T cells that are interacting with the infected cell. Specific viral gene products block (red bars) this process at almost every step along the pathway. Green arrows indicate stimulation. In the nucleus, transcription of MHC class I genes can be blocked by E1A or Tat at the promoter (P), as indicated. HSV, herpes simplex virus; hCMV, human cytomegalovirus; mCMV, mouse cytomegalovirus; HIV, human immunodeficiency virus.

proteins, as well as costimulatory molecules present on the surfaces of infected cells, by adding ubiquitin to the cytoplasmic domains. This modification stimulates endocytosis of the marked proteins. The related MK3 protein is also an E3 ubiquitin ligase, but in this case, ubiquitinylation promotes retrotranslocation and proteosomal destruction of MHC class I proteins soon after they appear in the endoplasmic reticulum. The genome of myxoma virus encodes a similar RING finger E3 ligase called MV-LAP that also directs proteasomal destruction by a mechanism analogous to that used by the K5 protein. Importantly, while the effects of K5 protein upon human infections cannot be assessed, myxoma virus mutants that lack the MV-LAP gene are markedly attenuated in rabbits (the natural host).

Early observations indicated that Epstein-Barr virus-infected individuals do not produce CTLs capable of recognizing the viral protein EBNA-1. This phosphoprotein is found in the nuclei of latently infected cells, and is regularly detected in malignancies associated with the virus (see Tables 5.4 and 5.5). T cells specific for other Epstein-Barr virus proteins are made in abundance, indicating that EBNA-1 must possess some special features. Indeed, this protein contains an amino acid sequence with a remarkable activity that renders it invisible to the host proteasome so that relevant EBNA-1 peptides are not produced at all. This inhibitory sequence can be fused to other proteins to inhibit their processing and the subsequent presentation of peptide antigens normally produced from them. The biological relevance of this mechanism is evident after acute infection of B cells. The adaptive immune system kills all productively infected cells, sparing only rare cells that produce EBNA-1. These cells harbor a latent viral genome.

MHC Class II Modulation after Infection

In the exogenous pathway of antigen presentation, proteins are internalized and degraded to peptides that can bind to MHC class II molecules (Fig. 4.14). These complexes are transported to the cell surface, where they can be recognized by the $CD4^+$ T-cell receptor. Activated $CD4^+$ T-helper (Th) cells stimulate the development of CTLs and help coordinate an antiviral response to the pathogen. Any viral protein that modulates the MHC class II antigen presentation pathway would therefore interfere with Th-cell activation.

Many viral gene products modulate the MHC class II pathways. For example, the human cytomegalovirus US2 protein has been reported to promote proteasomal destruction of the class II DR-alpha and DM-alpha molecules. The Epstein-Barr virus BZLF2 protein interacts with intracellular and cell surface MHC class II molecules to block T-cell activation. Herpes simplex virus strain KOS infection results in removal of the MHC class II complex from the endocytic compartment. The human immunodeficiency virus Nef protein blocks the appearance of CD4 and MHC molecules on the cell surface. In endosomal compartments, Nef may interfere with acidification, affecting the loading of antigenic peptides onto MHC class II proteins.

Bypassing Deadly CTLs by Mutation of Immunodominant Epitopes

The Th and CTL populations found after some infections are surprisingly limited: the T cells respond to very few viral peptides. These peptides are said to be **immunodominant**. An extreme example of a limited CTL response is observed after infection of C57BL/6 mice with herpes simplex virus type 1. The virus-specific CTLs respond **almost entirely to a single peptide** in the viral envelope protein gB (the amino acid sequence of this peptide is SSIEFARL). Given that there are more than 85 open reading frames in the viral genome, it is remarkable that CTLs recognize only one peptide in this particular animal model of infection.

Focus of the T-cell response upon a small repertoire of viral peptides provides a ready opportunity to bypass T-cell recognition. A limited number of mutations in the coding sequence for these immunodominant peptides will render the infected cell invisible to the T-cell response produced early in infection. Viruses with these mutations are called **CTL escape mutants** and are thought to contribute to progressive accumulation of virus particles because of decreased clearing of infected cells. For example, CTL escape mutants, which are of central importance in human immunodeficiency virus pathogenesis, arise because of error-prone replication and the constant exposure to an activated immune response. In some well-documented cases, the T-cell peptide sequence is completely deleted from the viral protein synthesized by the CTL escape mutant. Understanding how immunodominant peptides are selected, maintained, and bypassed is essential if effective vaccines against human immunodeficiency virus are to be developed. For example, a vaccine directed toward a dominant T-cell peptide that is part of a critical structural motif in a viral protein may have value because CTL escape mutants will be less likely to survive and participate in subsequent spread of infection.

Immunodominant epitopes and CTL escape mutants play central roles in the increasingly common and dangerous infection caused by hepatitis C virus (more than 70 million people worldwide are infected [Appendix A]). The CTL response stimulated by acute infection is effective in less than 20 to 30% of individuals. An insidious persistent infection remains in the vast majority of patients.

After several years, this persistent infection can lead to serious liver damage and even fatal hepatocellular carcinoma. Persistently infected chimpanzees harbor viruses with CTL escape mutations in their genomes. In contrast, the viral population isolated from animals that resolved their acute infections rapidly included no such mutants. The conclusions from this work are clear: if CTL escape mutations occur early, a persistent infection is likely. If CTLs resolve the infection before escape mutants appear, no persistent infection is possible.

The CTL epitope need not be deleted or radically altered to escape CTL recognition. Indeed, when a T cell specific for a given viral peptide engages a similar, **but not identical**, peptide complexed to MHC class I, the T cell may respond partially or not at all. In this case, both mutant and parent viruses are likely to be maintained in the population. Altered viral peptides of this kind have been identified in viral isolates from persistent hepatitis B infections.

Killing Activated T Cells

Sometimes, when the CTL engages an infected cell, the CTL dies instead of its target. This unexpected turn of events is a remarkable example of viral defense. Activated T cells produce a membrane receptor called Fas on their surfaces. Fas is related to the Tnf family of membrane-associated cytokine receptors, and binds a membrane protein called Fas ligand (FasL). When Fas on activated T cells binds FasL on target cells, the receptor trimerizes, triggering a signal transduction cascade that results in apoptosis of the T cell. If viral proteins increase the quantity of FasL on the cell surface, any T cell (Th cell or CTL) that binds will be killed. Such a mechanism has been suggested to explain the relatively high frequency of "spontaneous" T-cell apoptosis in human immunodeficiency virus-infected patients. The viral Nef, Tat, and SU proteins, human T-lymphotropic virus Tax protein, and the human cytomegalovirus IE2 protein have all been implicated in increased production of FasL and resulting T-cell apoptosis. This seemingly unusual mechanism to kill T cells is not unique to viral infections. If it were, evolution would have removed the Fas system long ago. Indeed, Fas-mediated CTL killing is a normal activity in most complex organisms: its function is to remove T cells when they are no longer needed after infection, or when their presence in a tissue is detrimental. For example, certain delicate and irreplaceable tissues, such as the eye, remain free of potentially destructive T cells by maintaining a high concentration of FasL on cell surfaces.

Persistent Infections May Be Established in Tissues with Reduced Immune Surveillance

Cells and organs of the body differ in the degree of their immune defense. When virus particles infect such tissues, a persistent infection may be established if the organ is not otherwise compromised. Tissues with surfaces exposed to the environment (e.g., skin, glands, bile ducts, and kidney tubules) are exposed routinely to foreign matter, and therefore have a higher threshold for activating immune defenses. Persistent infections by members of the *Papillomaviridae* and the *Betaherpesvirinae* are common in these tissues. By replicating in cells present on lumenal surfaces of glands and ducts with poor immune surveillance (kidneys, salivary glands, and mammary glands), human cytomegalovirus particles are shed almost continually in secretions. Possibly the most extreme example of immune avoidance is represented by the papillomaviruses that cause skin warts. Productive replication of these infectious particles occurs only in the outer, terminally differentiated skin layer, where an immune response is impossible. Dry skin is continually flaking off, ensuring efficient spread of infection. This assertion can be verified by running a finger along a clean surface in the most hygienic hospital and noticing a white film, which is 70 to 80% keratin from human skin. Molecular biologists often discover this abundance of dried skin in the laboratory when examining silver-stained protein gels; the major band is often contaminating human keratin.

Certain compartments of the body, such as the central nervous system, vitreous humor of the eye, and areas of lymphoid drainage, are devoid of initiators and effectors of the inflammatory response, simply because these tissues can be damaged by the fluid accumulation, swelling, and ionic imbalances that characterize inflammation. In addition, because most neurons do not regenerate, immune defense by cell death is obviously detrimental. Accordingly, persistent infections of these tissues are common (Table 5.2).

Persistent Infections May Occur When Cells of the Immune System Are Infected

Many viruses infect cells of the immune system. Such infected lymphocytes and monocytes migrate to the extremes of the body, providing easy transport of virions to new sites of replication and shed. If these cells die or become impaired in an acute infection, the immune response could be ineffective, and a persistent infection may ensue. In the simplest case, the degree of immune response deficit would be directly proportional to the type and number of immune cells that are infected. We discuss the far-reaching consequences of systemic immunosuppression by viral infection in Chapter 4.

Human immunodeficiency virus provides a powerful reminder of how complicated and dynamic an infection of immune system cells can be (see Chapter 6). The virus infects not only $CD4^+$ Th cells, but also monocytes,

dendritic cells, and macrophages, all of which can transport virions to lymph nodes, the brain, and other organs. At first glance, one might expect the immune system to crash within a few days of the initial infection. However, this catastrophe does not happen, primarily because immune cells are continuously replenished. The new cells subsequently are infected and die, but on average, the immune system remains functional and does so for years. As a result, an untreated infected individual continues to produce prodigious quantities of virus particles in the face of a highly engaged immune system. The early stages of disease are characterized by continuous immune activation, and not by immunosuppression. It is only at the end stage of disease, when viral replication finally outpaces replenishment of immune cells, that massive immunosuppression occurs. Only then does the virus, as well as other unrelated secondary infections, spread in uncontrolled fashion, and the patient succumbs.

Two Viruses That Cause Persistent Infections

Measles Virus

Measles virus, a member of the family *Paramyxoviridae*, is a common human pathogen with no known animal reservoir (Appendix A). The genome organization and replication strategy are similar to those of the rhabdovirus vesicular stomatitis virus (Volume I, Appendix). Measles is one of the most contagious human viruses, with about 40 million infections occurring worldwide each year, resulting in more than 250,000 deaths (predominantly of children). This number is likely to be a substantial underestimate of the global burden, given the difficulties of record keeping in some countries. Normally, a single infection protects the individual for life. Consequently, the virus is maintained only in populations sufficient to produce a large number of new susceptible hosts (children). Population geneticists calculate that the critical community size required to maintain measles virus in the population is between 300,000 and 500,000.

Cellular receptors for measles virus include the CD46 and CD150 proteins. While CD150 is the receptor for all tested strains, CD46 is the predominant receptor for vaccine- and laboratory-adapted viruses. Many measles virologists think that other viral receptors must exist to account for the broad cellular tropism. After primary replication, measles virus infects local monocytes and lymphoid cells that migrate to draining lymph nodes. After replication in these tissues, a small proportion of monocytes, B cells, and T cells are infected and enter the circulation. Secondary infections of lymph tissues result in a secondary viremia, and replication continues in the epithelial cells of the lungs and the mouth. The characteristic mouth lesions, called Koplik's spots, are caused by a delayed-type hypersensitivity reaction, analogous to that responsible for the typical measles skin rash. The course of acute infection, so-called uncomplicated measles, runs for about 2 weeks (Fig. 5.7). An acute infection causes cough, fever, and conjunctivitis and confers lifelong immunity.

The vast majority of measles victims have an uneventful recovery, but a characteristic systemic immunosuppressive effect lasts for a week or two after the infection is resolved. Consequently, secondary infections by unrelated pathogens during this period may be uncontested by host defenses; the results may be serious or fatal if immediate intervention and care are not provided. The large number of children in the Third World who die after measles infection succumb to complications of secondary infections. Several mechanisms for immunosuppression have been proposed. Measles virus infects cells of the immune system, and this action may deregulate the immune response. Interleukin-12 is produced when Toll-like receptors on antigen-presenting cells are stimulated by infection. Infected T and B cells, as well as macrophages, are arrested in the late G_1 phase of the cell cycle and cannot perform their normal functions. Uninfected lymphocytes can also be affected by direct contact with viral membrane proteins present on the surface of infected cells.

On rare occasions, measles virus genomes and structural proteins are not cleared by the adaptive immune system, and may persist for years in an infected individual. The mechanisms responsible are only now being characterized. Some studies of humans infected with measles virus reported a positive correlation between antibody concentration and persistent infection. The significance of this correlation has been debated, but some insight has come from studying the effect of antibodies during infection of cultured cells. Measles virus-specific antibodies bind to viral membrane proteins present on cell surfaces to induce endocytosis and proteolysis of the antibody-protein complexes. Because one of these proteins, the viral fusion protein, is responsible for cell-cell fusion that causes cell death, exposure to antibodies effectively blocks this mechanism of cell killing, thereby allowing viral persistence in cultured cells.

An important finding is that measles virus can enter the brain in infected lymphocytes that traverse the body during the viremia following primary infection. Such a secondary infection of a tissue with reduced immune surveillance has a number of consequences. One is **acute postinfectious encephalitis**, which occurs in about 1 in 3,000 infections. The other is a rare, but delayed and often lethal, brain infection called **subacute sclerosing panencephalitis (SSPE)**. This disease is a manifestation of a slow infection, an unusual variation of a persistent infection (see "Slow

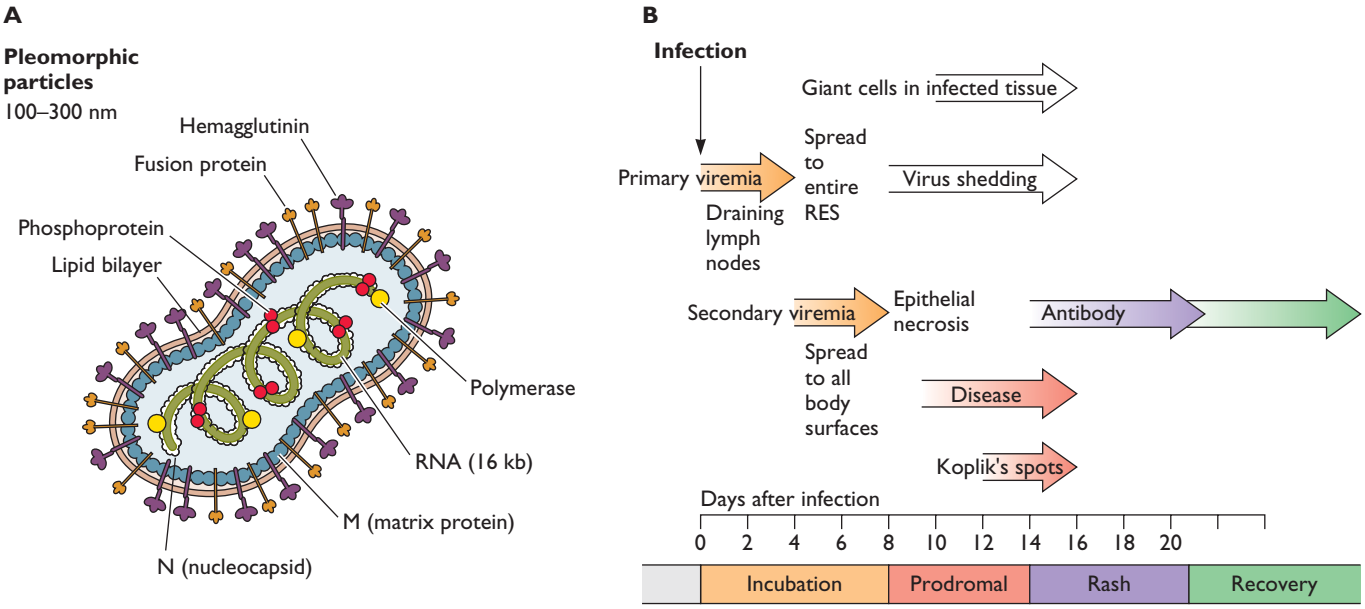


Figure 5.7 Infection by measles virus. (A) Diagrammatic representation of the structure of the pleomorphic measles virion. **(B)** Course of clinical measles infection and events occurring in the spread of the infection within the body. Four clinically defined temporal stages occur as infection proceeds (illustrated at the bottom). Characteristic symptoms appear as infection spreads by primary and secondary viremia from the lymph node to the entire reticuloendothelial system (RES) and finally to all body surfaces. The timing of typical reactions that correspond to the clinical stages is shown by the colored arrows. The telltale spots on the inside of the cheek (Koplik's spots) and the skin lesions of measles consist of pinhead-sized papules upon a reddened, raised area. They are typical of immunopathology produced in response to measles virus proteins. Redrawn from A. J. Zuckerman et al., *Principles and Practice of Clinical Virology*, 3rd ed. (John Wiley & Sons, Inc., New York, NY, 1994), with permission.

Infections: Sigurdsson's Legacy" below and Fig. 5.1). After young adults and children contract measles, about one in a million develop SSPE, with a 6- to 8-year incubation period. SSPE is more likely to occur if children are infected in their first year or two of life than if they are infected later. This disease begins in rare infected cells of the brain. In these cells, viral gene expression, especially synthesis of envelope proteins, is reduced. In addition, fully assembled particles cannot be detected in brains of afflicted patients. Alterations in the matrix (M) protein may lead to ineffective virion assembly. Even though particle assembly is not observed, nucleoprotein complexes are produced, and infectious genomes spread between synaptically connected neurons. The mechanism of such spread in the absence of assembled virions is of some interest because it does require the viral fusion protein, but not viral receptors.

We lack testable hypotheses that relate these provocative findings to the mechanism of persistent infection. More critical perhaps is that it is difficult, if not impossible, to test these ideas in human infections. Nevertheless, with appropriate animal models, we may be able to answer some fundamental questions. Does transient immunosuppression during an acute infection facilitate infection of the brain? Are defects in M protein synthesis and particle assembly necessary and sufficient to cause disease, or are they effects of other selection processes in the brain? Are the defects in viral gene expression the cumulative results of selection after years of exposure to host defenses? Transgenic mice that produce the human measles virus receptors are available, and should allow these important questions to be addressed in a rigorous and controlled fashion.

Lymphocytic Choriomeningitis Virus

Lymphocytic choriomeningitis virus, a member of the family *Arenaviridae,* was the first virus associated with aseptic meningitis in humans. Perhaps more importantly, in recent years its study has illuminated fundamental principles of immunology and viral pathogenesis, particularly those that underlie persistent infection and CTL recognition and killing. It was noted early on that the infection spreads from rodents (the natural host) to humans, in whom it can cause severe neurological and developmental damage (an example of a zoonotic infection [Chapter 10]). Infected rodents normally excrete large quantities of virions in feces

and urine throughout their lives without any apparent detrimental effect. These mice are called "carriers" because of such lifelong production of virions. The carrier state is established for two reasons: infection is not cytopathic, and, if mice are infected congenitally or immediately after birth, viral proteins are not recognized as "foreign" and so the infection is not cleared by the immune system. However, if virions are injected into the brains of healthy adult mice, the mice die of acute immunopathological encephalitis. This disease is similar to that contracted by humans.

In the mouse model, CTLs are required both for clearing virus and for the lethal response to intracerebral infections. If adult mice are depleted of CTLs, injection of virus particles into the brain is no longer fatal. Instead, the mice produce virions throughout their lifetimes, precisely as seen in persistent infections of neonates. When virus-responsive CTLs are added back to persistently infected neonates, the infection is cleared after several weeks. In the case of neonatal persistent infection, the brain is not infected, so no encephalitis is promoted by the activated T cells. How the effector system is prevented from clearing the infection in these circumstances is currently under investigation. Recent experiments have implicated an active process of clonal deletion of T lymphocytes that are capable of recognizing the dominant lymphocytic choriomeningitis virus peptides.

Latent Infections

General Properties

Latent infections are characterized by three general properties: viral gene products that promote productive replication are not made, or are found only in low concentrations; cells harboring the latent genome are poorly recognized by the immune system; and the viral genome persists intact so that at some later time a productive infection can be initiated to ensure the spread of viral progeny to new hosts (Fig. 5.1). The latent genome can be maintained as a nonreplicating chromosome in a nondividing cell like a neuron (e.g., herpes simplex virus, varicella-zoster virus), or as an autonomous, self-replicating chromosome in a dividing cell (e.g., Epstein-Barr virus or cytomegalovirus), or be integrated into a host chromosome, where it is replicated in concert with the host genome (e.g., adeno-associated virus).

Such "long-term parking" of a viral genome in the latent infection is noteworthy for its stability: a balance among the regulators of viral and cellular gene expression must be maintained. There is no one mechanism to establish and maintain a latent infection, but one principle emerging is that epigenetic alterations of viral genomes play central roles. Generally, only a restricted set of viral gene products are found in latently infected cells. For neurons harboring latent herpes simplex virus (an alphaherpesvirus), a unique RNA transcript, but no viral proteins are synthesized. In other cases, latently infected cells synthesize a small subset of viral proteins required for productive replication, a pattern exemplified by varicella-zoster virus, an alphaherpesvirus of humans (Fig. 5.4). Epstein-Barr virus (a gammaherpesvirus) latent infection of B cells is more complicated. At least nine viral proteins and small viral RNAs are required to support replication of the latent viral genome and modulate the host immune response (Table 5.4). For betaherpesviruses such as cytomegalovirus, viral micro-RNAs may function to establish a latent infection.

If latency is to have any value as a survival strategy, a mechanism for reactivation must exist so that infectious virions can spread to other hosts. Reactivation may be spontaneous or may follow trauma, stress, or other insults, conditions that may mark the host as unsuitable for continued latent infection.

Herpes Simplex Virus

Over three-quarters of all adults in the United States have antibodies to herpes simplex virus type 1 or 2 and harbor latent viral genomes in their peripheral nervous systems. Approximately 40 million infected individuals will experience recurrent herpes disease due to reactivation of their own personal viruses sometime in their lifetimes. Many millions more carry latent viral genomes in their nervous systems, but never report reactivated infections. Herpes simplex virus is an example of a well-adapted pathogen, as demonstrated by its widespread prevalence in humans, its only known natural hosts. However, why some people are more likely than others to be infected by this ubiquitous virus is poorly understood (Box 5.5). No animal reservoirs are known, although several laboratory animals, including rats, mice, guinea pigs, and rabbits, can be infected. The alphaherpesviruses, of which herpes simplex virus type 1 is the type species, are unique in establishing latent infections predominantly in terminally differentiated, nondividing neurons of the peripheral nervous system. Indeed, most other neurotropic viruses (e.g., rabies virus) initiate infections of peripheral tissue and spread to the central nervous system to cause devastating disease or death.

The Primary Infection

Herpes simplex virus infections usually begin in epithelial cells at mucosal surfaces (Fig. 5.8). Virions are released from the basal surface in close proximity to sensory nerve endings. Because sensory terminals are abundant, they are easily infected, but other autonomic nerve terminals also may be infected if deeper layers of the epithelium are involved. For example, endothelial cells of capillaries or cells surrounding hair follicles are in contact with sympathetic

Table 5.4 Epstein-Barr virus gene products synthesized in the latent infection

Gene product	Function
EBNA-1	Maintains replication of the latent Epstein-Barr virus genome during S phase of the cell cycle. It is a sequence-specific DNA-binding protein and binds to a unique origin of replication called *oriP* that is distinct from the origin used in the productive replication cycle.
EBNA-2	A transcriptional regulator that coordinates Epstein-Barr virus and cell gene expression in the latent infection by activating the promoters for the LMP-1 gene and cellular genes like CD23 (low-affinity immunoglobulin E Fc receptor) and CD21 (the Epstein-Barr virus receptor, CD23 ligand, and receptor for complement protein C3d)
EBNA-LP	Required for cyclin D2 induction in primary B cells in cooperation with EBNA-2
EBNA-3A and EBNA-3C	Play important roles early in the establishment of the latent infection
LMP-1	An integral membrane protein required to protect the latent infected B cell from the immune response. LMP-1 stimulates the synthesis of several surface adhesion molecules in B cells, a calcium-dependent protein kinase, and the apoptosis inhibitor Bcl2.
LMP-2	An integral membrane protein required to block the activation of the *src* family signal transduction cascade; an inhibitor of reactivation from latency. Two spliced forms exist: LMP-2A and LMP-2B; LMP-2B lacks receptor-binding domain and may act to modulate LMP-2A.
EBER-1 and EBER-2	Nonpolyadenylated, small RNA molecules that do not encode proteins; transcribed by RNA polymerase III; 166 and 172 nucleotides in length, respectively
miRNAs	At least 20 different miRNAs are processed from two viral transcripts, one set in the BART gene and one set near the BHRF1 cluster

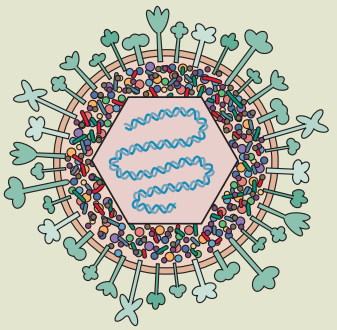
BOX 5.5 DISCUSSION

The hygiene hypothesis: why people vary in susceptibility to herpes simplex virus infection

More than 80% of the adult population in the developed world harbor latent herpesviral genomes in their peripheral nervous system. Some individuals suffer from lesions after reactivation while some never report symptoms or lesions. What accounts for the high infectivity yet marked diversity in host response to infection?

Hypothesis: The effectiveness of innate immunity to stimulate appropriate adaptive immune responses (Th1 versus Th2) is conditioned by the individual's exposure to microbes early in life. In the extreme case, early life in a highly sanitized environment leads to reduced stimulation of innate immunity. One consequence is inadequate development of the adaptive immune system and reduced capacity to control infections later in life.

According to this hypothesis, the rising incidence of allergy and asthma, as well as of herpes simplex virus infections, in Western societies results from



"hypersanitized" living conditions. Such conditions include use of sterilized baby food, excessive application of germicidal soaps and cleaners, and limited exposure of newborns to relatives and friends of the family. Individuals who had limited exposure to microbes in early life will experience more reactivations of latent herpesvirus with severe symptoms because of their inability to mount an effective Th1-dominated response. Instead, with inadequate early stimulation of innate immunity by microbial infections, subsequent exposure to foreign antigens may stimulate an inappropriate Th2 response. Testing the hygiene hypothesis is not an easy matter; many observations that apparently support or refute the hypothesis are anecdotal or poorly controlled. Nevertheless, the idea has stimulated considerable research and debate.

Camateros, P., J. Moisan, J. Henault, S. De, E. Skamene, and D. Radzioch. 2006. Toll-like receptors, cytokines and the immunotherapeutics of asthma. *Curr. Pharm. Des.* **12:**2365–2374.

Rouse, B. T., and M. Gierynska. 2001. Immunity to herpes simplex virus: a hypothesis. *Herpes* **8**(Suppl. 1)**:**2A–5A.

Strachan, D. 1989. Hay fever, hygiene, and household size. *Br. Med J.* **299:**1259–1260.

Zock J., E. Plana, D. Jarvis, et al. 2007. The use of household cleaning sprays and adult asthma: an international longitudinal study. *Am. J. Respir. Crit. Care Med.* **176:**735–741.

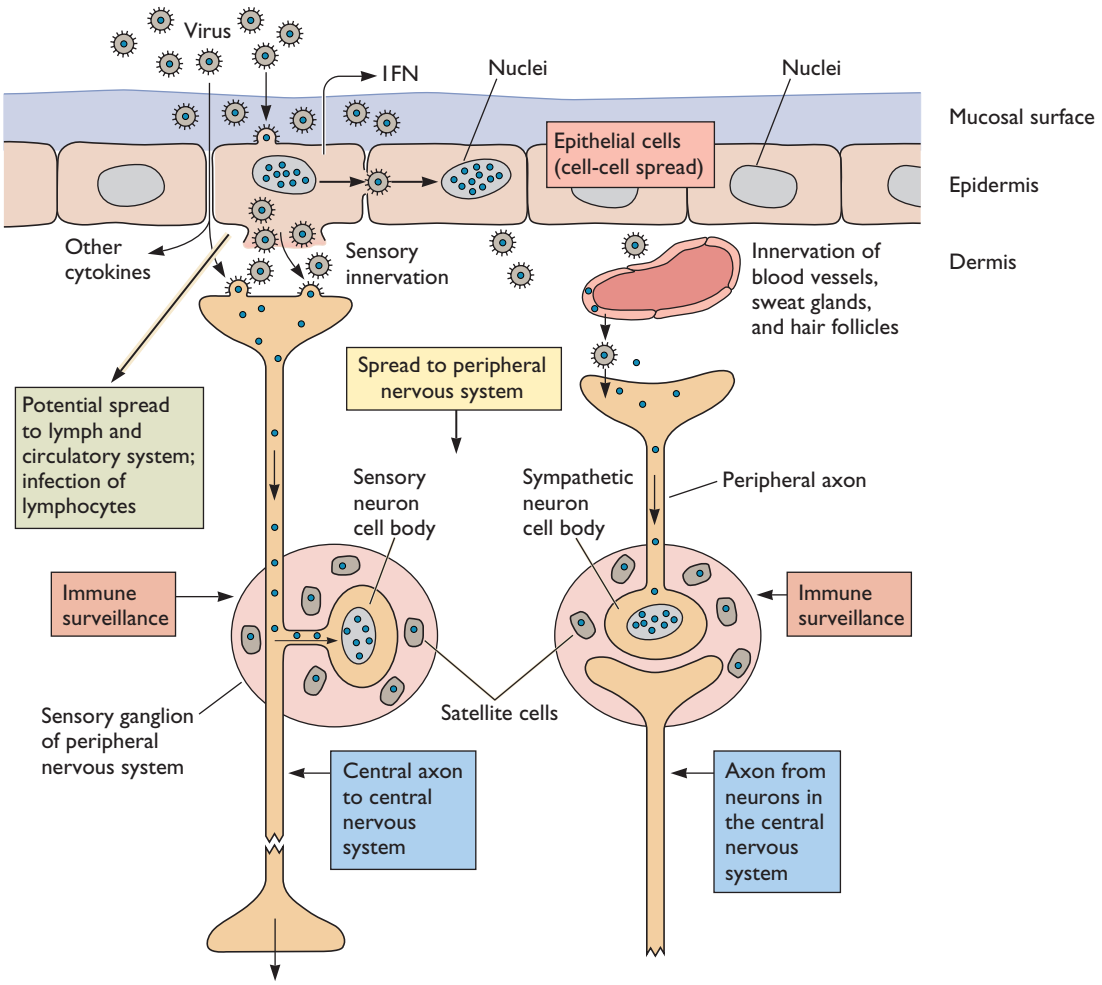


Figure 5.8 Herpes simplex virus primary infection of sensory and sympathetic ganglia. Viral replication occurs at the site of infection, usually in the mucosal epithelium; the infection may or may not manifest as a lesion. Host intrinsic and innate defenses, including IFN and other cytokines, normally limit the spread of infection at this stage. Virions may infect local immune effector cells, including dendritic cells and infiltrating natural killer cells. The infection also spreads locally between epithelial cells and may spread to deeper layers to engage fibroblasts, capillary endothelial cells, sweat glands, and other dermal cells such as those present in piloerector muscles around hair follicles. Particles that are released from basal surfaces infect nerve terminals in close contact. These axon terminals can derive from sensory neurons in dorsal root ganglia (left) or from autonomic neurons in sympathetic ganglia (right). Viron envelopes fuse with neuron axonal membranes, and the nucleocapsid with outer tegument proteins is transported within the axon to the neuronal cell body by microtubule-based systems (dynein motors), where it delivers the viral DNA to the nucleus. Spread of productive infection to the central nervous system from these peripheral nervous system ganglia is rare. Unlike the brain and spinal cord, peripheral nervous system ganglia are in close contact with the bloodstream and are exposed to lymphocytes and humoral effectors of the immune system (immune surveilance). Consequently, infected ganglia become inflamed and populated with lymphocytes and macrophages. Infection of the ganglion is usually resolved within 7 to 14 days after primary infection, virus particles are cleared, and a latent infection of some neurons in the ganglion is established (see also Fig. 5.9).

nerve endings and also may be exposed to virus. If infection occurs in the eye or other facial tissues, parasympathetic and cranial nerve endings may be invaded. Fusion of the virion envelope with any of these nerve endings releases the nucleocapsid with inner tegument proteins into the axoplasm. Dynein motors then move the internalized nucleocapsid on microtubules over long distances to the particular neuronal cell bodies that innervate the infected peripheral tissue. A productive infection may be initiated in these neurons when the viral DNA enters the nucleus.

Establishment and Maintenance of the Latent Infection

Soon after this acute infection begins in neurons, the viral genome is silenced and coated with nucleosomes (Volume I, Chapter 8). The nucleosome-covered viral genome is tethered in some fashion in the nucleus to cellular chromatin. Only limited transcription occurs, and a quiescent, latent infection is established. As we will see, the establishment of this latent state is likely to depend on both viral regulatory proteins and RNA, as well as the intrinsic and innate immune defenses that protect these tissues.

In general, most neurons neither replicate their DNA nor divide, and so once a silenced viral genome is established in the nucleus, no further viral replication is required for it to persist. Standard antiviral drugs and vaccines are not able to cure a latent infection. Consequently, latency is absolute persistence, or, as one herpesvirologist put it, "herpes is forever."

Despite many years of study, many details of the molecular aspects of herpes simplex virus latency await discovery and explanation, but the general pathway is well established. The outline of possible regulatory steps necessary for the establishment, maintenance, and reactivation of a viral infection is shown in Fig. 5.9. A typical primary infection of a mouse, showing the time course of production of infectious virus and establishment of a latent infection, is illustrated in Fig. 5.10.

An often unappreciated fact is that in several animal models, peripheral ganglia undergo a rather robust acute infection with substantial production of virions followed by a strong inflammatory response. Nevertheless, after 1 or 2 weeks, infectious particles can no longer be isolated from the ganglia, the operational definition of an established latent infection. If the animal survives the primary infection, establishment of the latent infection is inevitable. The time frame for this process varies depending on the animal species, the concentration and genotype of the infecting virus, and the site of primary infection. Inflammatory cells may persist in the latently infected ganglia for months or years, perhaps as a result of continuous or frequent low-level reactivation and production of viral proteins in latently infected tissue.

Many questions remain. We do not understand why neurons are the favored site for a latent infection. Under particular laboratory conditions, it is possible to establish a quiescent infection in nonneuronal cells, but these conditions apparently are not available in natural infections. It is difficult to understand how neurons in ganglia survive the primary infection by this markedly cytolytic virus. Evidence suggests that the productive-cycle gene expression pathway is turned off **after** it has started and cells are subsequently purged of infection by local innate defenses. In addition, we do not understand why the infection stops in the first-order neurons of the peripheral nervous system and rarely spreads to the central nervous system, which is in direct synaptic contact with peripheral neurons.

The Latency-Associated Transcripts

Many latently infected neurons synthesize RNA molecules termed **latency-associated transcripts** (LATs) (discussed in Volume I, Chapter 8). Some researchers argue that all latently infected neurons synthesize LATs, while others report that only 5 to 30% do so. As in many studies of the herpes simplex latent state, the results depend on the animal model.

After infection of rabbits, herpes simplex virus type 1 mutants that do not synthesize LATs establish a latent infection, but spontaneous reactivation is markedly reduced. Despite this important finding, identifying functions for the LATs continues to be a challenge. The major LAT contains two prominent open reading frames with potential to encode two proteins, but there is little evidence that these proteins are produced during latency. Moreover, disruption of these open reading frames induces no latency phenotypes, and the sequences are not conserved in the closely related herpes simplex type 2 genome.

Remarkably, and in contrast, human ganglia latently infected with varicella-zoster virus, a distantly related alphaherpesvirus, do not synthesize a single LAT. Rather, at least five distinct viral transcripts are found. Despite considerable effort, scientists cannot ascribe functions of these viral transcripts or the corresponding proteins in either the establishment or the maintenance of the varicella-zoster latent infection. Suffice it to say that while alphaherpesvirus latency in neurons is a common feature of these viruses, it is probably not achieved by a single mechanism.

If the herpes simplex virus LATs are not translated, then the RNA molecules themselves may have biological activity. One idea is that they are micro-RNA precursors leading to degradation or reduced translation of host messenger RNAs (mRNAs). Micro-RNAs may be one common feature of herpesvirus latency systems, as they now are suspected to be important for latent infections by the betaherpesviruses and gammaherpesviruses. Another idea is that the herpes simplex virus type 1 LATs block apoptosis upon primary infection of neurons (or upon reactivation). Evidence exists indicating that they maintain the latent state through antisense inhibition of translation of ICP0 (a crucial transcription activator). A hypothesis with some support is that herpes simplex virus type 1 LATs mediate the transition to latency by altering chromatin structure, perhaps by a process similar to mammalian X-chromosome inactivation by the Xist RNA.

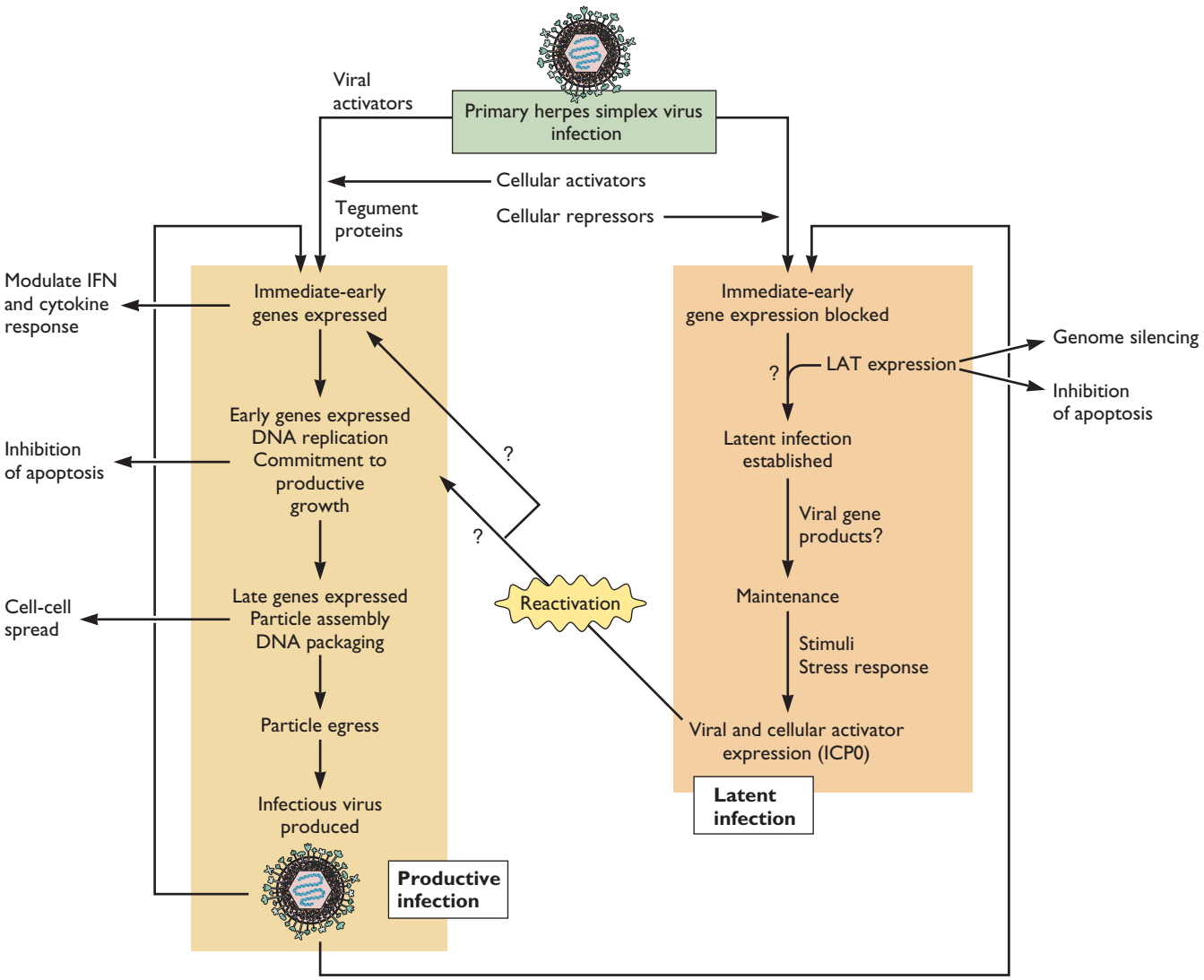


Figure 5.9 General flowchart for establishment, maintenance, and reactivation of a latent infection by herpes simplex virus. The green box at the top indicates the primary infection by virus particles at mucosal surfaces. The productive infection is shown by the pathway on the left, and the latent infection is indicated by the pathway on the right. The question marks indicate our lack of knowledge concerning synthesis and function of viral proteins at the indicated steps. Infectious particles produced by the productive pathway may infect other cells and enter either the productive or latent pathway as indicated. Infection can also spread from cell to cell without release of particles. Apoptosis induced by infection is inhibited by viral gene products. In addition, antiviral effects of IFN and other cytokines are modulated by viral gene products. The contribution of these processes in establishing the latent infection is not well understood. Reactivation is indicated by the diagonal arrow from the latent state to the start of the productive infection. The question marks note the current controversy as to whether reactivation requires "return to go" (immediate-early gene expression) or "start in the middle" (expression of early genes required for DNA replication). Experimental data indicate that synthesis of the immediate-early protein ICP0 is sufficient to activate latent infection. Adapted from M. A. Garcia-Blanco and B. R. Cullen, *Science* **254:**815–820, 1991, with permission.

Larger Numbers of Nonneuronal Cells than Neurons in Peripheral Ganglia

While it is commonplace to focus on neurons in this pattern of infection by herpes simplex virus, only 10% of the cells in a typical sensory ganglion are neurons; the remaining 90% are nonneuronal satellite cells and Schwann cells associated with a fibrocollagenous matrix. These nonneuronal cells are in intimate contact with ganglionic neurons. Some of the nonneuronal cells are infected during initial invasion of the ganglion, and may

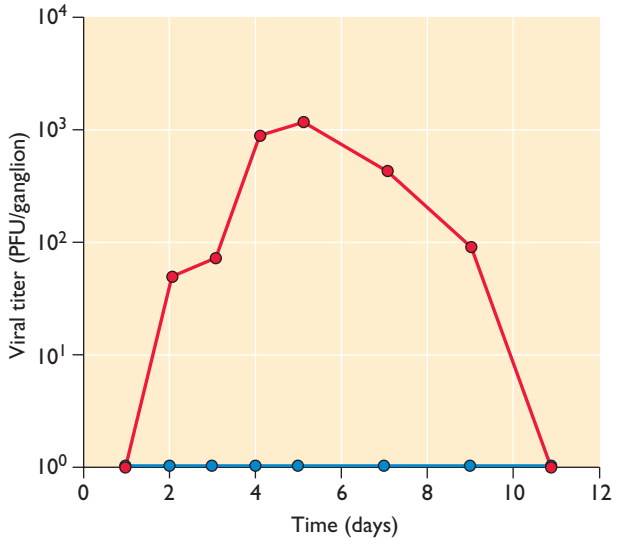


Figure 5.10 Replication of infectious herpes simplex virus type 1 in mouse trigeminal ganglia during acute infection. Mice were anesthetized and infected by a standard strain of herpes simplex virus by dropping approximately 10^5 plaque-forming units (PFU) onto the cornea of one eye that had been lightly scratched with a sterile needle. After a few minutes, the liquid was blotted and the animal was allowed to recover. At selected time points, animals were euthanized and the trigeminal ganglia were removed quickly and frozen. Each point on the graph (red line) represents the geometric mean titer in PFU from eight individual ganglia from two different experiments tested at the indicated time after infection. Uninfected animal controls are indicated by the blue line.

be the major source of infectious particles isolated from infected ganglia. These infected support cells also produce prodigious amounts of cytokines that can promote an antiviral response in the entire ganglia. In addition, in contrast to the brain and spinal cord, the peripheral nervous system is accessible to antibodies, complement, cytokines, and lymphocytes of the innate and adaptive immune system.

The intimate contact of peripheral neurons with epithelial cells (sites of primary infection) enables movement of virus particles in and out of the nervous system without exposure to circulating antibodies. Murine models demonstrate efficient establishment of latency in neurons even in the face of a robust antibody response in vaccinated animals or in animals that receive passive immunization with virus-specific antibodies prior to infection. This rather curious twist of immune avoidance presents extreme difficulties to those who strive to produce alphaherpesvirus vaccines for humans.

Reactivation

After reactivation of a latent infection in sensory ganglia, virions appear in the mucosal tissues innervated by that particular ganglion. This outcome is an effective means of ensuring transmission of virions after reactivation, because mucosal contact is widespread among affectionate humans. However, two apparently contradictory facts should be obvious. First, reactivation takes place **in an immune individual**, and second, an individual must be actively producing sufficient virions to infect another person. Both facts are true, and the contradiction is explained by the simple fact that the immune response reacts more slowly than shedding of infectious virions. The spread of infection among epithelial cells after reactivation may be facilitated by action of the viral protein ICP47. This protein blocks MHC class I presentation of viral antigens to T cells. Such activity may provide sufficient time for a few rounds of replication before elimination of the infected cell by activated CTLs.

The immune response after reactivation is usually robust and clears the infected epithelial cells in a few days, but not before virions are shed. The typical "cold sore" lesion of herpes labialis is the result of the inflammatory immune response attacking the infected epithelial cells that were in contact with axon terminals of reactivating neurons. Some individuals with latent herpes simplex virus experience reactivation every 2 to 3 weeks, while others report only rare or no episodes of reactivation. Importantly, reactivation may result in shedding infectious virions in the absence of obvious lesions or symptoms (Fig. 5.1).

Reactivation: Not "All or None"

The signaling mechanisms that reactivate the latent infection are under active study. Sunburn, stress, nerve damage, depletion of nerve growth factor, steroids, heavy metals, and trauma (including dental surgery) all promote reactivation. Despite the apparent systemic nature of most reactivation stimuli, when reactivation does occur in animal models, only about 0.1% of neurons in a ganglion containing the viral genome synthesize viral proteins and virions. Multiple levels of regulation must be operating, and the overwhelming thrust must be to maintain the latent state. The regulatory network employed is not an "on or off" circuit affecting all latently infected neurons. Its nonlinear response may be the result of some nonuniformity within the latent population, or of the signal transduction process. Not only are different types of neurons infected in peripheral ganglia, but also the number of viral genomes in a given neuron varies dramatically (Box 5.6). It is likely that one facet of competency for reactivation is the number of viral genomes within a given neuron: more genomes, more likely to reactivate.

Signaling Pathways in Reactivation

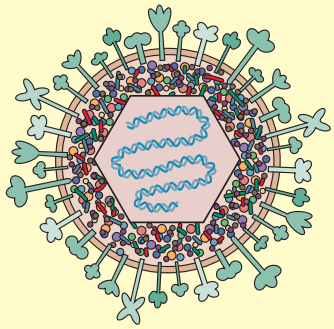
At first glance, the diversity of potential reactivation signals may be surprising. However, it is likely that they all converge to stimulate production or action of specific

BOX 5.6 EXPERIMENTS

Neurons harboring latent herpes simplex virus often contain hundreds of viral genomes

The number of neurons in a ganglion that will ultimately harbor latent genomes following primary infection depends upon the host, the strain of virus, the concentration of infecting virions, and the conditions at the time of infection. A mouse trigeminal ganglion contains about 20,000 neurons. It is possible to infect as few as 1% to as many as 50% of the neurons in a ganglion. In controlled experiments with mice, the number of latently infected neurons increases as the titer of infecting virions increases.

Many infected neurons contain multiple copies of the latent viral genome, varying from fewer than 10 to more than 1,000; a small number have more than 10,000



copies. This variation in copy number has been enigmatic. Does it reflect multiple infections of a single neuron, or is it the result of replication in a stimulated permissive neuron after infection by one particle? If it is the latter, how does the neuron recover from what should be an irreversible commitment to the productive cycle?

When viral replication is blocked by mutation or antiviral drugs, the number of latently infected neurons with multiple genomes is reduced significantly. Therefore, in a natural infection, a single neuron may be infected by multiple virus particles, each of which participates in the latent infection.

Sawtell, N. M. 1997. Comprehensive quantification of herpes simplex virus latency at the single-cell level. *J. Virol*. **71:**5423–5431.

cellular proteins needed for transcription of the herpes simplex virus immediate-early genes and consequently activate the productive transcriptional program. Indeed, all of these exogenous signals have the capacity to induce the synthesis of cell cycle and transcription regulatory proteins that may render neurons permissive for viral replication. It is known that synthesis of the viral immediate-early protein ICP0 is sufficient to reactivate a latent infection in model systems (Fig. 5.9). The ICP0 protein and LATs appear to have opposing functions in modulating chromatin structure, leading to active transcription or gene silencing, respectively. In a single latently infected neuron, reactivation may be an all-or-none process requiring but a single reaction such as chromatin structural changes to "flip the switch" that triggers the cascade of gene expression of the productive pathway. Glucocorticoids are excellent examples of such activators, as they stimulate transcription rapidly and efficiently while inducing an immunosuppressive response. These properties explain the observation that clinical administration of glucocorticoids frequently results in reactivation of latent herpesvirus.

Latent infections may reactivate in the absence of obvious stress or activation signals. Such spontaneous reactivation appears to result from random, low-concentration signals that impinge on the neurons from the circulation (cytokines) or from the innervated tissue (local trauma and consequent nerve firing). These stress signals are sensed by individual neurons and lead to a small burst of viral transcription in only one or a few neurons. When the stimulus is strong enough, such sporadic transcription ultimately passes a threshold, resulting in replication and reactivation. This idea is consistent with the very low levels of immediate-early and early transcripts that can be detected in latently infected ganglia. In turn, the immune system would be stimulated constantly by these low-level, nonproductive events. One thought is that the continuously activated immune system would be able to provide rapid control of massive reactivation, should it occur.

One question remains unanswered: what prevents an apparently systemic reactivation signal from turning on **all** latently infected neurons? Why are so few neurons activated at any given time even when glucocorticoids or trauma must affect all ganglia? One idea is that massive reactivation of latency would be met instantly with a lethal immune response. Killing all your sensory or sympathetic neurons is unlikely to be a selective force in evolution! The selective advantage may be that by ensuring reactivation in only a small fraction of latently infected neurons, the functional life of these important peripheral nervous system tissues is preserved so that the host can survive. This hypothesis begs the fundamental question of how reactivation of all latently infected neurons in an individual is avoided.

Epstein-Barr Virus

Epstein-Barr virus is named after Michael Epstein and Yvonne Barr, who, along with Bert Achong, discovered it in 1964. Epstein-Barr virus, also called human herpesvirus type 4, is the type species of the gamma subfamily of herpesviruses. It is one of the most common viral infections

of humans (its only host). Indeed, in the United States, up to 95% of adults between the ages of 35 and 40 are seropositive and carry the viral genome in latently infected B cells. Two strains of Epstein-Barr virus are recognized that differ in their terminal internal repeats, as well as in production of nuclear antigens and small RNAs during the latent infection. Epstein-Barr virus 1 is about 10 times more prevalent in the United States and Europe than is Epstein-Barr virus 2, while both strains are equally represented in Africa. Most people are infected with the virus

Figure 5.11 Epstein-Barr virus primary and persistent infection. (Left) Primary infection. Epstein-Barr virus infects epithelial cells in the oropharynx (e.g., the tonsils). Virions produced can infect resting B cells in the lymphoid tissue. Virus-infected B cells express the full complement of latent viral proteins and RNAs and are stimulated to enter mitosis and replicate. They produce antibody and function as B cell blasts. The latently infected B cells are attacked by natural killer cells and CTLs. **(Right)** Persistent infection. Most infected B cells are killed as a result of innate and immune defenses, but a few (approximately 1 in 100,000) persist in the blood as small, nonproliferating memory B cells that synthesize only LMP-2A mRNA. These memory B cells are presumably the long-term reservoir of Epstein-Barr virus *in vivo* and the source of infectious virus when peripheral blood cells are removed and cultured. A limited immune response to these infected B cells leads to self-limiting proliferation, infectious mononucleosis, or unlimited proliferation (polyclonal B-cell lymphoma). When stimulated or propagated in culture, viral proteins needed to replicate and maintain the viral genome are again produced. Some latently infected B cells traffic to lymphoid tissues in close proximity to epithelial cells in the oropharynx. Here the B cells are stimulated to produce virions capable of infecting and replicating in epithelial cells. Infectious virions are produced and shed into the saliva for transmission to another host.

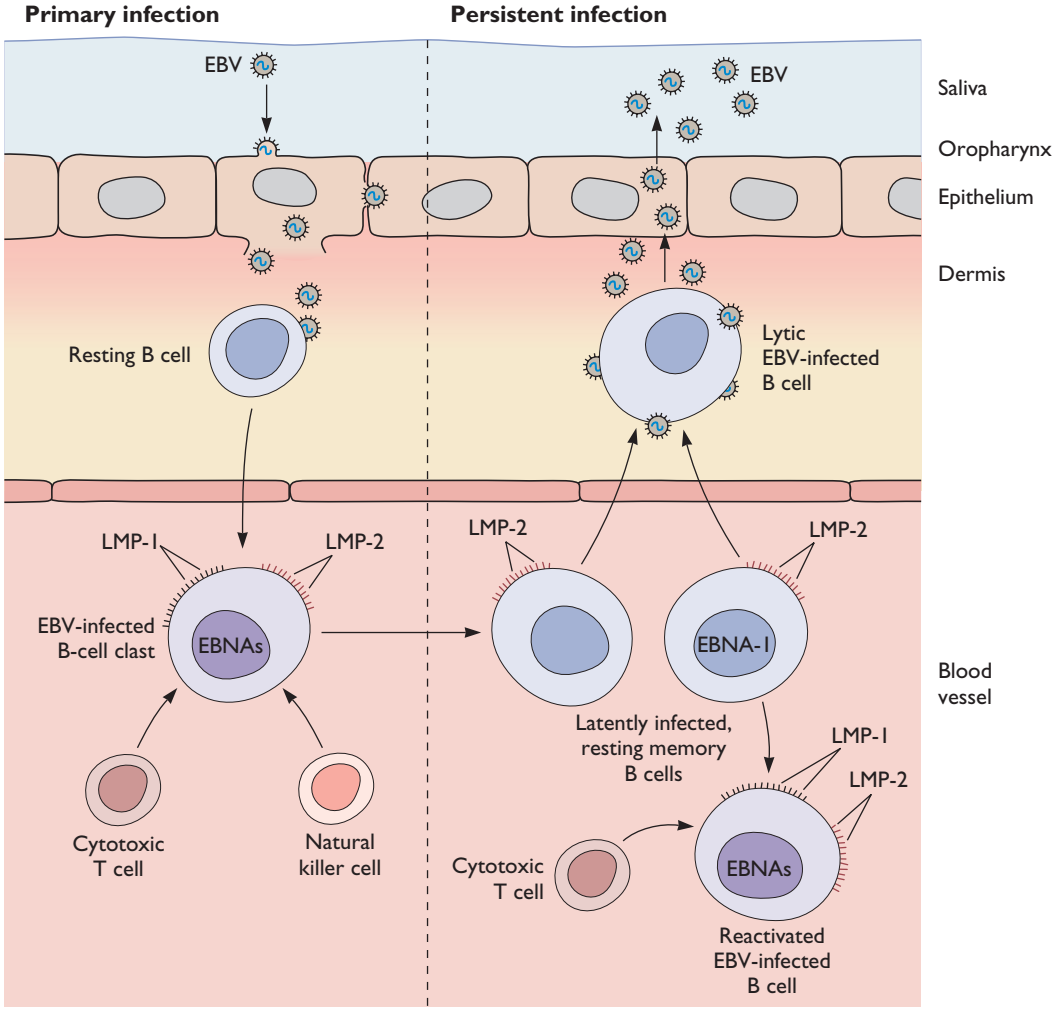


Table 5.5 Diseases associated with Epstein-Barr virus infections[a]

Disease	Characteristics
Acute infection	
Infectious mononucleosis	The best-known clinical presentation of infection; resolves in 1–2 wk,but fatigue symptoms may last longer
Oral hairy leukoplakia	Primary infection leading to a wartlike lesion of epithelial cells of the tongue seen in AIDS patients and transplant recipients
Abnormalities of latent infection, lymphoproliferative disorders, and malignancies	
B-lymphoproliferative disease	Frequently observed in individuals experiencing a primary viral infection following tissue transplantation; initially benign; if untreated, can lead to B-cell lymphoma
X-linked lymphoproliferative syndrome	Certain males have X-linked mutations that lead to a severe immunodeficiency after primary viral infection
Burkitt's lymphoma	The most common childhood cancer in equatorial Africa; cells from Burkitt's B-cell lymphomas exhibit a reciprocal translocation involving the *c-myc* locus on the long arm of chromosome 8 and one of the immunoglobulin loci on chromosome 2, 14, or 22; newly explanted B cells from tumors produce only EBNA-1
Hodgkin's disease	Mixed cells in tumor; 1–2% are malignant, and the remaining cells are infiltrating lymphocytes; association of virus with Hodgkin's lymphomas varies with geography; newly explanted B cells from tumors produce EBNA-1, LMP-1, and LMP-2A
Nasopharyngeal carcinoma	A cancer of epithelial cells and one of the most common cancers in China; tumor cells produce EBNA-1, LMP-1, and LMP-2, but not EBNA-2

[a]Data from G. C. Faulkner et al., *Trends Microbiol*. **8:**185–189, 2000.

at an early age and have no symptoms, but some develop **infectious mononucleosis** ("mono").

Epstein-Barr virus establishes latent infections in B lymphocytes (Fig. 5.11). It is one of the human herpesviruses consistently associated with human cancers (Table 5.5; Appendix A). As we will learn in Chapter 7, such viral oncogenesis is a by-product of the mechanisms by which a latent infection is established. In contrast to the nonpathogenic latent state of herpes simplex virus, the latent state of Epstein-Barr virus is implicated in several important diseases.

The Primary Infection

Epstein-Barr virions have the capacity to shuttle between epithelial cells and B cells. One line of research suggests that a different viral ligand complex on virions engages different entry receptors on either cell type. Other data are more consistent with a common receptor. In any case, infection initiates in epithelial cells, usually those of the mucosal epithelia in the oropharygeal cavity. The replication in epithelia is reminiscent of papillomavirus replication in that the infection is not completed in basal cells, but finishes in the more superficial differentiated spinous and granular layers. These tissues are the sites for shedding of infectious virions. Lingual epithelium and tonsil tissue are rich in lymphoid cells and provide the perfect milieu for the next stage of infection. After productive infection of epithelial cells, released virions infect B lymphocytes in closely associated lymphoid tissue via a pathway different from that used to infect epithelial cells. These infected B cells do not produce infectious virions, because an entirely different transcriptional program ensues that leads to establishment of a unique latent infection. The viral genome exists as a circular, self-replicating episome in the B-cell nucleus (Volume I, Chapter 9). The viral episome becomes associated with nucleosomes and undergoes progressive methylation at CpG residues. When latently infected B cells come in close contact with epithelial cells, the latent infection may be reactivated, resulting in production of more infectious virions capable of infecting epithelial cells. Infectious particles are shed predominantly in the saliva, but shedding from lung and cervical epithelia has also been reported.

The Persistent Infection Is a Dynamic State

A dynamic state of latent and productive infection exists in infected individuals. Despite the presence of latently

infected B cells, infectious virions are still produced, virus-specific CTLs circulate in the blood, and antibodies specific for viral proteins are produced in relatively large quantities. How latency is maintained in the face of an active immune response is an important question.

Children and teenagers are commonly afflicted, usually after oral contact (hence the name "kissing disease"). The acute infection requires expression of most viral genes and rapidly stimulates a strong immune response. Spread of infection to B cells in an individual with a normal immune system induces the infected B cells to divide, leading to substantial immune and cytokine responses. The resulting disease is called infectious mononucleosis. The ensuing immune response destroys most infected cells, but approximately 1 in 100,000 infected B cells survive. They persist as small, nonproliferating memory B cells that make only latent membrane protein 2A (LMP-2A) mRNA. They home to lymphoid organs and bone marrow, where they are maintained. These cells do not produce the B7 coactivator receptor, and therefore are not killed by CTLs (see Chapter 4). They proliferate indefinitely when stimulated or when propagated as cultured cells. They are the progenitors of the B-cell lines that grow out of peripheral blood of an infected patient.

When peripheral blood of an infected individual is cultured, growth factors in the media stimulate replication of the rare latently infected B cells, while the uninfected B cells die. It is important to understand that these cultured immortal lymphoblasts are most assuredly **not** the same as latently infected cells that circulate *in vivo*. Nevertheless, this class of virus-infected B cells often yields immortalized progeny capable of being propagated indefinitely in the laboratory. Consequently, these laboratory-produced cells are the best-understood models of Epstein-Barr virus latent infection. These cells synthesize a set of at least 10 gene products, including six nuclear proteins (termed EBNAs), three viral membrane proteins (LMPs) that are important in altering the properties of the cells, small RNA molecules called EBER-1 and EBER-2, and at least 20 micro-RNAs (Table 5.4). While considerable effort has focused on understanding the mechanism of transformation of B cells in culture, the viral genes so identified often are not expressed in human cancers associated with viral infection.

Three Programs of Viral Gene Expression Produce Different Phenotypes of Latent Infection

At least three distinct phenotypes or programs can be distinguished by the viral gene products made in an infected B cell. These are called latency 1 (EBNA-1, Bam A RNAs, EBER RNAs 1 and 2), latency 2 (EBNA-1, LMP-1, LMP-2, Bam A RNAs, EBER RNAs, micro-RNAs), and latency 3 (all the latent-cycle proteins and the RNAs). Viral infection stimulates B cells to divide rapidly and continuously. These B-cell blasts express all of the latency-associated genes (latency 3) (Fig. 5.11; Table 5.4). The viral proteins are required to establish the latent infection and to promote growth of the infected cell. This phenotype is also characteristic of B-cell lymphomas of immunodeficient patients. The latency 3 phenotype is similarly characteristic of nasopharyngeal carcinoma, Hodgkin's disease, and T-cell lymphomas. B cells expressing the latency 1 program (EBNA-1 only) are found in Burkitt's lymphoma, and have been difficult to detect in virus-infected individuals.

The Complicated Collection of Different B-Cell Phenotypes Is Best Understood in the Context of Normal B-Cell Biology

To enter the resting state and become a memory cell, an uninfected B cell must have bound its cognate antigen and received appropriate signals from helper T cells in germinal centers of lymphoid tissue. Remarkably, during latent infection, the viral LMP-1 and LMP-2a proteins mimic **all** of these steps such that the infected B cell is able to differentiate into a memory cell in the absence of other cues.

The Equilibrium Established between Active Immune Elimination of Infected Cells and Viral Persistence Is Noteworthy

Although immunocompetent individuals maintain CTLs directed against many of the viral proteins synthesized in latently infected B cells, these cells are not eliminated. Some viral proteins, such as LMP-1, inhibit apoptosis or immune recognition of latently infected cells. Moreover, EBNA-1 peptides are not presented to T cells, as discussed above. When the equilibrium between proliferation of latently infected B cells and the immune response that kills them is altered (e.g., after immunosuppression), the immortalized B cells can form lymphomas (Fig. 5.11; see also Chapter 7). It is a matter of debate if any viral protein is synthesized in the infected, nondividing B cell. Certainly, virus-infected proliferating cells produce viral proteins and are superb targets for the host's immune system. It is likely that the normal immune response selects nonproliferating B cells as the survivors of infection, and ensures that the latent infection is benign in the majority of cases.

Reactivation

The signals that reactivate latent Epstein-Barr virus infection in humans are not well understood, but considerable information has been obtained from studies of

cultured cells. Certain signal transduction cascades or production of an essential viral transcriptional activator, Zta (Z or zebra protein; see Volume I, Fig. 8.18, and below), induces the productive infection. It has been established that Zta induces the full productive program only when crucial promoters are methylated at CpG residues. Recall that in latently infected cells, the viral genome slowly acquires methylated cytosine residues, thus facilitating reactivation when Zta is made. Indeed, Zta, like phorbol esters, increases transcription of viral lytic genes. This protein also represses the latency-associated promoters and is responsible for recognition of the viral lytic origin of replication.

Many signal transduction pathways efficiently reactivate Epstein-Barr virus from the latent state. Such pathways are activated by various interactions, including clustering of the B-cell antigen receptor CD21 after interaction with anti-immunoglobulin antibodies (activation of tyrosine kinases), binding of phorbol esters (stimulation of protein kinase C), and introduction of calcium ionophores. Therefore, it is surprising that latent infection is so stable. We now know that virus-encoded LMP-2A makes an important contribution to maintaining the latent infection by inhibiting tyrosine kinase signal transduction pathways. It is the first example of a viral protein that blocks reactivation of a latent infection. As reactivation occurs efficiently, a second signal transduction pathway that bypasses the LMP-2A block must exist, but such a pathway has not been identified.

Slow Infections: Sigurdsson's Legacy

Many fatal brain diseases, characterized by ataxia (movement disorders) or dementia (severe cognitive impairment), stem from another extreme variation of persistent infection, a pattern called slow infection (Fig. 5.1). It may be **years** from the time of initial contact of the infectious agent with the host until the appearance of recognizable symptoms. Such long incubation periods are remarkable (Table 5.1). Once symptoms appear, death usually follows quickly. Viruses such as measles virus, the polyomavirus JC virus, and retroviruses such as human immunodeficiency virus and human T-lymphotropic virus can establish slow infections with severe nervous system pathogenesis at the end stage of disease. In many cases, the persistent infection is maintained in peripheral compartments with no apparent effect, and only enters the brain after many years.

Elucidating the molecular mechanisms responsible for an infectious-disease process of such long duration is a formidable challenge. Experimental analysis of these unusual diseases began in the 1930s, when a flock of Karakul sheep was imported from Germany to Iceland, where they infected the native sheep, causing a disease called maedi/visna. Thanks to the many years of careful work by Bjorn Sigurdsson and colleagues, we now know that the maedi/visna syndrome is caused by a lentivirus that is like human immunodeficiency virus. The striking feature that Sigurdsson discovered is the slow progression to disease after primary infection, often taking more than 10 years. He developed a framework of experimentation for studying the slow, relentless, usually progressive and fatal brain infections, including those now proposed to be caused by prions (see Appendix B).

Abortive Infections

In an abortive infection, virions infect susceptible cells or hosts, but replication is not completed, usually because an essential viral or cellular gene is not expressed. Clearly, an abortive infection is nonproductive. Even so, it is not necessarily uneventful or benign for the infected host. Viral interactions at the cell surface and subsequent uncoating can initiate membrane damage, disrupt endosomes, or activate signaling pathways that cause apoptosis or cytokine production. In some instances, abortively infected cells may not be recognized by the immune system, and if they do not divide, the viral genome may persist as long as the cell survives. In some cases, an infection may proceed far enough that the infected cell is recognized by CTLs. Such an infection would probably induce an IFN, as well as an inflammatory response that may damage the host if sufficient cells participate. Recently, it was discovered that the human immunodeficiency virus structural protein Vpr can damage cells when it is associated with particles that contain noninfectious genomes. When such noninfectious particles bind to T cells, Vpr induces G_2 arrest. It has been suggested that, although most of the particles in an infected individual are replication defective, they participate in immune suppression because of the presence of virion Vpr.

With the advent of modern viral genetics, virologists can construct defective viral genomes, which in the absence of a complementing gene product initiate an abortive infection. One popular idea is to use such defective genomes as vectors for gene therapy or as vaccines. To be effective, cytopathic genes of a prospective viral vector certainly must be eliminated. Many of the well-known, defective viral vectors lack essential genes and are designed to express only the therapeutic cloned gene. Care must be taken to ensure that the infection is truly noncytotoxic. Given that intrinsic and innate defenses can be activated by replication-defective particles, prudence in assuming the safety of viral vectors is essential (see Box 4.7).

Cytotoxicity and inflammatory host responses are of particular concern if the therapeutic gene is to be delivered to a substantial number of cells, a process that requires administration of many virus particles.

Transforming Infections

A transforming infection is a special class of persistent infection. A cell infected by certain DNA viruses or retroviruses may exhibit altered growth properties and begin to proliferate faster than uninfected cells. In some cases, this change is accompanied by integration of viral genetic information. In others, replication is in concert with the cell. Virus particles may no longer be produced, but some or all of their genetic material generally persists. We characterize this pattern of persistent infection as transforming because of the change in cell behavior. It is also considered oncogenic because some transformed cells cause cancer in animals. This important infection pattern is discussed in detail in Chapter 7.

Perspectives

All patterns of infection most likely are established during the clash between host defense and viral countermeasures in the first minutes to hours after the initial infection. The selection pressures are strong, and one might expect wide variation in successful host and virus populations. For the viruses, it appears that two distinct strategies of propagation that correlate with patterns of infection have emerged during evolution: one produces large numbers of progeny (high reproductive output or ***r*-replication strategies; acute infections**) and one results in a lower reproductive output, but better competition for resources (***K*-replication strategies; persistent infections**).

Acute infections occur primarily because host defenses are modulated passively or actively, at least for a short time. Acute infections progress beyond physical, intrinsic, and innate defenses only to be blocked and cleared by the adaptive immune response. Large numbers of new hosts are required to sustain the acute pattern of infection.

Persistent infections result because essentially **all defenses**, including the adaptive immune system, are ineffective, often for long periods. Ineffective does not always mean nonfunctional. For example, in some persistent infections, a low rate of viral replication is equal to the rate of immune elimination. This particular persistent infection pattern can be characterized as "smoldering," as it continues for very long periods in the face of active host defenses. When the rate of viral replication is very low or zero, but the viral genome is not eliminated, immune defenses may be effectively blinded. Success is ensured only if a mechanism exists for periodic production of virions and their transmission to new hosts.

The existence of only two primary patterns of infection confront us with several questions. A particular pattern can be a defining characteristic of a virus family (e.g., influenza virus always produces an acute infection; herpes infections are forever). Why has one particular pattern been selected over another? We know that acute and persistent infections are determined by properties of both host and virus. The patterns are not mutually exclusive, as some infections exhibit both acute and persistent phases. Some of the answers are discussed in Chapter 10, where we point out that viral populations emerge and prosper as a consequence of selection pressures. The selective advantages or disadvantages of rapid or limited replication manifest themselves quickly. Those host and viral genomes that can adapt survive to carry on the relationship another day.

The role of pathogenesis as a selective force in the establishment and maintenance of viral infections is a subject of research and debate. One hypothesis is that successful patterns result in symbiosis, neither helping nor harming the host. In this context, as suggested by Lewis Thomas, pathogenesis is an aberration of symbiosis, an overstepping of boundaries. Benign symbiosis is a recipe for stability, but many apparently successful viral infections are far from stable. Accordingly, another hypothesis posits that pathogenesis is a necessary survival feature of the viral population, and is selected during evolution of the relationship. Some individuals may be harmed in the short run to achieve long-term survival of the virus population. We continue the discussion of the evolution of new viral infections and pathogenesis in Chapter 10.

Selection works in unexpected ways. In laboratory situations, adaptation to r-selection conditions (low MOI, rapid growth) yields viral populations that are less fit when exposed to K-selection conditions (high MOI, reduced growth) and *vice versa*. This fact may underlie the stability of one pattern compared to the other. Another obvious but poorly understood fact is that pathogen interactions in an infected individual or tissue are complex and provide unexpected selection conditions. Infections with one virus can influence those of completely unrelated viruses by systemic or local immunosuppression (see Chapter 4). In addition, some viral infections are advantageous to the host, a fact well established for lysogens of enteric bacteria. Lysogens outcompete nonlysogens in chemostat growth experiments. This concept of a "good infection" certainly tests our current understanding of the complexities of the evolution of patterns of infection and host-virus interactions.

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Liebert, U., K. Baczko, H. Budka, and V. ter Meulen. 1986. Restricted expression of measles virus proteins in brains from cases of subacute sclerosing panencephalitis. *J. Gen. Virol*. **67:**2435–2444.

Slow Viral Infections

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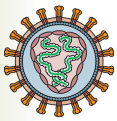
Lutley, R., G. Petursson, P. Aalsson, G. Georgsson, J. Klein, and N. Nathanson. 1983. Antigenic drift in visna: virus variation during long-term infection of Icelandic sheep. *J. Gen. Virol*. **64:**1433–1440.

Poiesz, B., F. Ruscetti, M. Reitz, V. Kalyanaraman, and R. Gallo. 1981. Isolation of a new type C retrovirus (HTLV) in primary uncultured cells of a patient with Sezary T-cell leukaemia. *Nature* **294:**268–71.

Sigurdsson, B. 1954. Rida, a chronic encephalitis of sheep with general remarks on infections which develop slowly and some of their special characteristics. *Br. Vet. J.* **110:**341–354.

Ter Meulen, V., J. R. Stephenson, and H. W. Kreth. 1983. Subacute sclerosing panencephalitis. *Comp. Virol*. **18:**105–159.



6

Human Immunodeficiency Virus Pathogenesis

Nature is not human-hearted.
LAO TZU
Tao Te Ching

Introduction

Worldwide Scope of the Problem

Acquired immunodeficiency syndrome (AIDS) is the name given to end-stage disease caused by infection with human immunodeficiency virus (HIV). By almost any criteria, HIV qualifies as one of the world's deadliest scourges. First recognized as a clinical entity in 1981, by 1992 AIDS had become the major cause of death in individuals 25 to 44 years of age in the United States. Although the rate of increase has been reduced since 2000, the current worldwide statistics are still staggering, with the developing countries of Africa and parts of Asia being especially hard-hit (Fig. 6.1). An end-of-year report from the United Nations' AIDS program estimated the number of new HIV infections in 2007 to be 2.5 million, bringing the total number of infected people worldwide to approximately 33.2 million. This number corresponds to almost 1 in every 100 adults aged 15 to 49 in the world's population. Although the recent availability of drugs to treat HIV infection has decreased the annual death toll in wealthy countries, HIV/AIDS is still the leading cause of death in sub-Saharan Africa, with 1.6 million fatalities in 2007 alone. In certain parts of this region, 25 to 30% of the adult population has become infected. It is estimated that one-third of the children under 15 years of age in these areas have lost one or both parents to AIDS. In these places a whole generation of human beings has succumbed to this fatal disease. AIDS kills more people than any other infectious disease, and HIV continues to spread faster than any known persistent infectious agent in the last half century. The clinical emergence of this virus is likely to be the consequence of a number of political, economic, and societal changes, including the breakdown of national borders, economic distress with the migration of large populations, and the ease and frequency of travel throughout the world. International efforts have focused on bringing funds and expertise to bear on the HIV/AIDS pandemic in Africa and elsewhere, but the task is enormous.

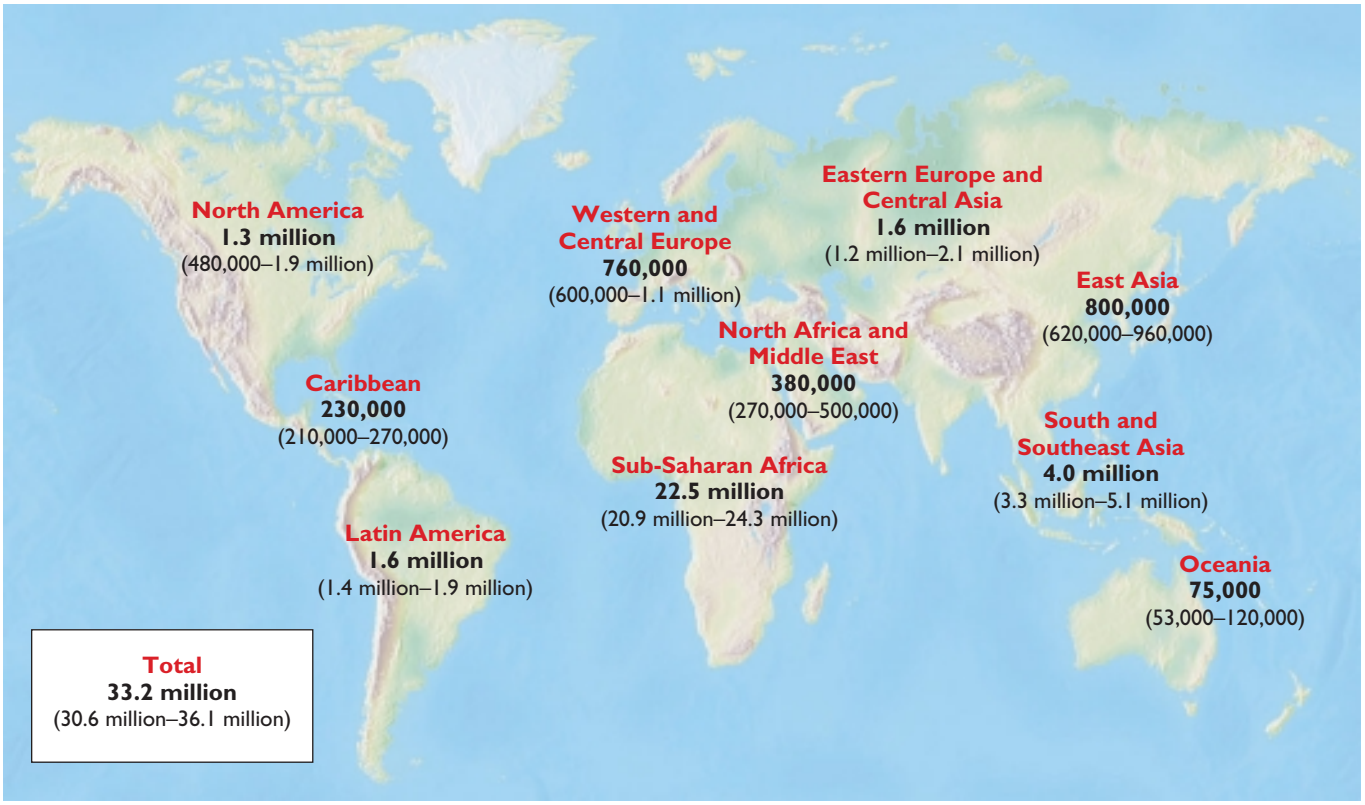


Figure 6.1 Estimated number of people living with HIV infection worldwide, 2007. Data from the Joint United Nations Programme on HIV/AIDS, November 2007.

Because of its medical importance, HIV has also become the most intensely studied infectious agent. Research on the virus has contributed to our understanding of AIDS and related veterinary diseases; provided new insights into virology, cellular biology, and immunology; and allowed researchers to develop strategies for its prevention and control of HIV infection. This chapter describes the many facets of HIV-induced pathogenesis and what has been learned through its analysis. The complexities illustrate the enormous scope of the challenges faced by biomedical researchers and physicians in their efforts to control this virus, which strikes at the very heart of the body's defense systems.

HIV Is a Lentivirus

Discovery and Characterization

The first clue to the etiology of AIDS came in 1983 with the isolation of a retrovirus from the lymph node of a patient with lymphadenopathy at the Pasteur Institute in Paris. Although not fully appreciated initially, the significance of this finding became apparent in the following year with the isolation of a cytopathic, T-cell-tropic retrovirus from combined blood cells of AIDS patients by researchers at the U.S. National Institutes of Health and of a similar retrovirus from blood cells of an AIDS patient at the University of California, San Francisco. Although the National Institutes of Health isolate was later shown to originate from a sample received from the Pasteur Institute (Box 6.1), the virus isolated at the University of California, San Francisco, and subsequent isolates at the National Institutes of Health laboratory were unique. As commonly happens, each laboratory gave its isolate a different name: LAV (lymphadenopathy-associated virus), HTLV-III (human T-cell lymphotropic virus type III), and ARV (AIDS-associated retrovirus). Electron microscopic examination revealed that these viruses were morphologically similar to a known group of retroviruses, the lentiviruses, and further characterization confirmed this relationship. In 1986, the International Committee on Taxonomy of Viruses recommended the current name, human immunodeficiency virus.

Lentiviruses comprise a separate genus of the family *Retroviridae* (Table 6.1). The equine infectious anemia lentivirus was one of the first viruses to be identified. Discovered

BOX 6.1 DISCUSSION

Lessons from discovery of the AIDS virus(es)

The first AIDS virus was obtained from a patient with lymphadenopathy by Françoise Barré-Sinoussi in collaboration with Jean-Claude Chermann and Luc Montagnier at the Pasteur Institute (1983). The isolate, named Bru, grew only in primary cell cultures. We now know that Bru belonged to a class of slow-growing, low-titer viruses that are common in early-stage infection.

Between 20 July and 3 August 1983, Bru-infected cultures at the Pasteur Institute became contaminated with a second AIDS virus, called Lai, which had been isolated from a patient with full-blown AIDS and which belonged to a class of viruses that grow well in cell culture. HIV-1 Lai rapidly overtook the cultures.

Unaware of this contamination, Pasteur scientists subsequently sent out virus samples from these cultures as "Bru" to several laboratories, including those of Robin Weiss in Britain and Malcolm Martin and Robert Gallo in the United States. Unlike earlier samples of Bru, this virus grew robustly in the laboratories to which it was distributed. Indeed, Lai was later discovered to have contaminated some AIDS patient "isolates" obtained by Weiss. In retrospect, such contamination is not surprising, as biological containment facilities were limited at the time, with the same incubators and hoods being used for maintaining HIV stocks and making new isolates.

Lai also contaminated cultures of blood cells combined from several AIDS patients in the Gallo laboratory at the National Institutes of Health. Because the properties of this virus were found to be different from those described for Bru, Gallo and coworkers reported the discovery of a second type of AIDS virus, which they believed to have originated from one of their AIDS patients.

This second claim, a race to develop blood screening tests, and the later revelation from DNA sequence analyses that the French and the Gallo viruses were one and the same (Lai) led to a much publicized scientific controversy with significant political overtones. Simon Wain-Hobson and colleagues at the Pasteur Institute eventually sorted out the chain of events in 1991 by comparing nucleotide sequences of stored samples of the original stocks of Bru and Lai. The controversy has since subsided—what remains are important lessons in virology: that contamination can be a real problem, that passage in the laboratory tends to select for viruses that replicate rapidly, and that rigorous characterization (nowadays by genome sequencing) is a prudent safeguard against costly mistakes.

Goudsmit, J. 2002. Lots of peanut shells but no elephant. A controversial account of the discovery of HIV. *Nature* **416:**125–126.

Wain-Hobson, S. J. P. Vartanian, M. Henry, N. Chenciner, R. Cheynier, S. Delassus, L. P. Martins, M. Sala, M. T. Nugeyre, D. Guetard, et al. 1991. LAV revisited: origins of the early HIV-1 isolates from Institut Pasteur. *Science* **252:**961–965.

Weiss, R., and M. Martin. Personal communication.

in 1904, this virus causes episodic autoimmune hemolytic anemia in horses. Lentiviruses of sheep (visna/maedi virus) and goats (caprine arthritis-encephalitis virus) have also been known for many years. All these viruses are associated with long incubation periods and are therefore called **slow viruses** (Chapter 5). The discovery of HIV led to a search for additional lentiviruses and their subsequent isolation from cats (feline immunodeficiency virus) and a variety of nonhuman primates (simian immunodeficiency virus [SIV]). In 1986, a distinct type of HIV that is prevalent in certain regions of West Africa was discovered. It was called HIV-2. Individuals infected with HIV-2 also develop AIDS, but with a longer incubation period and lower morbidity.

Many independent isolates of both HIV-1 and HIV-2 have been characterized over the last decade. Nucleotide sequence comparisons allow us to distinguish two major groups among HIV-1 isolates: group M includes most HIV-1 isolates, and group O represents what appear to be relatively rare "outliers" (Box 6.2). Nine distinct subtypes are currently recognized in group M (called **clades** A to K, except E, which was found to be a recombinant), each of which is prevalent in a different geographic area. For example,

Table 6.1 Lentiviruses[a]

Virus	Host infected	Primary cell type infected	Clinical disorder(s)
Equine infectious anemia virus	Horse	Macrophages	Cyclical infection in the first year, autoimmune hemolytic anemia, sometimes encephalopathy
Visna/maedi virus	Sheep	Macrophages	Encephalopathy/pneumonitis
Caprine arthritis-encephalitis virus	Goat	Macrophages	Immune deficiency, arthritis, encephalopathy
Bovine immunodeficiency virus	Cow	Macrophages	Lymphadenopathy, lymphocytosis
Feline immunodeficiency virus	Cat	T lymphocytes	Immune deficiency
Simian immunodeficiency virus	Primate	T lymphocytes	Immune deficiency and encephalopathy
Human immunodeficiency virus	Human	T lymphocytes	Immune deficiency and encephalopathy

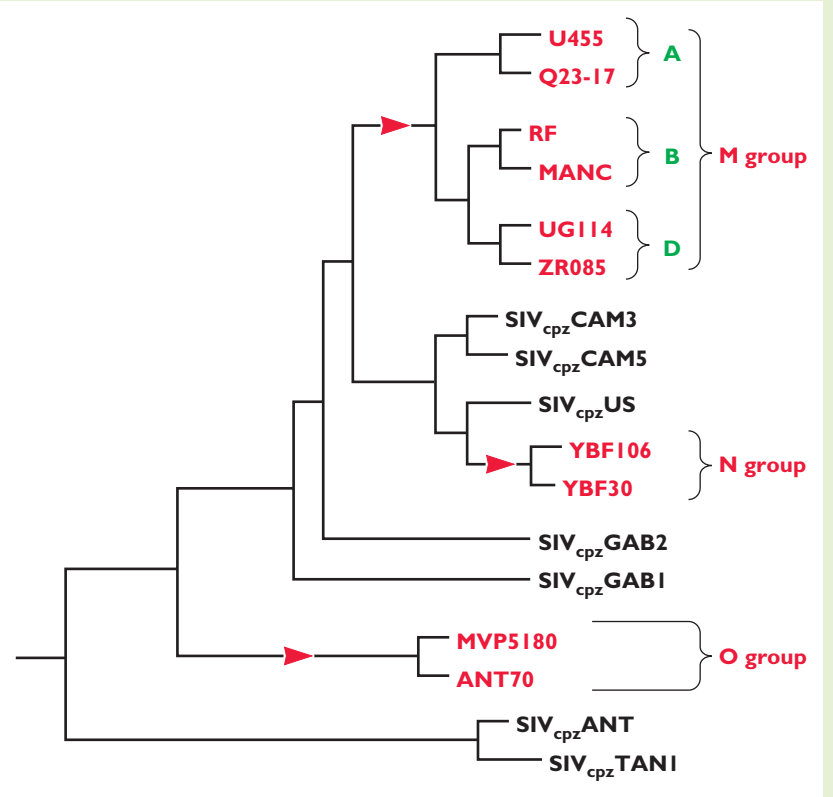
[a]Adapted from Table 1.1 (p. 2) of J. A., Levy, *HIV and the Pathogenesis of AIDS,* 3rd ed. (ASM Press, Washington, DC, 2007), with permission.

BOX 6.2 BACKGROUND

The earliest record of HIV-1 infection

The earliest record of HIV-1 infection comes from a serum sample obtained in 1959 from a Bantu male in the city now known as Kinshasa, in the Democratic Republic of Congo. Phylogenetic analyses place the viral sequence (ZR59) near the ancestral node of clades B and D. As this is not at the base of the M group, this group must have originated earlier (red arrowhead near top of figure), and back calculations suggest that the M group of viruses arose via transspecies transmission from a chimpanzee into the African population around 1930. Its rapid evolution, giving rise to at least 10 subtypes (clades A to K), seems to have occurred near the end of or just after World War II. Separate transspecies transmissions (red arrowheads in bottom half of figure) account for the origin of the N and O groups.

Sharp, P. M. 2002. Origins of human virus diversity. *Cell* **108**:305–312.



clade B is the most common subtype in North America and Europe. A new group, N, was proposed for HIV-1 in 1998 based on a virus isolate, YBF30, obtained from an AIDS patient in Cameroon. The nucleotide sequence of this virus is more closely related to group M than to group O. Identification of related strains in Cameroon supports a three-pronged radiation of HIV-1 groups. Eight distinct groups of HIV-2 have also been identified. Of these, groups A and B (found in different parts of West Africa) account for most infections worldwide.

Figure 6.2 shows the phylogenetic relationships among the lentiviruses, based on sequences of their *pol* genes. The African monkey and ape isolates are endemic to each of the species from which they were obtained and do not appear to cause disease in their native hosts (Box 6.3). However, a fatal AIDS-like disease is caused by infection of Asian macaques with virus originating from the African sooty mangabey (SIV_{smm}). Close contact between sooty mangabeys and humans is common, as these animals are hunted for food and kept as pets. Such interaction and the observation that several isolates of HIV-2 are nearly indistinguishable in nucleotide sequence from SIV_{smm} support the hypothesis that HIVs emerged via interspecies transmission from nonhuman primates to humans. This hypothesis is supported further by recent studies indicating that the known HIV-1 groups arose via at least three independent transmissions from chimpanzees. The strains of SIV_{cpz} from the chimpanzee *Pan troglodytes troglodytes* are closest in sequence to HIV-1, implicating this subspecies as the origin of the human virus. In this chapter we use the abbreviation HIV to describe properties shared by HIV-1 and HIV-2, and specify the type when referring to one or the other.

As summarized in Table 6.1, lentiviruses cause immune deficiencies and disorders of the hematopoietic and central nervous systems and, sometimes, arthritis and autoimmunity. Lentiviral genomes are relatively large, with more genes than those of simpler retroviruses (Fig. 6.3).

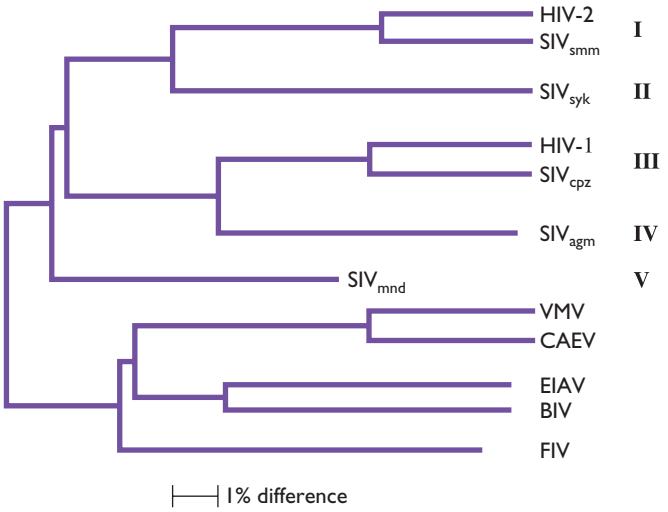


Figure 6.2 Phylogenetic relationships among lentiviruses. Representative lentiviruses were compared by using *pol* gene nucleotide sequences for establishing phylogenetic relationships. Five groups of primate lentiviruses (labeled I through V) are shown: HIV-1, HIV-2, SIV from the sooty mangabey monkey (SIV_{smm}), SIV from Sykes' monkey (SIV_{syk}), SIV from the chimpanzee (SIV_{cpz}), SIV from the African green monkey (SIV_{agm}), and SIV from the mandrill (SIV_{mnd}). Nonprimate lentiviruses are visna/maedi virus (VMV), caprine arthritis-encephalitis virus (CAEV), equine infectious anemia virus (EIAV), bovine immunodeficiency virus (BIV), and feline immunodeficiency virus (FIV). The scale indicates the percentage difference in nucleotide sequences in the *pol* gene. The branching order of the primate lentiviruses is controversial. Adapted from Fig. 2 of P. A. Luciw, p. 1881–1952, *in* B. N. Fields et al. (ed.), *Fields Virology*, 3rd ed. (Lippincott-Raven, Philadelphia, PA, 1996), with permission.

In addition to the three structural polyproteins Gag, Pol, and Env, common to all retroviruses, lentiviral genomes encode a number of additional **auxiliary proteins**. Two HIV auxiliary proteins (Table 6.2), Tat and Rev, perform **regulatory** functions that are essential for viral replication. The remaining four, Nef, Vif, Vpr, and Vpu, are not essential for viral reproduction in most immortalized T-cell lines and hence are known as **accessory** proteins. However, these proteins do modulate virus replication, and they are essential for efficient virus production *in vivo* and the ensuing pathogenesis.

Distinctive Features of the HIV Replication Cycle and the Roles of Auxiliary Proteins

Much of what we know about the function of the auxilliary proteins of HIV comes from studies of their effects on cells in culture, often produced transiently from plasmid expression vectors in the absence of other viral components (Volume I, Box 8.8). Although these methods are simple and sensitive, they do not necessarily reproduce conditions similar to those that occur upon viral infection. Preparation and analysis of viral mutants have also been used to investigate the functions of these proteins in cell culture. However, as the hosts for this virus are humans, it is difficult to evaluate the significance of many of the functions deduced from cell culture to pathology in the whole organism.

cis-Acting Regulatory Sequences and Tat and Rev

Tat interacts with TAR sequences in the long terminal repeat. As in all retroviruses, expression of integrated HIV DNA is regulated by sequences in the transcriptional control region of the viral long terminal repeat (LTR), which are recognized by the host cell's transcriptional machinery. The HIV-1 LTR functions as a promoter in a variety of cell types, but its basal level is very low. As described in Volume I, Chapter 8 (Fig. 8.10), the LTR of HIV includes an enhancer sequence that binds a number of cell-type-specific transcriptional activators, for example, Nf-κb (Volume I, Fig. 8.11). The release of Nf-κb from its cytoplasmic inhibitor in activated T-cells may explain why HIV replication requires T-cell stimulation.

Just downstream of the site of initiation of transcription in the HIV LTR is a unique viral regulatory sequence, TAR (Fig. 6.4). As described in Volume I, Chapter 8 (Fig. 8.13), TAR RNA forms a stable, bulged stem-loop structure that binds the regulatory protein Tat (Table 6.2), together with a number of host proteins, to stimulate transcription. In the absence of Tat, viral transcription usually terminates prematurely. The principal role of Tat is to enhance the processivity of transcription and thereby facilitate the elongation of viral RNA.

The Tat protein is released by infected cells and can be taken up by other cells and influence their function. Tat can act as a chemoattractant for monocytes, basophils, and mast cells. It also induces expression of a variety of important proteins in the cells that it enters, and some of these proteins can have a profound effect on virus spread and immune cell function. For example, in transient-expression assays Tat can up-regulate the expression of genes encoding the CXCr4 and CCr5 coreceptors in target cells and can enhance the expression of a number of chemokines. The Tat protein is reported be cytotoxic to some cultured cells and is neurotoxic when inoculated intracerebrally into mice. It has also been reported that transgenic expression of Tat in mice causes a disease that resembles Kaposi's sarcoma. Although the human disease is almost certainly caused by a herpesvirus, Tat contributes to the aggressive nature of this malignancy in AIDS patients by promoting the growth of spindle cells in the Kaposi's sarcoma lesions.

BOX 6.3 DISCUSSION

TRIM5 restriction, an example of coevolution of viral and host genes?

The tripartite motif (Trim) 5α protein was identified in a screen for "factors" that might be responsible for the inability of HIV-1 to replicate in the cells of Old World monkeys, including those of rhesus macaques. Trim5α is translated from a spliced macaque TRIM5 mRNA. The macaque protein has no apparent effect on the replication of SIV_{mac}, but blocks HIV-1 replication shortly after entry by binding to the capsid protein and mediating the degradation or premature disassembly of the infecting particle.

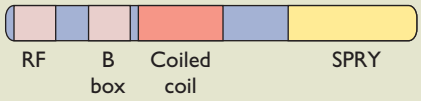
TRIM5 is a member of a large, multigene family that has proliferated during evolution and has been under positive selection for about 35 million years. There are hundreds of copies of TRIM genes in the primate genomes; 80 members of this family have been identified in humans. However, each primate species encodes a TRIM5 gene with different antiviral specificity, consistent with differences in the viral capsids with which the proteins interact.

One current theory is that TRIM5 genes evolved independently in each species to protect against particular endemic viruses. This idea is supported by the finding that human Trim5α blocks replication of a 4-million-year-old endogenous virus (PtERV1) that was resurrected from the genome of the chimpanzee *Pan troglodytes*.

Susceptibility to retroviral infection is dependent on a number of host-virus interactions, and it is not yet clear how the antiviral activities of TRIM5 orthologs may affect the resistance or sensitivity of each primate species. However, it has been suggested that selective changes in the TRIM5 gene that occurred in the human lineage in response to PtERV1, or a related ancient virus, may have left our species more susceptible to HIV-1.

There is currently great interest in elucidating the antiviral mechanism of TRIM5 and related proteins. Such knowledge should uncover new vulnerabilities of HIV and may suggest additional targets for antiviral drug development.

The rhesus macaque Trim5α protein contains a RING domain (RF), a B box, and a coiled-coil domain. Many Zn-binding RING domains have E3 ubiquitin ligase activity, and Trim5α can mediate RING-dependent auto-ubiquitinylation *in vitro*. This property supports the hypothesis that the antiviral activity of Trim5α is mediated by ubiquitinylation of itself and associated viral proteins, leading to degradation in the proteosome. The B box domain also has a Zn-binding motif, is likely to be involved in protein-protein interactions, and may contribute to ubiquitin ligation specificity. The SPRY domain interacts with the HIV-1 capsid protein and is responsible for antiviral activity. Trim5α exists as a trimer with the coiled-coil facilitating homo- and heteromultimerization with related Trim proteins.



Kaiser, S. M., H. S. Malik, and M. Emerman. 2007. Restriction of an extinct retrovirus by the human TRIM5α antiviral protein. *Science* **316:**1756–1758.

Luban, J. 2007. Cyclophilin A, TRIM5, and resistance to human immunodeficiency virus type 1 infection. *J.Virol.* **81:**1054–1061.

Stremlau, M., C. M. Owens, M. J. Perron, P. Kiessling, P. Autissier, and J. Sodroski. 2004. The cytoplasmic body component TRIM5α restricts HIV-1 infection in Old World monkeys. *Nature* **427:**848–853.

Towers, G. 2007. The control of viral infection by tripartite motif proteins and cyclophilin A. *Retrovirology* **4:**40.

Multiple splice sites and the role of Rev. Unlike those of the simpler oncogenic retroviruses, the full-length HIV transcript contains numerous 5′ and 3′ splice sites. The regulatory proteins Tat and Rev and the accessory protein Nef are synthesized early in infection from multiply spliced messenger RNAs (mRNAs) (Fig. 21, appendix in Volume I). As Tat then stimulates transcription, these mRNAs are found in abundance at this early time. However, the accumulation of Rev protein brings about a change in the pattern of mRNAs, leading to a temporal shift in viral gene expression.

Figure 6.3 Organization of HIV-1 and HIV-2 proviral DNA. Vertical positions of the colored bars denote each of the three different reading frames that encode viral proteins. The LTRs contain sequences necessary for transcriptional initiation and termination, reverse transcription, and integration. Adapted from Fig. 1 of M. Emerman, *Curr. Biol.* **6:**1096–1103, 1996, with permission.

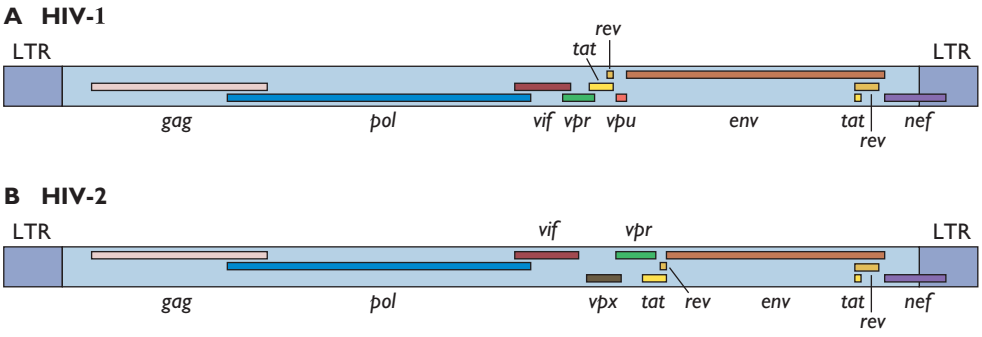
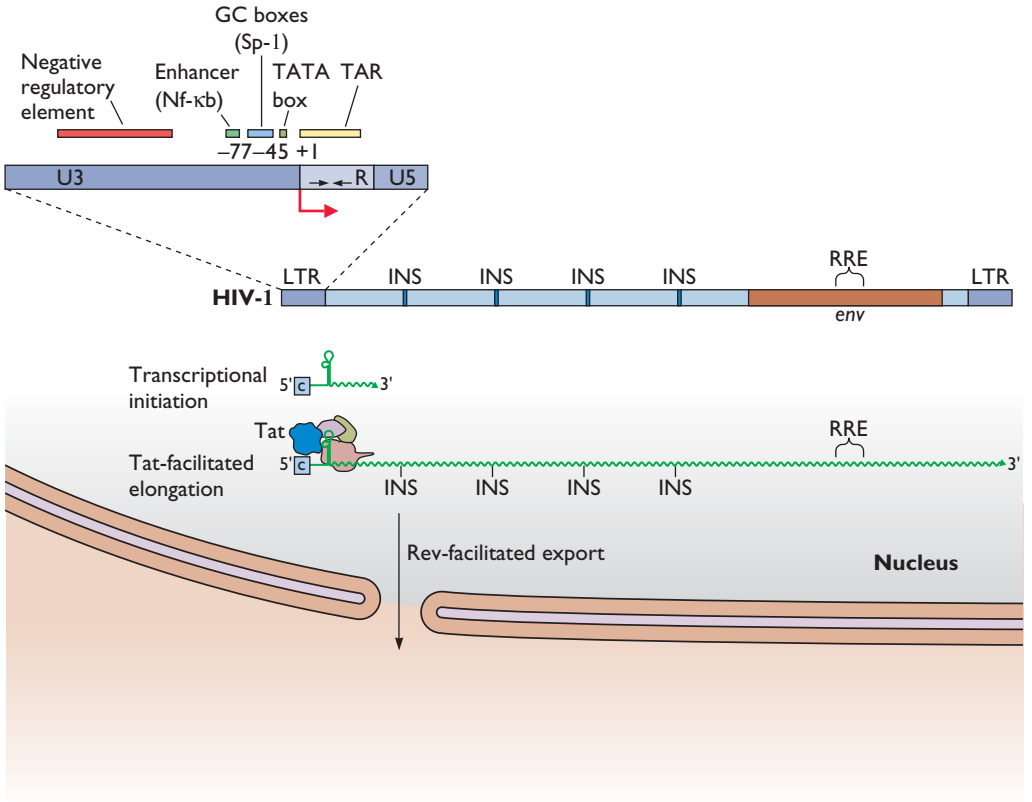


Table 6.2 HIV auxiliary proteins[a]

Protein[b]	Size (kDa)	Function	Location
Regulatory			
Tat	14	Transactivation; binds TAR to facilitate initiation and elongation of viral transcription	Primarily in cell nucleus
Rev	19	Regulation of viral mRNA expression; binds RRE and facilitates nuclear export of unspliced or singly spliced RNAs	Primarily in cell nucleus
Accessory			
Nef	27	Pleiotropic, can increase or decrease virus replication; down-regulates MHC-I and the CD4 receptor; influences T-cell activation; enhances virion infectivity	Cell cytoplasm, plasma membrane
Vif	23	Increases virus infectivity; helps in virion assembly and in viral DNA synthesis	Cell cytoplasm
Vpr	15	Helps in virus replication; causes G_2 arrest; facilitates nuclear entry of preintegration complex	Virion
Vpu[c]	16	Helps in virus release; disrupts Env-CD4 complexes; causes CD4 degradation	Integral cell membrane protein
Vpx[d]	15	Nuclear entry of preintegration complexes	Virion

[a]Adapted from Table 1.5 (p. 10) of J. A. Levy, *HIV and the Pathogenesis of AIDS,* 3rd ed. (ASM Press, Washington, DC, 2007), with permission.
[b]See Figure 6.3 for location of the viral genes on the HIV genome.
[c]Present only with HIV-1. Expression appears regulated by Vpr.
[d]Encoded only by HIV-2. May have originated via a duplication of Vpr.

Figure 6.4 Mechanisms of Tat activation. Some regulatory sequences in the HIV LTR are depicted in the expanded section at the top. The numbers refer to positions relative to the site of initiation of transcription. The opposing arrows in R represent a palindromic sequence that folds into a stem-loop structure (TAR) in the transcribed mRNA to which Tat binds (center). Tat is required for efficient elongation during HIV-1 RNA synthesis. The position of the RRE in the *env* transcript and the presence of *cis*-acting repressive sequences, also known as instability elements (INS), are also illustrated.



The Rev protein (Table 6.2) is an RNA-binding protein that recognizes a specific sequence within a structural element in the *env* region of the elongated transcript called the **Rev-responsive element (RRE)** (Fig. 6.4). As discussed in Volume I, Chapter 10 (Fig. 10.14 to 10.16), Rev mediates the nuclear export of any RRE-containing RNA. As the concentration of Rev increases, unspliced or singly spliced transcripts containing the RRE are exported from the nucleus. In this way, Rev promotes synthesis of the viral structural proteins and enzymes and ensures the availability of full-length genomic RNA for incorporation into new virus particles. The accessory proteins Vif, Vpr, and Vpu (for HIV-1) or Vpx (for HIV-2) are also expressed later in infection from singly spliced mRNAs that are dependent on Rev for export to the cytoplasm (Fig. 22, appendix in Volume I).

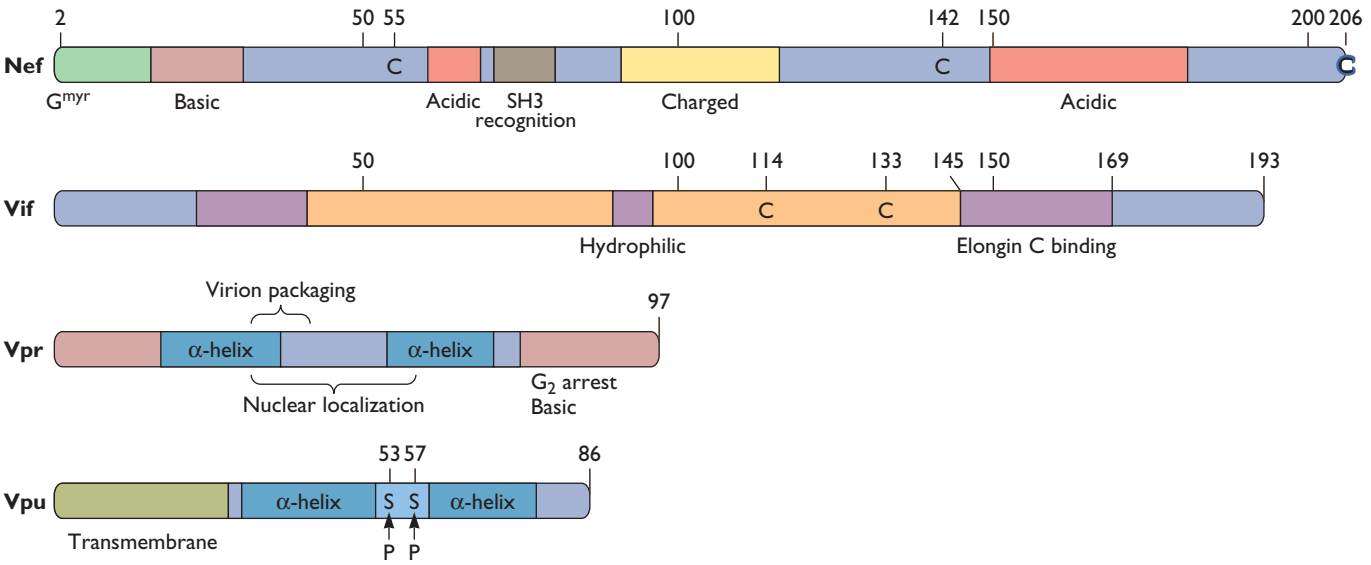
The dependence of HIV gene expression on Rev is due in part to *cis*-acting repressive sequences, also called **instability elements**, present in the unspliced or singly spliced transcripts. These sequences, some of which are characterized by a high A+U content, lie within regions in *gag* and *pol* mRNAs. Mutations in these sequences increase the stability, nuclear export, and translatability of the transcripts in the absence of Rev. The response to their presence appears to be cell dependent, but the mechanism(s) by which these sequences act, and exactly how Rev counteracts their effects, are not understood. This phenomenon does, however, provide an explanation for the puzzling failure of early attempts to express individual HIV-1 structural proteins and enzymes in primate or human cells from mRNAs that did not also encode Rev.

The Accessory Proteins

A very large number of seemingly disparate functions have been attributed to the accessory proteins of HIV. Because in many cases activities have been observed under conditions in which the proteins are overproduced from plasmid vectors in cultured cells, the biological significance of some of the proposed functions is not always clear. However, recent studies have uncovered a common mechanism for many of the activities of the accessory proteins: all seem to act as **adapter proteins,** partnering with multicomponent cellular complexes that target proteins for degradation.

Vif protein. Vif stands for viral infectivity factor. This protein (Table 6.2 and Fig. 6.5) accumulates in the cytoplasm and at the plasma membrane of infected cells. Early studies showed that mutant viruses lacking the *vif* gene were approximately 1,000 times less infectious than the wild type in certain $CD4^+$ T-cell lines and peripheral blood lymphocytes and macrophages. However, direct cell-to-cell transfer was only slightly lower than normal. Virions produced in the absence of Vif are therefore defective.

Figure 6.5 Organization of known or presumed functional regions in HIV-1 accessory proteins. Locations of conserved and critical cysteine residues are indicated in Nef and Vif. Serines that require phosphorylation for protein function are noted in Vpu. Magenta regions in Vif are conserved, but their functions are unknown. Numbering corresponds to amino acid positions in proteins of the laboratory strain of HIV-1, NL43. Where known, functions are indicated. G^{myr} is the myristoylated glycine residue at position 2 in Nef, which facilitates membrane binding.



Production of Vif from a plasmid vector in susceptible host cells does not compensate for its absence in the cell that produces virions. Rather, Vif is needed at the time of virus assembly in the producing cells.

Vif is an RNA-binding protein. Small amounts can be detected in HIV particles and also in heterologous retroviral particles produced by cells that contain a Vif-expressing plasmid. Virions produced from *vif*-defective HIV genomes contain the normal complement of progeny RNA, and they are able to enter susceptible cells and to initiate reverse transcription, but full-length double-stranded viral DNA is not detected. These observations indicate that Vif is required in a step following virus entry that is essential for completion of reverse transcription. The requirement for Vif is strikingly cell type dependent. Experiments in which cells that are permissive for *vif* mutants were fused with cells that are nonpermissive established that the nonpermissive phenotype is dominant; the infectivity of virions produced in such heterokaryons was enhanced by Vif production. This observation suggested that Vif protein may suppress a host cell function that otherwise inhibits progeny virus infectivity.

All of these seemingly unusual properties were demystified with the discovery that Vif plays a critical role for the virus by blocking the antiviral action of members of an RNA-binding family of cellular cytidine deaminases, called apoplipoprotein B mRNA editing enzyme catalytic peptides 3 (Apobec3). These enzymes are synthesized in nonpermissive cells and incorporated into virus progeny via interactions with the viral RNA and possibly NC protein. Apobec3G (A3G) was the first family member to be identified as a Vif target. It was subsequently shown that Vif prevents its incorporation into virions by binding to A3G and mediating its depletion. In this role, Vif partners with cellular proteins (Cul5, elongins B ad C, and Rbx1) in an E3 ubiquitin ligase complex that polyubiquitinates A3G, leading to its degradation in proteosomes (Fig. 6.6). Vif expression also blocks the antiviral activities of human Apobec3F and 3C, which like A3G are produced in abundance in lymphoid cells, presumably by a similar mechanism.

A3G appears to exert a number of antiviral activities. It has been proposed that its binding to viral RNA may account, in part, for its inhibition of reverse transcription in newly infected cells. In addition, the enzyme catalyzes the deamination of deoxycytidine to form deoxyuridine (dU) in the first (–) strand of viral DNA to be synthesized by reverse transcriptase. The dU is a substrate for the cellular uracil-DNA glycosylase, and the abasic sites produced by its action are likely targets for endonucleolytic digestion in the newly formed (–) DNA strands. If dU is not removed, the (+) strand complement of the deaminated (–) strand would contain deoxyadenosine in place of the normal deoxyguanosine at such sites (Fig.6.6). The frequency of G→A transitions is abnormally high in the genomes of *vif*-defective virions produced in nonpermissive cells, and incomplete protection from Apobec3 proteins by Vif may explain why such transitions are the most frequent point mutations in HIV genomes. It has been suggested that the Apobec3 proteins represent an ancestral mode of intrinsic cellular defense against retroviruses (see Chapter 3).

Vpr protein. The viral protein R, or Vpr (Table 6.2 and Fig. 6.5), derives its name from the early observation that it affects the rapidity with which the virus replicates in, and destroys, T cells. Most T-cell-adapted strains of HIV-1 carry mutations in *vpr*. The Vpr protein is encoded in an open reading frame lying between *vif* and *tat* in the genomes of primate lentiviruses (Fig. 6.2). The SIV and HIV-2 genomes include a second, related gene, *vpx,* which appears to have arisen as a duplication of *vpr*. The other lentiviruses do not contain sequences related to *vpr* but do include small open reading frames that might encode proteins with similar functions.

Vpr and Vpx are incorporated into virions. Vpr incorporation is dependent on specific interactions with a proline-rich domain at the C terminus of the Gag polyprotein. Vpr protein, in turn, mediates virion incorporation of the host's uracil DNA glycosylase, Ung2. About 100 to 200 molecules of Vpr are present in nucleocapsids. Its presence in virions is consistent with the observation that Vpr function is required at some early stage in the virus replication cycle.

Two principal functions have been recognized for HIV-1 Vpr. The protein causes a G_2 cell cycle arrest and may promote entry of viral nucleic acids into the nucleus. In HIV-2 these functions are segregated into Vpr and Vpx, respectively. Although Vpr itself does not damage host DNA, expression of the isolated gene in cultured cells elicits a response similar to the response to DNA damage, resulting in G_2 arrest and apoptosis. It has recently been discovered that Vpr, like Vif, is an adapter protein, which hijacks another ubiquitin ligase complex. In the case of Vpr, the complex includes the scaffold protein Cul4A, Rbx1 E2 ligase, and damaged DNA-binding protein 1 (Ddb1); targets for polyubiquitinylation and subsequent degradation include the Ung2 protein mobilized by Vpr binding. It has been proposed that binding of Vpr to the Ddb1-containing complex could prevent DNA repair, leading to the accumulation of damaged DNA, subsequent activation of the DNA damage response, and G_2 arrest. The biological advantage of preventing infected cells from entering mitosis is not clear, but the increased activity of the LTR promoters in the G_2 phase of the cell cycle may lead to enhanced virus production. The fact that other viruses, including paramyxovirus 5 and hepatitis B virus, encode proteins that

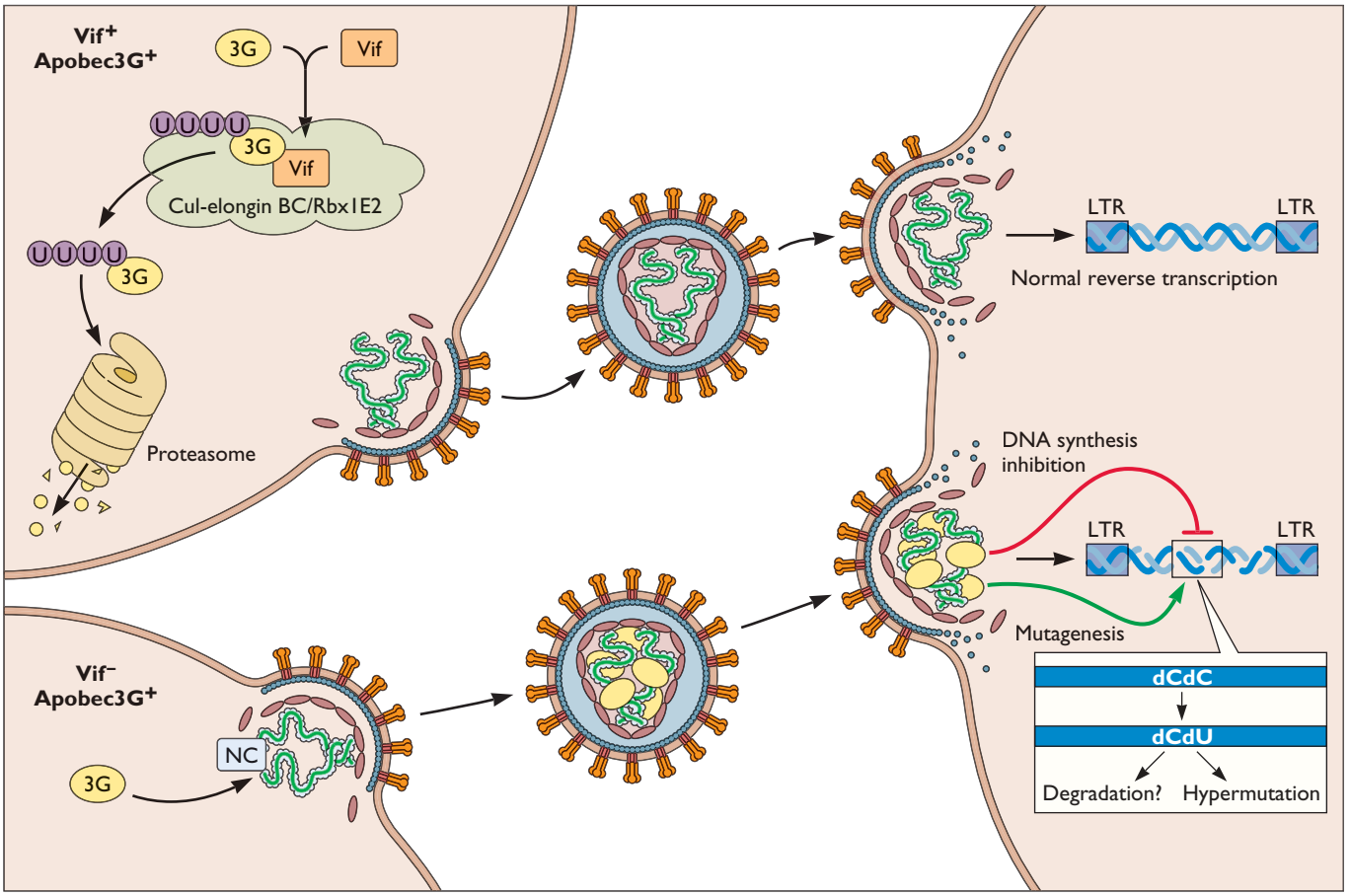


Figure 6.6 Mechanisms of action of Vif and Apobec3G. (Top) Vif counteracts the antiviral affects of Apobec3G (3G) by mediating its polyubiquitinylation, which leads to proteosomal degradation. **(Bottom)** In the abscence of Vif, 3G is incorporated into newly formed virions through interaction between viral RNA and NC protein. In the newly infected cell, viral RNA reverse transcription is inhibited by 3G and cytosines in the newly synthesized DNA are converted to uracil, causing cDNA degradation or hypermutation through eventual C to A transversions. Adapted from B. Cullen, *J. Virol.* **80:**1067–1076, 2006, with permission.

also target Ddb1 suggests that this response may provide some physiological advantage to viral replication.

Vpr has been shown to bind to nuclear pore proteins. As noted in Volume I, Chapter 5, although not essential, these interactions may facilitate docking of the HIV-1 preintegration complex at the nuclear pore in preparation for import. Studies with SIV-infected macaques indicate that deletion of *vpr* attenuates viral pathogenicity. However, deletion of both *vpr* and *vpx* does reduce virus replication in these animals. It seems likely, therefore, that HIV-1 Vpr, which combines the functions of the two SIV gene products, is crucial to HIV pathogenesis.

Vpu protein. The small Vpu protein is unique to HIV-1 and the related SIV_{cpz} (Fig. 6.2), hence the name viral protein U (Vpu). The predicted sequence of Vpu includes an N-terminal stretch of 27 hydrophobic amino acids that comprises a membrane-spanning domain (Fig. 6.4). Biochemical studies show that Vpu is an integral membrane protein that self-associates to form oligomeric complexes. In infected cells, the protein accumulates in the perinuclear region.

Synthesis of Vpu is required for the proper maturation and targeting of progeny virions and for their efficient release (Table 6.2). In its absence, virions containing multiple cores are produced and budding is targeted to multivesicular bodies rather than to the plasma membrane. Vpu also reduces the syncytium-mediated cytopathogenicity of HIV-1, perhaps because the efficient release of virions prevents the accumulation of sufficient Env protein at the cell surface to promote cell fusion.

The Vpu protein has structural and biochemical features similar to those of the influenza virus M2 protein. As noted in Volume I, Chapter 12, M2 is an ion channel protein that modulates the pH in the Golgi compartment, thereby

protecting the newly formed hemagglutinin protein from changing conformation prematurely in the secretory pathway. Vpu appears to oligomerize in lipid bilayers, forming channel-like structures which are thought to play a role in virion release. In this connection, it is interesting that synthesis of Vpu also enhances the release of virions produced by other retroviruses, such as Moloney murine leukemia virus, and the lentiviruses visna/maedi virus and HIV-2. Recent studies suggest that Vpu enhances virus particle release by counteracting the activity of one or more cellular proteins that trap assembled virus particles at the cell surface, leading to their sequestration in endosomes.

A second function of Vpu is the degradation of CD4. Vpu traps newly formed CD4 receptor in the endoplasmic reticulum via specific interactions with its cytoplasmic domain. In this role Vpu, like Vif and Vpr, acts as an adapter protein that links the receptor to the Scf ubiquitin ligase complex (which includes Cul1, Skp1, and Roc1) mediating the entry of CD4 into the endoplasmic reticulum-associated proteasome degradation pathway. These two activities of Vpu are distinct, as the stimulation of virion release is independent of Env or CD4 expression.

Nef protein. Most laboratory strains of HIV-1 that have been adapted to grow well in T-cell lines contain deletions or other mutations in the *nef* gene. Restoration of *nef* reduces the efficiency of virus replication in these cells, hence the name "negative factor." Multiple functions have been attributed to Nef (Table 6.2 and Fig. 6.7), and it is now clear that Nef does exert pleiotropic effects on infected cells. The functions reported for the Nef protein vary with different strains of the virus and with different cell types.

As noted above, Nef is translated from multiply spliced early transcripts. The 5′ end of Nef mRNA includes two initiation codons, and, as both are utilized, two forms of Nef are produced in infected cells. The apparent size of these proteins can vary because of differences in posttranslational modification. Nef is incorporated into the virion, as with Vpr, via interaction with the p6 domain at the C terminus of the Gag polyprotein. Virion-incorporated Nef appears to contribute to virion disassembly and may also enhance reverse transcription. Nef is synthesized in large quantities after proviral DNA integration. The protein is myristyolated posttranslationally at its N terminus (Fig. 6.5 and 6.6) and thereby anchored to the inner surface of the plasma membrane, probably in a complex with a cellular serine kinase. There are numerous potential threonine and serine phosphorylation sites in Nef, and the protein is phosphorylated, but the significance of such modification is as yet unknown.

Nef includes a protein-protein interaction domain (SH3), which mediates binding to components of intracellular signaling pathways, eliciting a program of gene expression similar to that observed after T-cell activation. Such expression may provide an optimal environment for viral replication. Among the best-studied and possibly most physiologically relevant activities of Nef are its downregulation of surface concentrations of CD4 and major

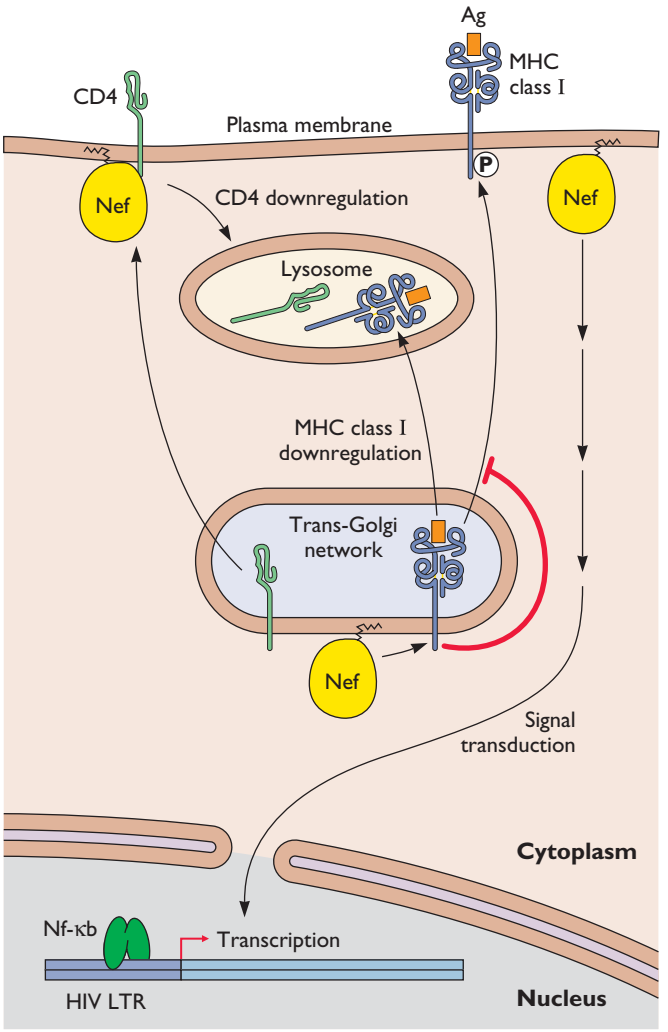


Figure 6.7 Intracellular functions attributed to Nef. Nef is myristoylated posttranslationally; the jagged protrusion represents myristic acid covalently linked to the glycine residue at position 2. Myristoylation enables Nef to attach to cell membranes, where it can interact with membrane-bound cellular proteins. Nef reduces the cell surface expression of CD4 by binding to sequences in the cytoplasmic domain of this receptor and enhancing clathrin-dependent endocytosis and the subsequent degradation of CD4 within lysosomes (left). In contrast, MHC class I expression on the cell surface is reduced by Nef binding in the membrane of the trans-Golgi network. This interaction interferes with the normal vesicular sorting required for passage of the receptor to the cell surface, and MHC class I is directed to the lysosome for degradation (right). Nef also affects signal transduction by increasing the activity of the cellular transcriptional activator Nf-κb and perhaps other cellular transcription proteins.

histocompatibility complex (MHC) class I molecules. As noted above, the former activity is shared with Vpu.

Nef binds to the cytoplasmic tail of CD4 and links this receptor to components of a clathrin-dependent trafficking pathway at the plasma membrane, leading to its internalization and delivery to lysosomes for degradation (Fig. 6.7, left). Reducing the amount of CD4 at the cell surface limits superinfection by HIV-1. It also limits the loss of Env protein via CD4 binding, thereby enhancing infectious-particle production. Nef decreases cell surface expression of MHC class I molecules by a different pathway that involves engagement with these molecules in the *trans*-Golgi network prior to their transport to the cell surface (Fig. 6.7, right). These MHC class 1 molecules are also directed to lysosomes for degradation, reducing their concentration on the cell surface. As a strong cytotoxic T-lymphocyte (CTL) response against viral infection requires recognition of viral epitopes presented by MHC class I molecules, this inhibitory activity of Nef allows infected cells to evade lysis by CTLs and could be a major factor contributing to HIV-1 pathogenesis. Nef-mediated down-regulation of a number of other cell surface molecules such as CD28, the costimulatory molecule for T-cell activation, MHC class II, and CCr5, might also affect the outcome of infection. Many other activities reported for Nef could also contribute to pathogenesis. For example, Nef is reported to enhance interaction and virus transmission between dendritic cells and T cells, and Nef-up-regulation of Fas ligand signaling could protect infected cells by promoting apoptosis in attacking CTLs.

Although the initial cell culture experiments suggested a negative effect on virus production, subsequent experiments with animals showed that Nef augments HIV pathogenesis quite significantly. Rhesus macaques inoculated with a Nef-defective mutant of SIV had low virus titers in their blood during early stages of infection, and the later appearance of high titers was associated with reversion of the mutation. More importantly, adult macaques inoculated with a virus strain containing a deletion of *nef* did not progress to clinical disease and were, in fact, immune to subsequent challenge with wild-type virus. The observation that *nef* had been deleted in HIV-1 isolates from some individuals who remained asymptomatic for long periods and from transfusion recipients who did not develop AIDS also suggests that this viral protein can contribute significantly to pathogenesis. Initial hopes that intentional deletion of *nef* might facilitate the development of a vaccine strain for humans were dashed when it was discovered that the humans infected with *nef* deletion mutants eventually developed AIDS and that newborn offspring of the female macaques that had been immunized with the *nef*-minus strain of SIV developed an AIDS-like disease.

Cellular Targets

As discussed in Volume I, Chapter 5, virus attachment and entry into host cells are dependent on the interaction between viral proteins and cellular receptors. The major receptor for the HIV envelope protein, SU, is the cell surface CD4 molecule. The viral envelope protein must also interact with a coreceptor to trigger fusion of the viral and cellular membranes and gain entry into the cytoplasm. The ability to bind to specific coreceptors is a critical determinant of the cell tropism of different HIV-1 strains. For example, binding to the α-chemokine receptor CXCr4 is a definitive feature of strains that infect T-cell lines. Infection with these strains also causes T cells to fuse, forming syncytia. Binding to the β-chemokine receptor CCr5 is characteristic of non-syncytium-inducing monocyte/macrophage-tropic strains. However, some of these strains can also infect T cells. Strains of HIV that bind to CXCr4 or CCr5 coreceptors are commonly referred to as X4 and R5 strains, respectively. The importance of these two chemokine receptors to HIV pathogenesis is demonstrated by two findings. People who carry a mutation in the gene encoding CCr5, and produce a defective receptor protein, are resistant to HIV-1 infection. So too are individuals who carry a mutation in the gene for the ligand of CXCr4 (Table 3.7). The latter mutation may lead to increased availability of the ligand, which then blocks virus entry by competing for coreceptor binding. This idea is consistent with earlier studies showing that chemokine binding to the receptors inhibits the infectivity of specific strains of HIV in cell culture (Fig. 6.8). Cells of the hematopoietic lineage that bear CD4 and one or more of these chemokine receptors are the main targets of HIV infection, and they produce the highest titers of progeny virions.

Several additional coreceptors for HIV and SIV have been identified in cell culture experiments in various laboratories, but their roles in natural infection remain to be determined. These additional coreceptors may allow the virus to enter a broader range of cells than first appreciated. Some of them are found on cells of the thymus gland and the brain, and they could play a role in infection in infancy or of cells in the central nervous system. It has also been proposed that binding to these additional coreceptors may trigger signals that affect virus replication in target cells, or that harm nonpermissive cells, producing a "bystander" effect.

Cell culture studies have identified additional mechanisms by which HIV may enter cells. For example, the virus can be transmitted very efficiently through direct cell contact. In addition, cells may be infected by virus particles that are endocytosed after binding to cell surface galactosyl ceramide or to Fc receptors (as antibody-virus complexes). HIV can infect many different types of human

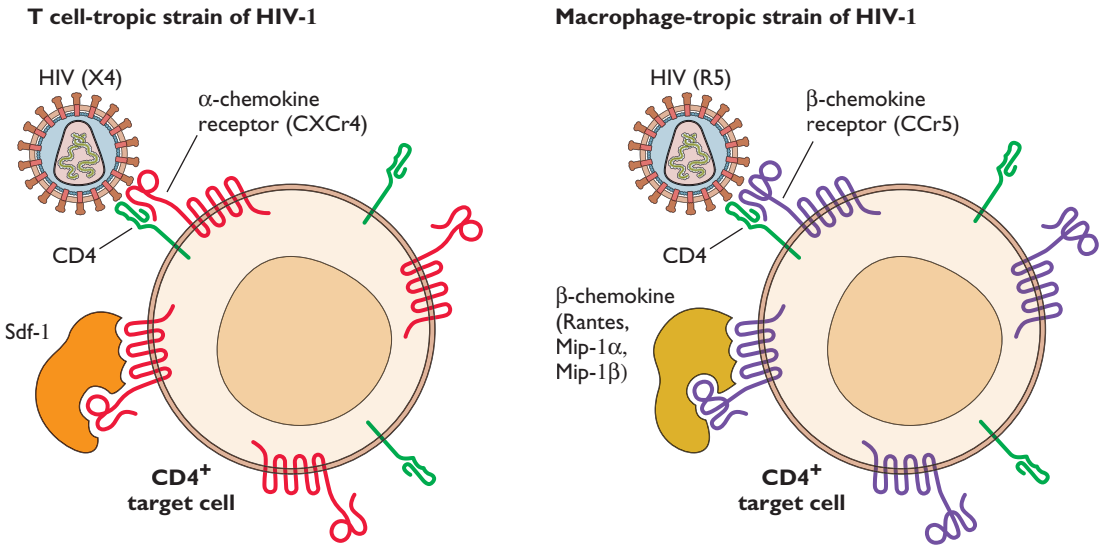


Figure 6.8 Coreceptors for T-cell- and macrophage/monocyte-tropic strains of HIV-1. CXCr4 is the major coreceptor for T-cell-tropic strains; entry of such strains (denoted X4) is inhibited by the receptor's natural ligand, Sdf-1. CCr5 is the major coreceptor for macrophage/monocyte-tropic strains (denoted R5), and their entry is inhibited by the receptor's natural ligands, Rantes, and the macrophage inflammatory proteins Mip-1α and Mip-1β. Primary T cells and monocytes produce both coreceptors; primary T cells are susceptible to both strains, but monocytes can be infected only by M-tropic strains for reasons that are not yet clear. Adapted from Fig. 3 of A. S. Fauci, *Nature* **384:**529–533, 1996, with permission.

cells in culture and has been found in small quantities in several tissues of the body. As discussed below, infection of these cells and tissues is likely to be relevant to HIV-1 pathogenesis in humans.

Routes of Transmission

Sources of Virus Infection

Even before HIV-1 was identified, epidemiologists had established the most likely routes of its transmission to be via sexual contact, via blood, and from mother to child. As might be anticipated, the efficiency of transmission is influenced greatly by the concentration of the virus in the body fluid to which an individual is exposed. Table 6.3 provides estimates of the percentage of infected cells and the concentration of HIV-1 in different body fluids. The highest values are observed in peripheral blood monocytes, in blood plasma, and in cerebrospinal fluid, but semen and female genital secretions also appear to be important sources of the virus.

Other routes of transmission are relatively unimportant or nonexistent, at least for HIV-1; among these are casual nonsexual contact, exposure to saliva or urine from infected individuals, and exposure to blood-sucking insects. Fortunately, HIV-1 infectivity is reduced upon air drying (by 90 to 99% within 24 h), by heating (56 to 60°C for 30 min), by exposure to standard germicides (such as 10% bleach or 70% alcohol), or by exposure to pH extremes (e.g., <6 or >10 for 10 min). This information and results from epidemiology studies have been used to establish safety regulations to prevent transmission in the public sector and in the health care setting.

Modes of Transmission

Modes of HIV-1 transmission vary in different geographic locations. In the United States, the major mode is via homosexual contact, although the number and relative proportion of heterosexual transmissions has increased since the mid-1990s; heterosexual transmission remains the most common world wide (Figure 6.9). A single contact can be sufficient for transmission of the virus if the infected partner is highly viremic. The presence of other sexually transmitted diseases also increases the probability of HIV-1 transmission, presumably because infected inflammatory cells may be present in both seminal and vaginal fluids. Genital ulceration and consequent direct exposure to infected blood cells also increase the likelihood of transmission. In both heterosexual and male homosexual contact, the recipient partner is the one most at risk.

Intravenous drug use is the next most common route of transmission, owing to the common practice of sharing contaminated needles and other drug paraphernalia. Here again, the probability of transmission is a function of the frequency of exposure and the degree of viremia among

Table 6.3 Isolation of infectious HIV-1 from body fluids[a]

Fluid	Virus isolation[b]	Estimated quantity of virus[c]
Cell-free fluid		
Cerebrospinal fluid	21/40	10–10,000
Ear secretions	1/8	5–10
Feces	0/2	None detected
Milk	1/5	<1
Plasma	33/33	1–5,000[d]
Saliva	3/55	<1
Semen	5/15	10–50
Sweat	0/2	None detected
Tears	2/5	<1
Urine	1/5	<1
Vaginal-cervical	5/16	<1
Infected cells		
Bronchial fluid	3/24	Not determined
PBMC	89/92	0.001–1%[d]
Saliva	4/11	<0.01%
Semen	11/28	0.01–5%
Vaginal-cervical fluid	7/16	Not determined

[a]Adapted from Table 2.1 (p. 28) of J. A. Levy, *HIV and the Pathogenesis of AIDS*, 3rd ed. (ASM Press, Washington, DC, 2007), with permission.

[b]Number of samples positive/number analyzed.

[c]For cell-free fluid, units are infectious particles per milliliter; for infected cells, units are percentages of total cells capable of releasing virus. Results from studies in the laboratory of J. A. Levy are presented.

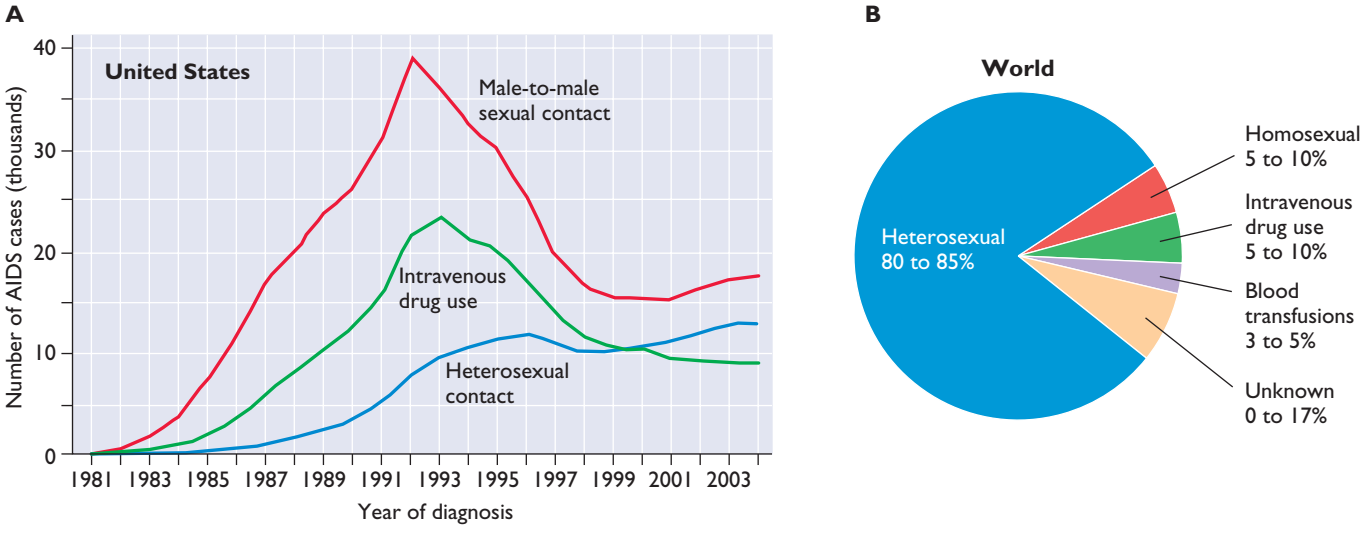
[d]High levels associated with acute infection and advanced disease (~5 × 10^6 PBMCs/ml/of blood).

a drug user's contacts. Of course, sexual partners of drug users are also at increased risk.

Until 1985, when routine HIV antibody testing of donated blood was established in the United States and other industrialized countries, individuals who received blood transfusions or certain blood products, such as clotting factors VIII and IX, were at high risk of becoming infected. Transfusion of a single unit (500 ml) of blood from an HIV-1-infected individual nearly always led to infection of the recipient. Appropriate heat treatment of clotting factor preparations and, more recently, their production by biotechnology have eliminated transmission from this source. This safeguard was small comfort to the many hemophiliacs who contracted the disease before this route was understood. Fortunately, other blood products, such as pooled immunoglobulin, albumin, and hepatitis B vaccine, were not implicated in HIV-1 transmission, presumably because their production methods include steps that destroy the virus.

Transmission of HIV from mother to child can occur across the placenta (5 to 10%) or at the time of delivery as a consequence of exposure to a contaminated genital tract (ca. 15%). The virus can also be transmitted via infected cells in the mother's milk during breast-feeding. Rates of transmission from an infected mother to a child range from as low as 11% to as high as 60%, depending on the severity of infection (i.e., the concentration of virus present) in the mother and the prevalence of breast-feeding (the frequency

Figure 6.9 Modes of transmission of HIV in the United States and worldwide. (A) Comparison of the number of AIDS cases in the United States by major transmission category and year of diagnosis. Adapted from Fig. 1 (p. 591) of Centers for Disease Control and Prevention, *Morb. Mortal. Wkly. Rep.* **55:**589–592, 2006. **(B)** Transmission of HIV worldwide, shown as percentage of cases in each major transmission category. From January 1998 data published by the Joint United Nations Programme on HIV/AIDS.



of the infant's exposure). Administration of antiviral drug therapy during pregnancy is an effective measure to reduce the amount of virus to which the newborn is exposed and, therefore, to reduce the frequency of transmission. Even a single treatment with an antiviral drug early in labor can reduce the incidence significantly. Unfortunately, because of the cost, this is not often an option in underdeveloped countries, where the risk is greatest. Campaigns focused on discouraging breast-feeding by infected mothers, although logical, have actually led to decreased infant survival, primarily because breast-feeding protects against a variety of other infections that are prevalent in these parts of the world. Each year an estimated 700,000 children are newly infected worldwide.

Mechanics of Spread

Except in cases of direct needle sticks or blood transfusion, HIV enters the body through mucosal surfaces, as do most viruses (see Chapter 1). In the case of sexual transmission, the initial target cells in the rectum or genital tract have not yet been identified. The most likely sources of transmission are virus-infected cells, as they can be present in much larger numbers than free infectious virus particles in vaginal or seminal fluids. Results of cell culture studies show that HIV-1 can be transferred directly to $CD4^-$ epithelial cells via cell-cell contact. Whether such transfer is relevant to natural transmission is unknown. Results from analyses of tissue biopsy specimens of bowel mucosae and cervical and uterine epithelia suggest that cells in these layers can be infected in the absence of any injury. As noted in the preceding section, this infection might occur by interaction of the virus with galactosyl ceramide or Fc receptors on the mucosal cells.

The main routes of initial infection are likely to include the acquisition of virus by cells of the mucosal and cutaneous immune system described in Chapter 4; these include M cells, present in the bowel epithelium; dendritic, antigen-presenting $CD4^+$ Langerhans' cells in the vaginal and cervical epithelia; and $CD4^+$ T cells present in the intestinal and genital mucosa. In addition, dendritic cells express a glycoprotein on their surface, called DC-Sign (dendritic-cell-specific, Icam-3-grabbing nonintegrin), that binds the HIV-1 envelope protein with high affinity and can stabilize the virus for several days until it encounters a susceptible T cell (Fig. 6.10). Activated T cells, whose numbers are usually elevated at sites of genital infections that cause lesions, are also likely targets. Although the insertive partner is at relatively low risk for infection, transmission to the male

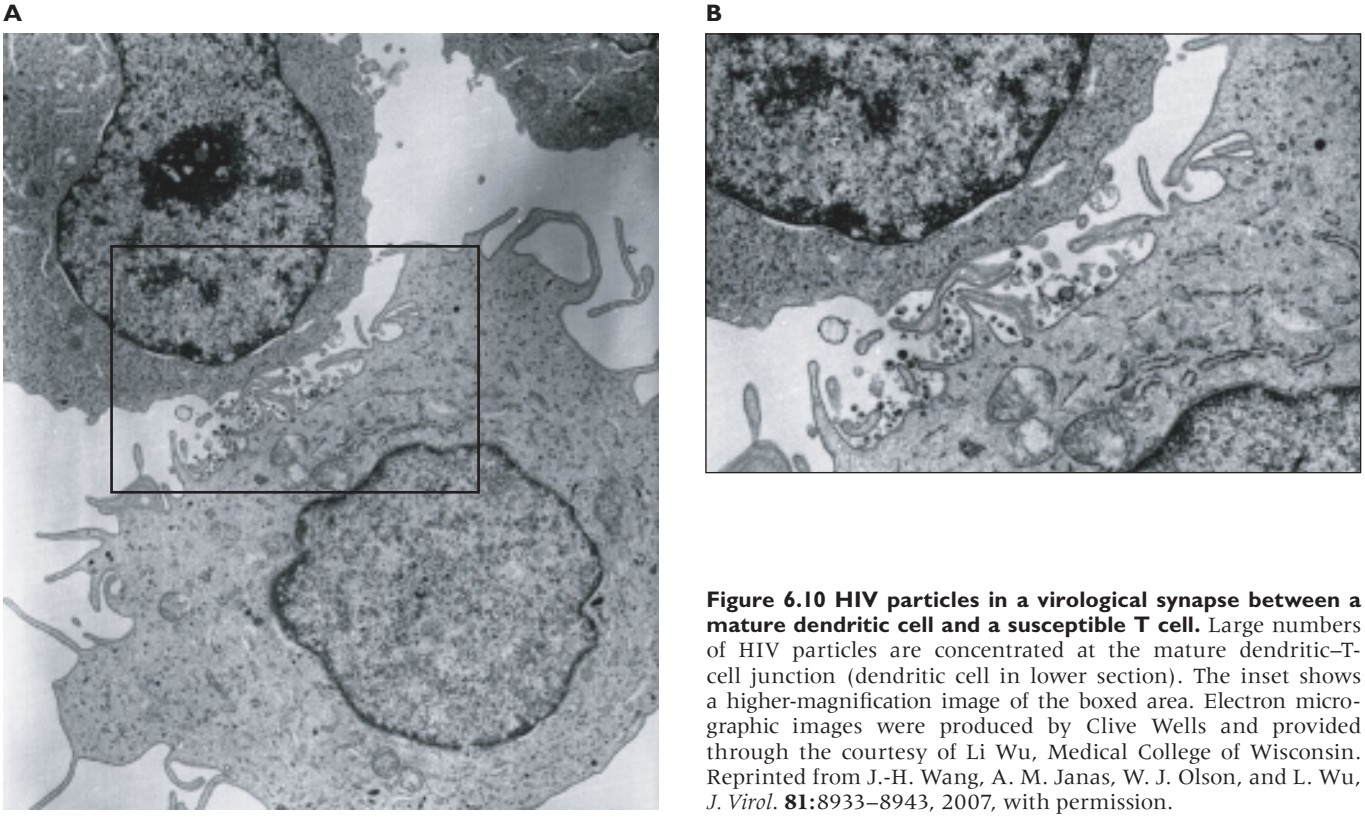


Figure 6.10 HIV particles in a virological synapse between a mature dendritic cell and a susceptible T cell. Large numbers of HIV particles are concentrated at the mature dendritic–T-cell junction (dendritic cell in lower section). The inset shows a higher-magnification image of the boxed area. Electron micrographic images were produced by Clive Wells and provided through the courtesy of Li Wu, Medical College of Wisconsin. Reprinted from J.-H. Wang, A. M. Janas, W. J. Olson, and L. Wu, *J. Virol*. **81:**8933–8943, 2007, with permission.

can occur through cells in the lining of the urethral canal of the penis, presumably from infected macrophages or Langerhans' cells in the cervix or the intestinal mucosa of the infected partner. Uncircumcised males have a twofold-increased risk of infection, suggesting that the mucosal lining of the foreskin may be susceptible to HIV infection. Both male and female hormones appear to facilitate HIV transmission by stimulating cell-cell contact (prostaglandins) or erosion of the vaginal lining (progestin).

Free virus, virus attached to dendritic cells, or virus-infected cells enter draining lymph nodes or the circulatory system, where they encounter the next major targets, namely, susceptible cells that bear the CD4 receptor. Non-activated peripheral blood mononuclear cells are not very permissive for infection, and few activated $CD4^+$ lymphocytes are circulating in the blood at any given time. The first $CD4^+$ cells in the blood to be infected are therefore probably macrophages. The macrophages are in a differentiated state, permissive for viral replication, and can pass progeny virions to activated lymphocytes in the lymph nodes. From this point the infection runs its protracted but usually inevitable course.

The Course of Infection

Patterns of Virus Appearance and Immune Cell Indicators of Infection

Pathological conditions associated with different phases in HIV-1 infection are summarized in Table 6.4.

The Acute Phase

In the first few days after infection, the virus is produced in large quantities by the activated lymphocytes in lymph nodes, sometimes causing the nodes to swell (lymphadenopathy) or producing flu-like symptoms. Virus released into the blood can be detected by infectivity with appropriate cell cultures or by screening directly for viral RNA or proteins (Fig. 6.11). As many as 5×10^3 infectious virions or 1×10^7 viral RNA molecules (i.e., ~5×10^6 particles) per ml of plasma can be found during this stage. During this time, some 30 to 60% of $CD4^+$ T cells in the gut are lost. The memory T cells in this location are most susceptible to infection, and a percentage of the surviving, quiescent memory cells harbor replication-competent proviruses that cannot be transcribed in these cells. They form a long-lived latent viral reservoir. Interaction of such memory cells with their cognate antigens, sometimes many years after the initial HIV infection, will lead to their activation and subsequent transcription of their latent provirus. If antiviral treatments have not been continued, these progeny viruses can initiate a new round of infection.

The initial peak of viremia is greatly curtailed within a few weeks after initial infection, as the susceptible T-cell population is depleted and a cell-mediated immune response is mounted. The number of CTLs increases before neutralizing antibodies can be detected. The inflammatory response that occurs upon primary infection stimulates the production of additional $CD4^+$ T cells, which stems the depletion of this population. The $CD4^+$ T-cell count returns to near normal levels, but these cells represent a

Table 6.4 Pathological conditions associated with HIV-1 infection[a]

Acute phase
Mononucleosis-like syndrome: fever, malaise, pharyngitis, lymphadenopathy, headache, arthralgias, diarrhea, maculopapular rash, meningoencephalitis
Asymptomatic phase
Often none, but patients may present sporadically with one or more of the following symptoms: fatigue, mild weight loss, generalized lymphadenopathy, thrush, oral hairy leukoplakia, shingles
Symptomatic phase and AIDS
200–500 $CD4^+$ T cells/ml
Generalized lymphadenopathy
Oral lesions (thrush, hairy leukoplakia, aphthous ulcers)
Shingles
Thrombocytopenia
Molluscum contagiosum
Basal cell carcinomas of the skin
Headache
Condyloma acuminatum
Reactivation of latent *Mycobacterium tuberculosis*
Fewer than 200 $CD4^+$ T cells/ml
Protozoal infections: *Pneumocystis jiroveci, Toxoplasma gondii, Isospora belli, Cryptosporidium*, microsporidia
Bacterial infections: *Mycobacterium avium-M. intracellulare, Treponema pallidum*
Fungal infections: *Candida albicans, Cryptococcus neoformans, Histoplasma capsulatum*
Viral infections and malignancies: cytomegalovirus, recurrent bouts of oral or genital herpes simplex virus infection, lymphoma (mostly Epstein-Barr virus some human herpesvirus 8), Kaposi's sarcoma (human herpesvirus 8), anogenital carcinoma (human papillomavirus)
Neurological symptoms: aseptic meningitis; myelopathies such as vacuolar myelopathy; pure sensory ataxia, paresthesia/dysesthesia; peripheral neuropathies such as acute demyelinating polyneuropathy, mononeuritis multiplex, and distal symmetric polyneuropathy; myopathy; AIDS dementia complex

[a] Adapted from Table 1 (p. 597) of A. S. Fauci and R. C. Desrosiers, *in* J. M. Coffin et al. (ed.), *The Retroviruses* (Cold Spring Harbor Laboratory Press, Plainview, NY) with permission.

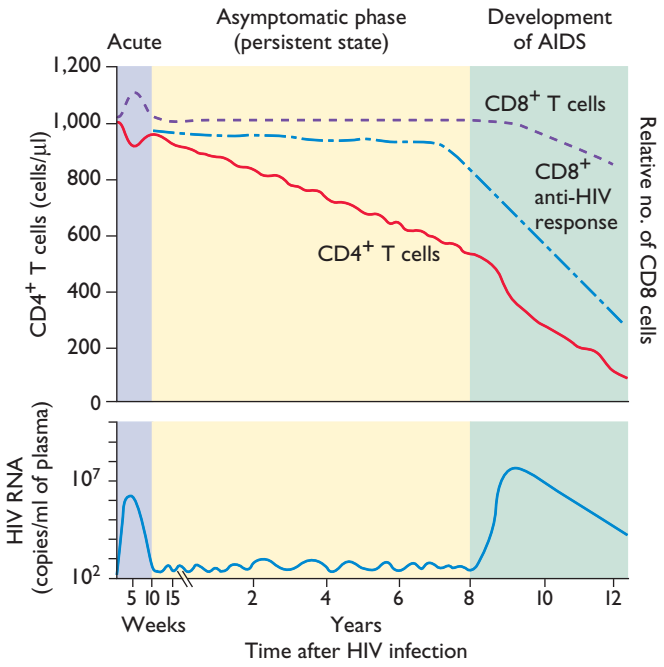


Figure 6.11 Schematic diagram of events occurring after HIV-1 infection. Adapted from Fig. 13.1 of J. A. Levy, *HIV and the Pathogenesis of AIDS*, 3rd ed. (ASM Press, Washington, DC, 2007), with permission.

new source of susceptible cells and their infection produces chronic immune stimulation.

During the period of acute infection, the virus population is relatively homogeneous. In most cases, it appears that the predominant virus was a minor variant in the population present in the source of the infection. These early-stage isolates are generally CCr5-macrophage tropic; the reason for such selective transmission is a topic of intense investigation, as it has great bearing on vaccine development.

The Asymptomatic Phase

By 3 to 4 months after infection, viremia is usually reduced to low levels, with small bursts of virus appearing from time to time. It is known that the degree of viremia at this stage of infection, the so-called **virologic set point**, is a direct predictor of how fast the disease will progress in a particular individual: the higher the set point, the faster the progression. During this time, CD4+ T cell numbers decrease at a steady rate, estimated to be approximately 60,000 cells/ml/year. Direct cytopathogenicity by the virus and apoptosis due to continued immune stimulation and inappropriate cytokine production seem likely explanations. In this protracted asymptomatic period, which can last for years, the CTL level remains slightly elevated, but virus replication continues at a low rate, mainly in the lymph nodes. In lymphoid tissues a relatively large, stable pool of virions can be detected bound to the surface of follicular dendritic cells. Small numbers of infected T cells are also observed. During this asymptomatic phase of persistent infection, known also as **clinical latency**, only 1 in 300 to 400 infected cells in the lymph nodes may actually release virus. It is thought that, as in acute infection, virus propagation is suppressed at this stage by the action of antiviral CTLs. The number of these specific lymphocytes decreases toward the end of this stage. During the asymptomatic phase, the virus population becomes more heterogeneous, probably because of continual selection for specific mutations as a result of immunological pressures (Chapter 4).

The Symptomatic Phase and AIDS

The end stage of disease, when the infected individual develops symptoms of AIDS, is characterized by a CD4+ T-cell count below 200 per ml and increased quantities of virus. The total CTL count also decreases, probably owing to the precipitous drop in the number specific for HIV. In the lymph nodes, virus replication increases with concomitant destruction of lymphoid cells and of the normal architecture of lymphoid tissue. The cause of this lymph node degeneration is not clear; it may be due directly to virus replication or may be an indirect effect of chronic immune stimulation.

In this last stage, the virus population again becomes relatively homogeneous and, generally, CXCr4 T-cell tropic. Properties associated with increased virulence predominate, including an expanded cellular host range, ability to cause formation of syncytia, rapid replication kinetics, and CD4+ T-cell cytopathogenicity. Late-emerging virus also appears to be less sensitive to neutralizing antibodies and more readily recognized by antibodies that enhance infectivity. In some cases, strains that have enhanced neurotropism or increased pathogenicity for other organ systems emerge. Where analyzed, these changes can be traced to specific mutations, for example, in the viral envelope gene or in a regulatory gene (e.g., *tat*).

Variability of Response to Infection

Studies of large cohorts of HIV-1-infected adults show that approximately 10% progress to AIDS within the first 2 to 3 years of infection. Over a period of 10 years, approximately 80% of infected adults show evidence of disease progression and, of these, 50% have developed AIDS. Of the remainder, 10 to 17% are AIDS free for over 20 years; a very small percentage of these individuals are completely free of symptoms, with no evidence of progression to disease. What are the parameters that contribute to such variability?

One parameter is the degree to which an individual's immune system may be stimulated by infection with other

pathogens. HIV-1 replicates most efficiently in activated T cells, and it is known that virus quantities increase when the immune system is activated by opportunistic infections with other microorganisms. Such activation can explain the fact that HIV-1 disease is generally more aggressive in sub-Saharan Africa, where chronic infection by parasites and other pathogens is frequent. As might be expected for an outbred population, variations in an individual's genetic makeup can modulate the immune response to infection and affect survival. As already noted, differences in chemokines or chemokine receptors and, probably, in any one of several components of the immune system can have an impact on the course of the disease. Genomic analyses are beginning to identify polymorphisms associated with variations in viral load in infected individuals, and, as might be expected, major histocompatibility alleles are among the genes affected.

Clearly, accumulation of mutations in the genomes of the virus also influences the course of the disease. As noted above, some long-term survivors of HIV-1 infection harbor viruses with deletions in the *nef* gene. Others appear to be infected with otherwise attenuated strains that produce low titers in cultured cells and have restricted cell tropism. Low virus titers and the presence of viral-neutralizing and not virus-enhancing antibodies are other characteristics of these infections. Further study of the rare, fortunate individuals who are nonprogressors or long-term survivors of HIV-1 infection should lead to a better understanding of viral pathogenesis and may suggest new strategies for effective prevention or therapy.

Origins of Cellular Immune Dysfunction

The defining feature of HIV disease is impaired immune cell function. This defect is eventually devastating because these defenses are vital in the body's battle against this virus as well as other pathogens. At first the immune system appears to keep the HIV infection in check. However, the virus is not eliminated, and the infection that persists in the asymptomatic stage leads to increasing dysfunction among immune cells. In the end, most AIDS patients actually succumb to opportunistic infections with microorganisms that are little threat to individuals with healthy immune systems. The impairment in immune cell function typical of HIV-1 disease results from direct attack of the virus on particular cell types and from the response of uninfected cells to viral gene products or specific proteins made in infected or stimulated cells (Table 6.5).

$CD4^+$ T Lymphocytes

The major reservoir of HIV in the peripheral blood is the $CD4^+$ T cell. Even before the profound depletion of these cells that is a signal of end-stage disease, abnormalities in $CD4^+$ T-cell functions can be detected. These abnormalities include decreased ability to form colonies when grown in tissue culture, decreased expression of the cytokine interleukin-2 (IL-2) and its receptor, and reduced proliferative response to various antigens. The precise causes of these impairments are unknown. Some loss of function can be explained by direct killing of cells. In addition, as surface expression of CD4 is reduced by some viral proteins (SU, Vpu, and Nef), MHC class II-CD4 interactions become less frequent, leading to a decrease in antigen-specific responses. Sequestration of infected $CD4^+$ lymphocytes in lymphoid organs may account for some of the decline. Destruction of infected $CD4^+$ lymphocytes in the circulation by anti-HIV-1 CTLs also reduces their total number. Noninfectious virions and viral proteins (e.g., Tat or SU) shed from infected cells can bind to or enter uninfected $CD4^+$ lymphocytes, triggering inappropriate responses. For example, HIV-1 envelope and Tat proteins have been reported to suppress proliferative responses of T cells to antigens and other mitogens. It is possible that SU bound to CD4 interferes sterically with its interaction with MHC class II molecules. Such binding may also disturb the normal signaling that must take place during antigen-specific responses. Exposure to HIV-1 SU protein also inhibits production of IL-2 and its receptor by uninfected T cells. Finally, changes in cytokine production by HIV-1-infected macrophages can trigger apoptosis in uninfected $CD4^+$ T cells.

Cytotoxic T Lymphocytes

As mentioned above, the number of CTLs is abnormally high following the acute phase and decreases precipitously during the end stage of the disease. The early increase may be the result of an imbalance brought about as the immune system attempts to achieve homeostasis of $CD8^+$ and $CD4^+$ cells while $CD4^+$ cells are being destroyed. The reduced numbers of anti-HIV-1 CTLs at late times can be explained in part by the direct infection and killing of their progenitors. Additionally, and most importantly, because CTL proliferation and function depend on inductive signals from $CD4^+$ T cells (see Fig. 4.6), the decline of the $CD4^+$ population also contributes to CTL dysfunction.

Monocytes and Macrophages

HIV-1-infected macrophages can be detected readily in tissues throughout the body of an infected individual. However, as only a small proportion of monocytes/macrophages in the blood are infected with the virus, it seems likely that the functional impairment seen in this population of cells is due to indirect effects. Monocyte/macrophage abnormalities include defects in chemotaxis, inability to promote T-cell proliferation, and defects in Fc receptor function and complement-mediated clearance. Results of cell culture

Table 6.5 Immune cell dysfunction associated with onset of AIDS

Affected cell type	Known or postulated causes		
	Dysfunction	Direct	Indirect
$CD4^+$ T cells	Colony formation ↓ Proliferative response to antigen ↓ Expression of IL-2 and IL-2R ↓ Total number in circulation ↓	Direct killing by HIV-1 CD4 is down-regulated in infected cells (by SU, Vpu, Nef) Trapping of infected cells in lymphoid organs Destruction of infected cells by anti-HIV-1 $CD8^+$ CTLs	SU component of noninfectious virions binds to cell surface CD4, suppresses immune response, and causes inappropriate intracellular signaling
$CD8^+$ T cells	Abnormally large numbers following acute phase Loss of anti-HIV CTL activity Loss in numbers toward end stage	Infection and killing of progenitor $CD4^+/CD8^+$ and $CD8^+$ immature thymocytes	Attempt of the immune system to establish homeostasis results in higher production of $CD4^+$ **and** $CD8^+$ cells as $CD4^+$ cells are depleted Loss of IL-2 production as $CD4^+$ pool is depleted
Monocytes (dendritic cells)/ macrophages	Defects in chemotaxis Monocyte-dependent T-cell proliferation ↓ Antigen-presenting cell activity ↓ Fc receptor function ↓ Complement C3 receptor-mediated clearance/ oxidative burst ↓ Decrease in numbers	Only a few circulatory cells are infected, but changes in cytokine production by such cells (e.g., Tnf-α; IL-1 ↓) can cause apotosis of $CD4^+$ cells and other abnormalities	Exposure to noninfectious virions (SU and TM), down-regulates chemotactic ligand receptors and causes abnormal secretion of certain chemokines Decreased expression of costimulatory molecules (e.g., B7) Cells killed by eliciting cytotoxic response in CTLs
B cells	Abnormal proliferation Hypergammaglobulinemia Poor response to additional antigen signals Production of autoantibodies		Exposure to noninfectious virions (TM) causes polyclonal activation Loss of $CD4^+$ T-cell helper function Molecular mimicry of host proteins by viral proteins
NK cells	NK cytotoxicity function ↓		Loss of IL-2 as $CD4^+$ pool is depleted

experiments suggest that some of these effects are caused by exposure to the viral envelope protein.

B Cells

HIV-1-infected individuals produce abnormally large quantities of immunoglobulin G (IgG), IgA, and IgD. Such production is indicative of B-cell dysfunction that may result from increased proliferation of cells of the lymph nodes. Infected individuals also show poor responses to primary and secondary immunization. During end-stage disease, there may also be a decrease in the total number of B cells. Some of the B-cell loss may be attributable to the decline in $CD4^+$ T-cell-helper function (see Fig. 4.6). B cells isolated from infected individuals proliferate in culture without stimulation and are also defective in their response to specific antigens or mitogens. Binding of viral proteins (e.g., TM) induces polyclonal B-cell activation, a property that might explain such apparent spontaneous proliferation. Infection by Epstein-Barr virus and human cytomegalovirus, common in AIDS patients, may also contribute to abnormal B-cell function.

Natural Killer Cells

Impairment of natural killer (NK) cell function is observed throughout the course of HIV-1 infection, becoming more severe during end-stage disease. As NK cell cytotoxicity depends on IL-2, these abnormalities may be a consequence of impaired $CD4^+$ T-cell function and the reduced production of this cytokine. The reduction in NK cell function cripples the innate immune response to infection by other microorganisms.

Autoimmunity

Because of the imbalance of the immune system, T-cell dysfunction, and abnormal B-cell activation described above, it is not surprising that immune disorders, such as a breakdown in the system's ability to distinguish self from nonself, accompany HIV-1 infection. In early studies,

antibodies against platelets, T cells, and peripheral nerves were detected in AIDS patients. Subsequently, autoantibodies to a large number of normal cellular proteins have been found in infected individuals (Table 6.6). The specific reason for the appearance of such antibodies is not clear, but their production might be stimulated in part by cellular proteins on the surface of viral particles or by viral proteins, regions of which may resemble cellular proteins (called molecular mimicry) (Chapter 4 and Table 6.7).

Immune Responses to HIV

Humoral Responses

Antibodies to HIV-1 can be detected shortly after acute infection, sometimes as early as a few days after exposure to the virus but generally within 1 to 3 months. These antibodies, which are secreted into the blood and are present on mucosal surfaces of the body, can be detected in genital and other body fluids. This phenomenon has been exploited in the design of home kits for detecting anti-HIV antibodies in the blood or urine. Among the various isotypes, IgG1 antibodies are known to play a dominant role at all stages of infection, giving rise to an antibody-dependent cellular cytotoxicity response, complement-dependent cytotoxicity, and neutralizing and blocking responses (Fig. 6.12; Chapter 4). Levels of other classes of antibody may vary at different clinical times, but there is no known correlation between the isotype and the clinical stage of disease.

Neutralizing antibodies are likely to play a role in limiting viral replication during the early, asymptomatic stage of infection. However, the titers of these antibodies are generally very low. This property is consistent with recent structural analyses of the HIV-1 envelope protein, which indicate that many neutralizing epitopes are hidden from the immune system. The low titer may favor selection of resistant mutants. Indeed, many individuals produce antibodies that neutralize earlier virus isolates but not those present at the time of serum collection, suggesting effective immune "escape" by the virus. Some studies show loss of neutralizing antibodies with progression to AIDS, but the clinical relevance of such antibodies during the later stages of infection remains obscure.

Neutralizing antibodies generally bind to specific sites on the viral envelope complex, SU-TM. Variable region 3 (V3) in HIV-1 SU is one of the initial targets. Anti-V3 antibodies appear to block coreceptor interactions that occur after the virus attaches to the CD4 receptor. Because of the high sequence variation within the V3 loop (hence the name "variable"), neutralizing antibodies to this region are usually strain specific. Consequently, despite its relatively strong antigenicity, V3 is not a good target for the development of vaccines or broadly specific antiviral drugs. Members of another class of neutralizing antibodies block the binding of SU to the CD4 receptor. These antibodies bind to numerous conserved sites on SU and usually react with many strains of HIV-1. Other conserved or variable regions on both SU and TM can be targets for neutralizing antibodies. Broad neutralizing activity against carbohydrate-containing regions of the viral envelope protein has also been detected. Even less well understood is the relative importance of antibodies to other proteins on the surface of virions, such as the adhesion molecules Lfa-1 and Icam. Antibodies to these cell surface molecules inhibit the formation of syncytia following infection, perhaps because antibody-treated cells are less likely to form aggregates. In some instances, these antibodies seem to

Table 6.6 Some autoantibodies detected in HIV infection[a]

Antibodies to:	Associated clinical condition
CD4	$CD4^+$ cell loss
Cellular components (Golgi complex, centriole, vimentin)	Immune disorder
Erythrocytes	Anemia
HLA	Lymphocyte depletion
Lymphocytes	Loss of $CD4^+$, $CD8^+$, and B lymphocytes
Myelin basic protein	Dementia, demyelination
Nerves (myelin)	Peripheral neuropathy
Neutrophils	Neutropenia
Nuclear protein (antinuclear antibody)	Autoimmune symptoms
Platelets	Thrombocytopenia
Sperm, seminal plasma	Aspermia

[a]Adapted from Table 10.4 (p. 253) of J. A. Levy, *HIV and the Pathogenesis of AIDS*, 3rd ed. (ASM Press, Washington, DC, 2007), with permission.

Table 6.7 Some regions of HIV that resemble normal cellular proteins and exhibit cross-reactivity[a]

Normal cellular protein	HIV protein(s)
Astrocyte protein	MA, TM
Brain cell protein	SU (V3 loop)
Epithelial cell protein	MA
Neuroleukin (phosphohexose)	SU
Platelet glycoprotein	SU
Platelet protein	CA
Thymosin	MA, TM
Vasoactive intestinal polypeptide	SU (peptide T)

[a]Adapted from Table 10.6 (p. 255) of J. A. Levy, *HIV and the Pathogenesis of AIDS*, 3rd ed. (ASM Press, Washington, DC, 2007), with permission.

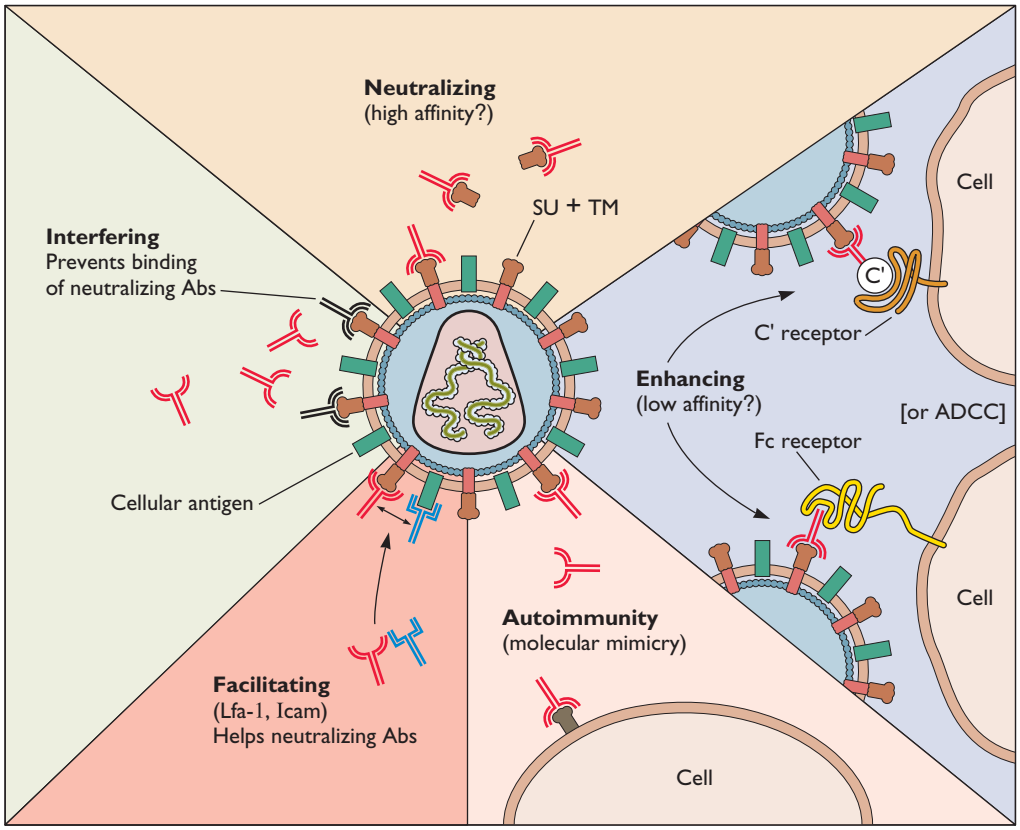


Figure 6.12 Antibody (Ab) responses to HIV infection. A summary of the various responses described in the text is presented. "Cellular antigen" refers to cellular membrane proteins that are incorporated in the virus envelope. One idea is that the relative affinities of the antibodies may be critical. According to this hypothesis, high-affinity antibodies neutralize the virus by binding tightly to SU, causing it to become detached from the virion; low-affinity antibodies bind to SU but not tightly enough to cause its detachment. Conformational changes in SU that might occur as a consequence of such low-affinity binding would then facilitate viral entry. C', complement; ADCC, antibody-dependent cellular cytotoxicity.

enhance neutralization of HIV-1 by antiviral antibodies (Fig. 6.12, bottom left).

Antibodies called **interfering antibodies** can bind to virions or infected cells and block interaction with neutralizing antibodies (Fig. 6.12, left). Others, called **enhancing antibodies**, can actually facilitate infection by allowing virions coated with them to enter susceptible cells (Fig. 6.12, right). In complement-mediated antibody enhancement, the complement receptors Cr1, Cr2, and Cr3 play a critical role in attaching virion-antibody complexes to susceptible cells. In Fc-mediated enhancement, attachment is via Fc receptors that are abundant not only on monocytes/macrophages and NK cells but also on other human cell types. As HIV-1 has been shown to replicate in cells that lack CD4 but express an Fc receptor, binding to the CD4 receptor is probably not required for Fc-mediated enhancement.

It is noteworthy that the same cellular receptors (for complement and Fc) are implicated in infection enhancement and the antibody-dependent cellular cytotoxicity response. In the case of enhancement, the receptors allow antibody-coated virions to enter susceptible cells bearing such receptors. In the case of antibody-dependent cellular cytotoxicity, such receptors on CTLs, NK cells, or monocytes/macrophages mediate the recognition and killing of antibody-coated infected cells.

Both neutralizing and enhancing antibodies recognize epitopes on SU and TM. Consequently it has been difficult to identify the features that specify either response. Indeed, polyclonal antibodies against SU possess both neutralizing and enhancing activities, and it has not been possible to decide how either effect might predominate. This idea and the distinctions between other classes of anti-HIV-1 antibodies are summarized in Fig. 6.12.

The clinical importance of antibody-dependent enhancement is uncertain, but the fact that circulating infectious virus-antibody complexes have been described suggests that this phenomenon may contribute to HIV-1 pathogenesis. In addition, infectivity-enhancing antibodies have been demonstrated in individuals who progress to disease. Results from studies of other lentiviral infections, as well as infections with other viruses (dengue viruses, coronaviruses, and others), show a correlation between increased symptoms of disease and increased quantities of enhancing antibodies. These observations, together with the finding that enhancing antibodies have been found in individuals who were vaccinated with Env from a different HIV-1 strain, certainly complicate strategies for the development of an effective vaccine.

The Cellular Immune Response

Antigen-specific cellular immune responses include activities of CTLs and T-helper cells. The great majority of CTLs are $CD8^+$, and their general role in limiting or suppressing viral replication is discussed in Chapter 4. CTLs can also block virus replication by producing cellular antiviral factors, including an unidentified protein called Caf, chemokines, and small peptides called defensins, that suppress transcription of viral genes.

CTLs programmed to recognize virtually all HIV-1 proteins have been detected in infected individuals. As noted above, there is a direct correlation among a good CTL response, a low virus load, and slower disease progression. Furthermore, a broadly reactive response appears to correlate with a less fulminant course of disease. End-stage disease is characterized by a rapid drop in the number of anti-HIV-1 CTLs. Results of animal studies with severe combined immunodeficient (SCID) mice that have been reconstituted with human lymphoid cells show that adoptive transfer of human anti-HIV-1 CTLs provides some protection against subsequent challenge with HIV. These findings all demonstrate a significant role for such lymphocytes in fighting HIV-1 disease. Why, then, do these CTLs eventually fail to control infection? While the answer to this most pressing question is not yet known, it seems quite likely that much of the failure is due to reduced cytokine expression from infected and dysfunctional $CD4^+$ T cells (Table 6.5). However, the T-cell receptor may not be able to recognize all viral peptides as the viral population becomes more diverse, or the CTLs may simply respond inadequately in the face of high rates of virus production in AIDS patients.

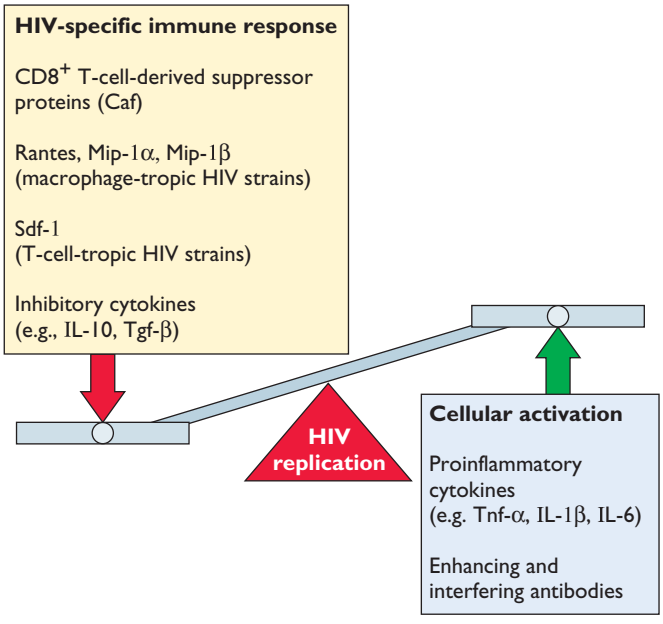
Summary: the Critical Balance

HIV-1 replication is controlled by what may be thought of as a finely balanced scale that can be tipped in either direction by a number of stimulatory or inhibitory host proteins. Among these, various cytokines can have important but opposing effects. Immune responses and the production of specific chemokines can inhibit virus replication, whereas immune cell activation and certain antibodies can be stimulatory (Fig. 6.13). The challenge of modern medicine has been to find practical interventions that will tip the balance in favor of patients' survival.

Dynamics of HIV-1 Replication in AIDS Patients

The availability of potent drugs that block HIV-1 replication by inhibiting the activity of the viral enzymes reverse transcriptase and protease made it possible to measure the dynamics of virus production in humans. Clinical studies performed with patients near end stage, whose $CD4^+$ T-cell counts are in decline (500 or fewer per ml), have revealed the magnitude of the battle between the virus and the immune system in HIV-1 disease. Within the first 2 weeks of treatment with a combination of these drugs, an exponential decline in viral RNA levels in the plasma is observed; this is followed by a second, slower decline. The initial drop represents clearance of free virus and loss of virus-producing $CD4^+$ lymphocytes from the blood. The most important contributor to the second drop is presumed to be the loss of longer-lived infected cells, such as tissue

Figure 6.13 Control of HIV-1 replication and the progress of HIV-induced disease by the balance of host proteins. Cellular activation and proinflammatory cytokines stimulate viral replication. These are counterbalanced by inhibitory proteins. Adapted from Fig. 4 of A. S. Fauci, *Nature* **384:**529–533, 1996, with permission.

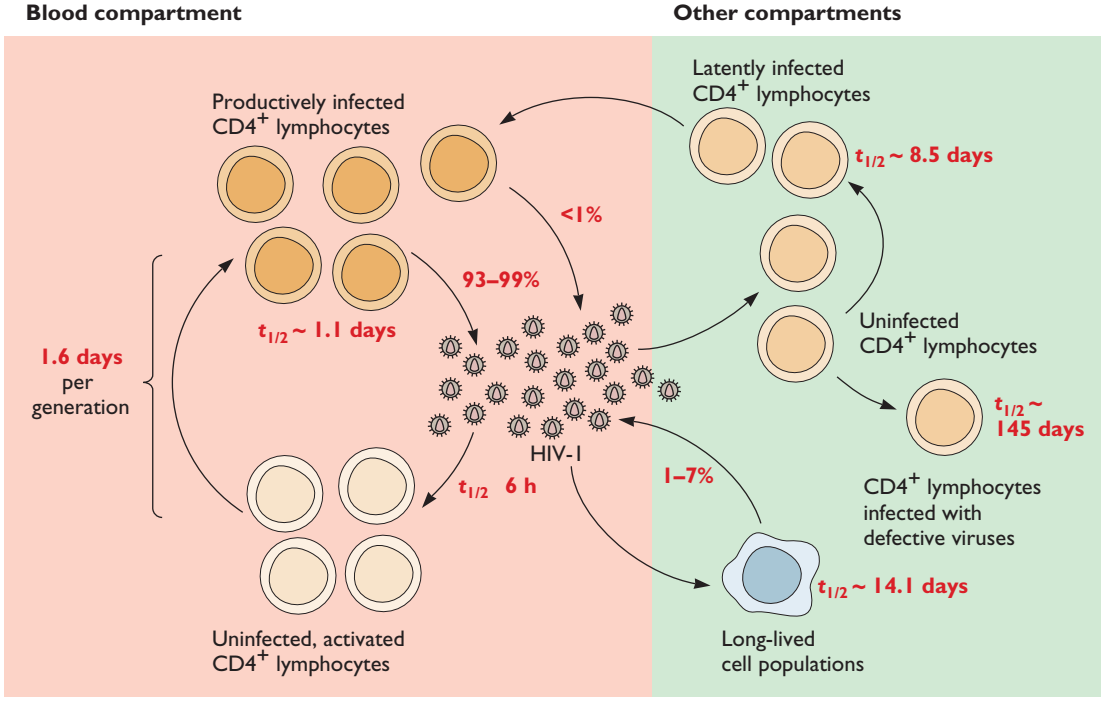


macrophages and dendritic cells, with a minor contribution from the clearance of latently infected, nonactivated T lymphocytes. The existence of the latter population, comprising infected $CD4^+$ T cells that have returned to a quiescent state, is the major barrier to eradication of the virus by treatment with antiviral drugs.

At steady state in the absence of drugs, the rate of virus production must equal the rate of virus clearance. Mathematical analyses of the data from clinical studies can therefore provide estimates of the rates of HIV-1 production in the blood and other compartments of the body, as well as the rate of loss of virus and virus-infected cells. The results calculated for HIV-1 production are nothing less than astonishing. The minimal rate estimated for release into the blood is on the order of 10^{10} virions per day. This minimal number computes to approximately 1 cycle per infected cell per day. Continuous high-replication capacity is undoubtedly the principal engine that drives viral pathogenesis at this stage. Because of the high mutation rate of HIV-1, on average every possible mutation at every position in the genome is predicted to occur numerous times each day. Based on the high rates of mutation and virus propagation, it has been estimated that the genetic diversity of HIV produced in a single infected individual can be greater than the worldwide diversity of influenza virus during a pandemic. This enormous variation and the continuous onslaught of infection must present a prodigious challenge to the immune system.

As illustrated in Fig. 6.14, more than 90% of the virus particles produced in the blood come from infected $CD4^+$ lymphocytes that have average half-lives of only ca. 1.1 days. A smaller percentage, approximately 1 to 7%, comes from longer-lived cells in other compartments, with half-lives from 8.5 to 145 days. Consequently, even if *de novo* synthesis of virus can be blocked **completely** by drug treatment, it could take approximately 3 to 5 years of treatment before these longer-lived compartments are free of cells with the potential to produce virus. Sadly, this can be considered only a minimal estimate. Complete eradication may not be possible, as some proviruses may still lurk in undetected "sanctuary" compartments, including long–lived quiescent memory T cells, or at sites that are not readily accessible to drugs, such as the brain. As all potent HIV-1 inhibitors produce some undesirable

Figure 6.14 Summary of kinetics of HIV-1 production in the blood and other compartments. The percentages indicate the relative quantities of virus calculated to be produced in blood plasma by the various cell populations illustrated. The average time in days for 50% of the cells in each population to be destroyed or eliminated is indicated as the half-life ($t_{1/2}$). The average time in hours (h) for 50% of the virus to be eliminated from the plasma ($t_{1/2}$) is also shown. Adapted from Fig. 1 of D. D. Ho, *J. Clin. Investig.* **99:**2565–2567, 1997, with permission.



side effects, long-term treatment will be a problem for many patients.

The other dramatic change that takes place in patients treated with antiviral drugs is a resurgence of the $CD4^+$ lymphocyte count in the blood. From the initial rates of recovery, it has been calculated that during an ongoing infection, as many as 4×10^7 of these cells are replaced in the blood each day. Lymphocytes in the peripheral blood comprise a relatively small fraction (ca. 1/50) of the total in the body, and lymphocyte trafficking, homing, and recirculation are complex processes. It is still uncertain whether such $CD4^+$ lymphocyte replacement following drug treatment represents new cells or a redistribution from other compartments. If one assumes that the increase in the circulation is proportional to the total, then as many as 2×10^9 new $CD4^+$ T cells are produced each day. This estimate is controversial because it seems to exceed significantly the normal proliferative capacity of these cells. However, some studies suggest that HIV-1-infected individuals who are treated with potent drug combinations do produce new $CD4^+$ T cells at rates higher than normal. Perhaps increased rates of both synthesis and redistribution contribute to the observed resurgence in $CD4^+$ T cells in the periphery.

Long-term studies of patients treated with highly active antiretroviral therapies (HAART) should help to clarify some of these issues. Some analyses indicate that a simple inverse correlation between virus load and number of $CD4^+$ cells is unlikely to exist. For example, in certain patients the virus reappears after 6 months, reaching levels near those recorded before treatment, even though $CD4^+$ T-cell counts remain high. Whether this situation can be explained by a change in the genetic makeup of virus populations or some sort of reactivation of the immune system remains to be determined.

Effects of HIV on Different Tissues and Organ Systems

Lymphoid Organs

The majority of the body's lymphocyte pool resides in lymphoid tissue (Chapter 4). Because these lymphocytes exist in a different environment from those in the periphery, their response to viral infection may also be different.

The function of these tissues is to filter invading pathogens, such as HIV, and to present them to immunocompetent cells. The majority of the viruses in lymph nodes are trapped within the germinal centers that comprise networks of follicular dendritic cells with long interdigitating processes that surround lymphocytes. Follicular dendritic cells also trap antibodies and complement and present antigens to B cells. The process of cellular activation that takes place in these tissues could make resident and recruited lymphocytes permissive for HIV-1 replication. Lymph nodes appear to contain a much larger percentage of virus-infected cells than the peripheral blood does.

Early in infection the germinal centers in lymph nodes appear to remain intact, although there is some proliferation of activated immune cells (Fig. 6.15). Early in the asymptomatic stage, infected individuals often have palpable lymphadenopathy at two or more sites as a result of follicular dendritic cell hyperplasia and capillary endothelial cell proliferation. Later, during an intermediate stage of disease ($CD4^+$ counts of 200 to 500 per ml), the nodes begin to deteriorate, there is evidence of cell death, and the trapping efficiency of the follicular dendritic cells declines. At a more advanced stage of disease (<200 $CD4^+$ cells per ml), the architecture of the lymphoid tissue is almost completely destroyed and the follicular dendritic cells disappear (Fig. 6.15). At this point, there is also a significant increase in the level of HIV-1 in the peripheral blood. Finally, because the lymphoid tissue is no longer able to mediate an immune response, there is an increased incidence of opportunistic infections.

The Nervous System

Approximately one-third of AIDS patients are diagnosed with neurological disorders at some time during the course of their disease. HIV-1 enters the nervous system early after infection, either as free virions in plasma or via infected monocytes or lymphocytes from the bloodstream. The most prevalent disorder associated with infection of the brain is subacute encephalitis, also called AIDS dementia complex; nearly two-thirds of all HIV-1-infected individuals ultimately develop AIDS dementia. The disease progresses slowly over a period of up to 1 year, but mean survival time from the onset of severe symptoms is less than 6 months. Several AIDS-associated opportunistic infections can also produce neurological damage, and neurological abnormalities in infected individuals can be caused by lymphomas of the central nervous system (Table 6.8).

In general, brain-derived isolates of HIV-1 are macrophage-tropic and distinct from blood-derived strains from the same individual. Results of viral genome sequencing suggest that the brain isolates represent a distinct lineage selected for neurotropism. The precise mechanisms by which these isolates cause neuronal abnormalities and damage are not known. In animal lentivirus infections, central nervous system disease is usually caused by direct infection of glial cells, astrocytes, and possibly brain macrophages called microglia. Infection of astrocytes and microglia also appears to be important in HIV-1 pathogenesis of the central nervous system. The virus can also be detected in brain capillary endothelial cells and has been shown to infect such cells in culture. Plausible hypotheses for how

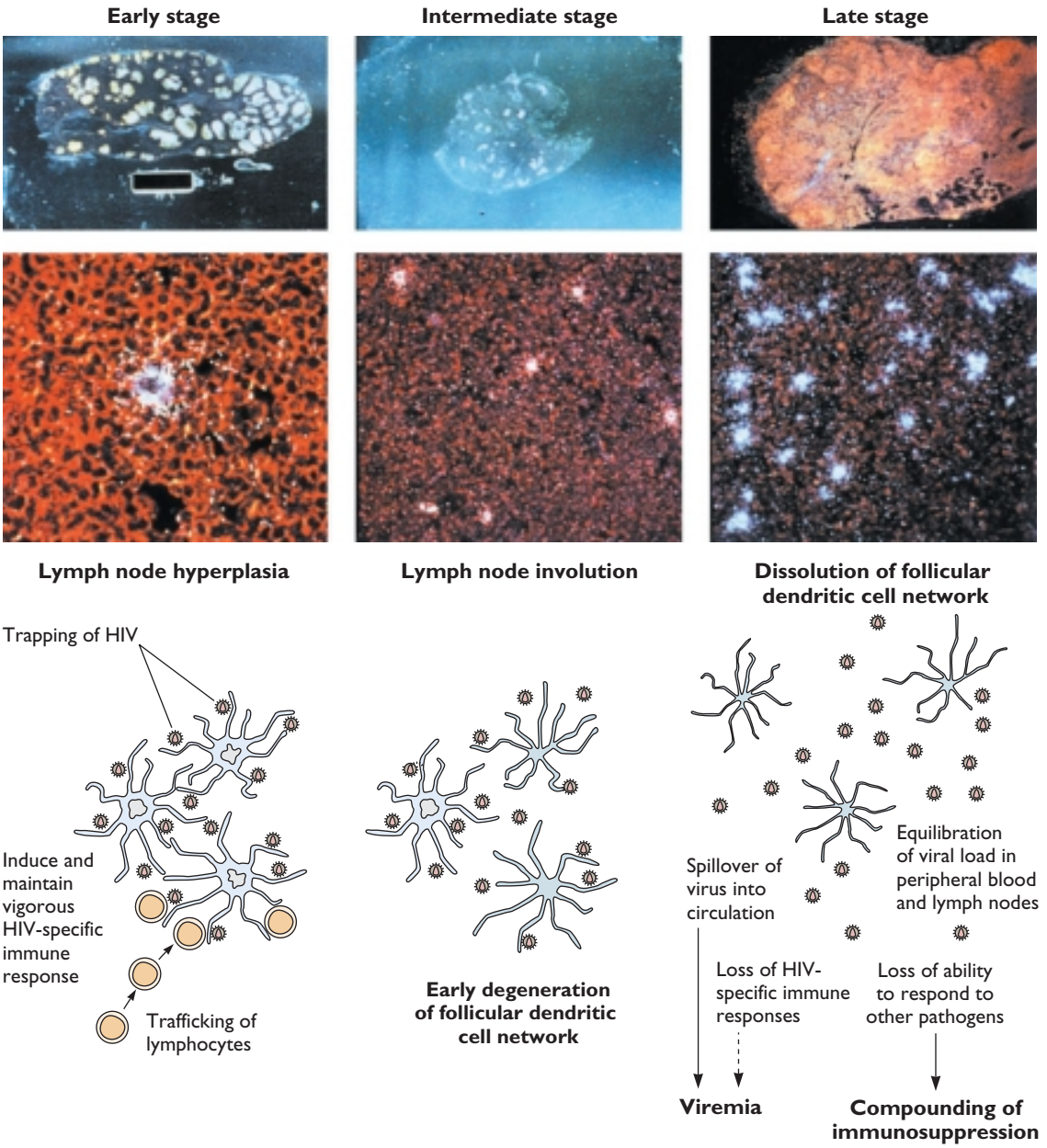


Figure 6.15 Effects of HIV-1 infection on lymphoid tissue. (Top) Changes in lymph node germinal centers as determined by selective staining (above) and by the location of viral replication, as blue-white dots, in lymph node tissue (below). HIV-1 replication was detected by polymerase chain reaction (PCR) procedures. The examples illustrate conditions in the early, intermediate, and late stages of HIV infection when connective tissue replaces much of the normal cell population. **(Bottom)** Events that take place in lymph node germinal centers during various stages of HIV-1 disease (see text). (Top) Reprinted from J. A. Levy, *HIV and the Pathogenesis of AIDS*, 3nd ed. (ASM Press, Washington, DC, 1998), with permission. (Bottom) Adapted from Fig. 10 of A. S. Fauci and R. C. Desrosiers, p. 587–635, *in* J. M. Coffin et al. (ed.), *The Retroviruses* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1998), with permission.

Table 6.8 Major neurological disorders associated with HIV-1 infection[a]

Disorder	Prevalence (%)	Clinical features
Opportunistic infections		
Toxoplasma gondii	10	Focal seizures, altered consciousness, CT ring-enhancing lesions
Cryptococcus neoformans	4–8	Headache, confusion, seizures, CSF cryptococcal antigen
Progressive multifocal altered leukoencephalopathy	1–4	Limb weakness, gait abnormalities, visual loss, mental state, CT-magnetic resonance imaging white matter lesions
Lymphoma	5–10	Diplopia, weakness, encephalopathy, CT contrast-enhancing lesions
HIV-1-related subacute encephalitis	90	Cognitive deficits, memory loss, psychomotor slowing, pyramidal tract signs, ataxia, weakness, depression, organic psychosis, incontinence, myoclonus, seizures
Peripheral neuropathies	10–50	
Chronic distal symmetric polyneuropathy		Painful dysesthesias, numbness, paresthesia, weakness, autonomic dysfunction
Chronic inflammatory myelinating polyneuropathy		Weakness, sensory deficits, mononeuropathy multiplex, cranial nerve palsies, hyporeflexia or areflexia, CSF pleocytosis
Vacuolar myelopathy	11–22	Gait ataxia, progressive spastic paraparesis, posterior column, deficits, incontinence
Aseptic meningitis	5–10	Headache, fever, meningeal signs, cranial nerve palsies, CSF pleocytosis

[a]Adapted from Table 4 of M. S. Hirsch and J. Curran, p. 1953–1995, *in* B. N. Fields et al. (ed.), *Fields Virology*, 3rd ed. (Lippincott-Raven, Philadelphia, PA, 1991), with permission.

[b]CSF, cerebrospinal fluid; CT, computed tomography.

infection of the central nervous system is initiated are shown in Fig. 6.16. As HIV-1 does not appear to infect neurons, direct replication is unlikely to explain their loss. It is more probable that the release of toxic cellular products and viral proteins (e.g., SU, TM, Nef, Rev, and Tat) from infected macrophages or microglial cells is responsible for the damage to neurons (Fig. 6.17). There is ample evidence from cell culture experiments that these viral proteins could contribute to neuropathogenesis. In addition, transgenic mice expressing HIV-1 *env* under the control of neuronal promoters show abnormalities in astrocytes, dendrites, and neurons similar to those seen in HIV-1-infected individuals. A role for HIV-1 proteins in neuropathogenesis is further supported by the observation that antiviral therapy can reduce the symptoms of AIDS dementia significantly in some patients.

The Gastrointestinal System

The more advanced stages of HIV disease are often associated with damage to the gastrointestinal system. Diarrhea and chronic malabsorption, with consequent malnourishment and weight loss, are frequently observed. In Africa, this has been called "slim disease." In some cases, the disorders are associated with opportunistic infections with other microbial agents, including human cytomegalovirus and herpes simplex viruses. Nevertheless, in cases where no opportunistic agent can be identified, HIV-1 itself may be the primary cause of gastrointestinal pathogenesis. As in the central nervous system, the tissue macrophage appears to be a major target cell of HIV-1 replication in the gastrointestinal system. HIV-1-inflicted damage is thought to result from virus replication, toxic effects of viral proteins, and/or indirect destructive effects of certain cytokines.

Other Organ Systems

HIV has been found in the lungs of patients with pneumonia, in the hearts of some with heart muscle dysfunction (cardiomyopathy), in the kidneys of some with renal injury, and in the joint fluid of patients with arthritis. It also has been identified in the adrenal glands of infected individuals. The role of the virus in these pathologies is not clearly understood. Opportunistic infection of the lungs is common; *Pneumocystis jiroveci*, a protozoan parasite that is usually dormant in the host lung, causes pneumonia in approximately 50% of AIDS patients. Other microorganisms, most notably *Mycobacterium tuberculosis, Mycobacterium avium,* fungi, and human cytomegalovirus, may also cause pulmonary infections. The known effects of SU on membrane permeability or other toxic effects of viral proteins might explain some of the lung damage and the electrophysiological abnormalities associated with heart disease. Direct infection of endothelial cells or other cells in the kidneys has been proposed as a potential cause of tubular

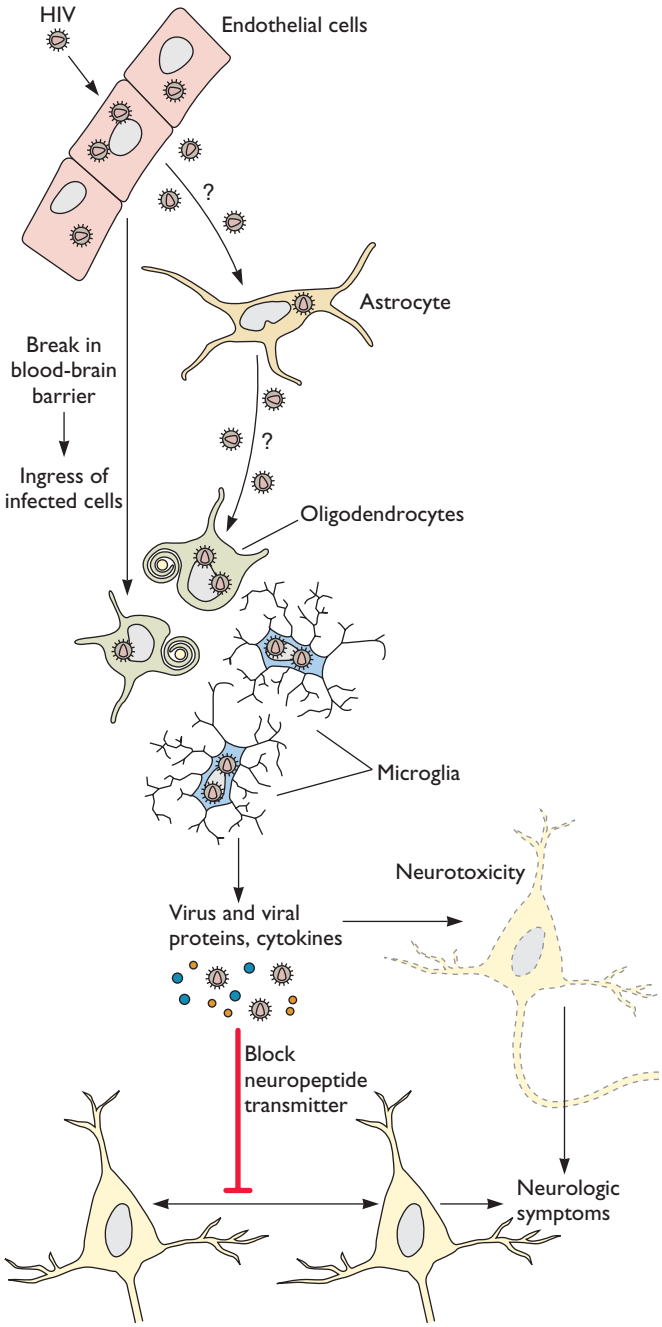


Figure 6.16 HIV-1 neuropathogenesis. It is proposed that the virus infects capillary endothelial cells of the brain and passes into the brain via the basolateral surface of these cells. Infection of both these cells and, perhaps, astrocytes could lead to a breakdown in the blood-brain barrier and ingress of infected T cells and macrophages or free virus from the blood. The ultimate result is an infection of other brain cells (e.g., oligodendrocytes and microglia). Production by these cells of HIV-1 and viral proteins ensues, as does the release of various cytokines and other cellular products. These products could lead to an interruption of cell-to-cell transmission by blocking the production of neurotropic factors. Direct infection of cells, as well as high levels of viral proteins (e.g., SU, TM, Tat, and Nef) and cytokines could lead to direct neurotoxicity through detrimental effects on the cell membrane. Neurotropic viral mutants that replicate well in microglia might later trigger the production of more virus, as well as harmful cytokines and other neurotoxic products. Adapted from Fig. 9.11 in J. A. Levy, *HIV and the Pathogenesis of AIDS*, 2nd ed. (ASM Press, Washington, DC, 1998), with permission.

destruction. Deposition of antigen-antibody complexes could also account for some kidney damage.

HIV and Cancer

HIV-1 infection leads to an increased incidence of neoplastic malignancies: some form of cancer eventually occurs in approximately 40% of infected individuals. The mechanism of oncogenesis in this case is quite different from that of other retroviruses, which can be explained either by direct viral transduction of oncogenes or by activation of cellular oncogenes or inactivation of tumor suppressor genes following retroviral DNA integration (Chapter 7). HIV-associated oncogenesis is an indirect effect of disregulation of the host's immune system. Contributory factors probably include the absence of proper immune surveillance directed against other (oncogenic) viruses or transformed cells. Also, high levels of cytokine production associated with HIV infection might induce inappropriate proliferation of cells, activate replication of oncogenic viruses, and promote the generation of blood vessels (angiogenesis) in developing tumors. Indeed, cancers that develop in HIV-infected individuals generally are more aggressive than those in uninfected individuals. These malignancies can develop in a number of tissues and organs, but certain types, such as Kaposi's sarcoma and B-cell lymphoma, are especially prevalent in AIDS patients. It may be important that in these cases the neoplastic cells are derived from the immune system. The endothelial cells thought to give rise to Kaposi's sarcoma can act as accessory cells in lymphocyte activation. One reasonable hypothesis is that proliferation of endothelial cells, B cells, and the epithelial cells that give rise to carcinomas may be promoted by cytokines produced by immune cells. As discussed in this section, many malignancies that develop in HIV-1-infected individuals are associated with infection by oncogenic viruses.

Kaposi's Sarcoma

Kaposi's sarcoma was first described by the Hungarian physician Moritz Kaposi in 1872. It is a multifocal cancer that differs from more common tumors in that the lesions contain many cell types; the dominant type is a called a spindle cell, thought to be of endothelial origin. The tumors contain infiltrating inflammatory cells and

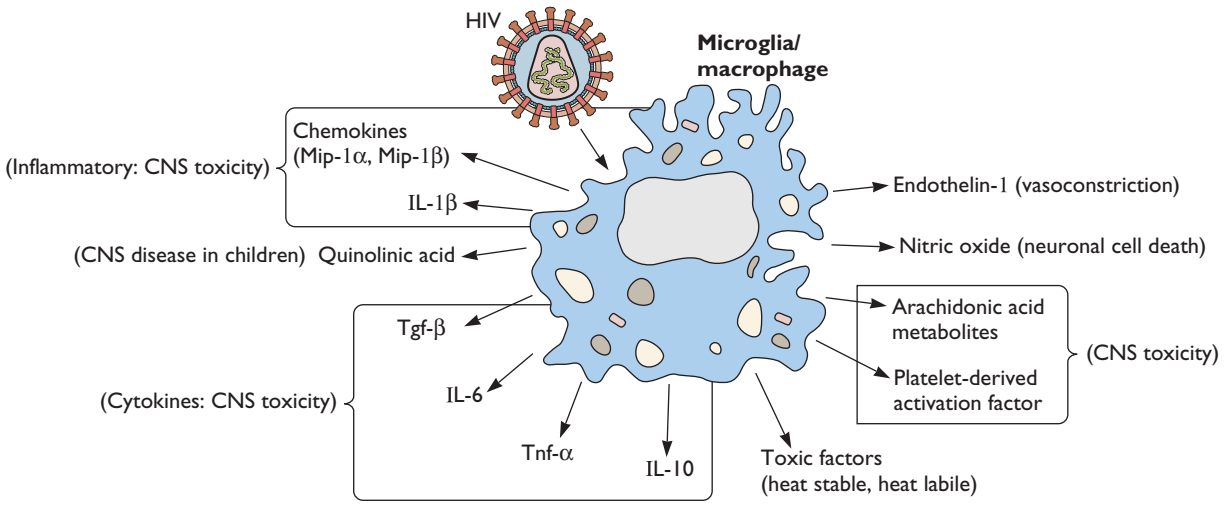
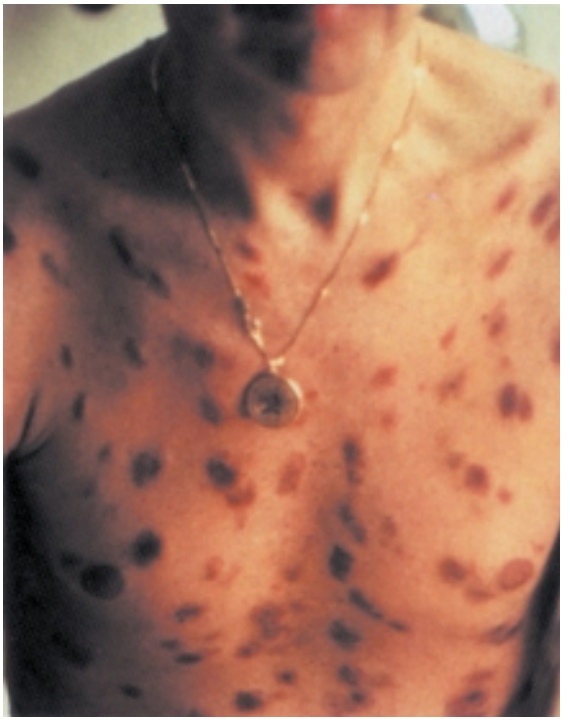


Figure 6.17 The role of infected microglia/macrophages in neural pathogenesis. After exposure to HIV or to viral envelope proteins, microglia/macrophages may be induced to produce cytokines and the macrophage inflammatory chemokines that can be toxic to cells of the central nervous system (CNS).

many newly formed blood vessels. Kaposi's sarcoma was typically found in older men from the Mediterranean rim region and eastern Europe. In these areas, and in immunocompetent individuals, Kaposi's sarcoma normally appears in a nonaggressive (classical) form confined to the skin and extremities, and is rarely lethal. The classical form, as well as a more aggressive, sometimes lethal form, is found in sub-Saharan Africa, where there are more immunocompromised individuals. In HIV-1-infected men, Kaposi's sarcoma appears in the more aggressive form, affecting both mucocutaneous and visceral areas. This disease occurs in about 20% of HIV-1-infected homosexual men (Fig. 6.18) but in only about 2% of HIV-1-infected women, transfusion-infected recipients, and blood product-infected hemophiliacs in the United States. This distribution suggests a specific type of sexual transmission.

Spindle cell cultures established from Kaposi's sarcoma tumors are not fully transformed according to the criteria defined in Chapter 7, but they do produce a variety of proinflammatory and angiogenic proteins. It is thought that these products are responsible for recruiting the other cell types in these tumors. Spindle cells from AIDS patients are also not infected with HIV-1, and the disease occurs in individuals who are not infected with the virus. HIV cannot therefore be the sole factor in the development of Kaposi's sarcoma. Epidemiologic studies suggested that another sexually transmitted virus was the inducing agent. Subsequently, a new member of the gammaherpesviruses was found to

Figure 6.18 Kaposi's sarcoma in a young man infected with HIV-1. Note the distribution of the lesions, suggesting lymphatic involvement. Reprinted from Color Plate 19 of J. A. Levy, *HIV and the Pathogenesis of AIDS*, 3rd ed. (ASM Press, Washington, DC, 2007), with permission. Photo courtesy of P. Volberding.



be associated with Kaposi's sarcoma. This new virus, called human herpesvirus 8, can infect spindle cells. The results of *in situ* hybridization studies show RNA transcripts from this virus in the vast majority of Kaposi's sarcoma lesions, irrespective of the presence or absence of HIV-1. Human herpesvirus 8 can also infect B cells and has been linked to certain AIDS-associated B-cell lymphomas (Fig. 6.19).

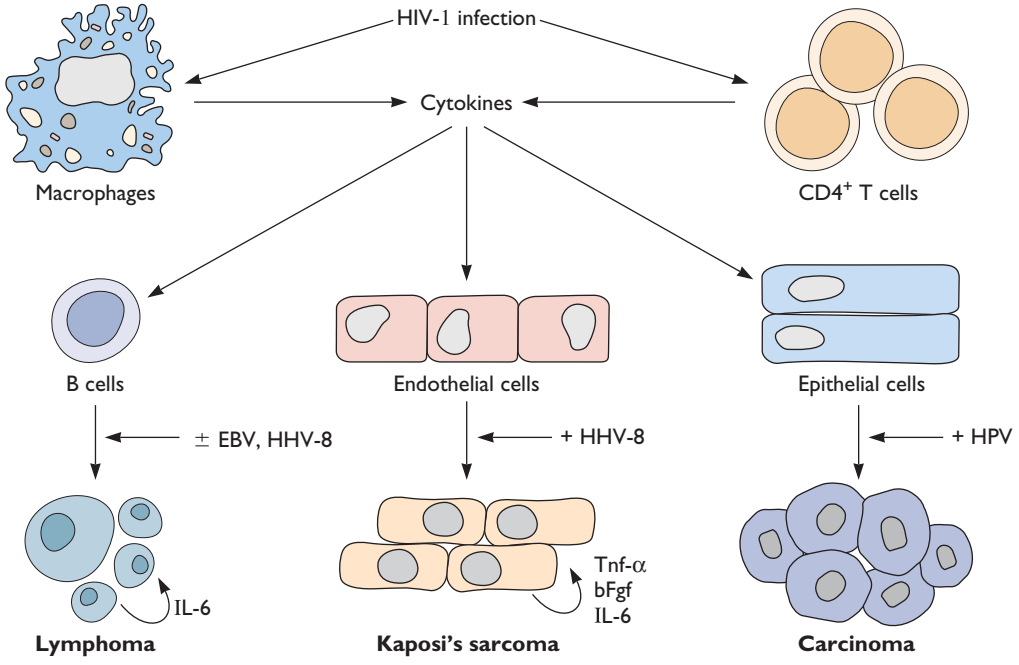
The evidence that infection with human herpesvirus 8 is necessary for the development of Kaposi's sarcoma is quite strong. Antibodies to this virus can be found in up to 6% of the general population but in 25% of the population in classic areas of endemic infection, indicating widespread exposure. Nevertheless, very few people develop this disease. Other parameters are therefore likely to be important in its etiology. The immune deficiency associated with HIV-1 is certainly one explanation for its prevalence in AIDS patients, and synthesis of viral proteins, such as Tat, may be another. The exact role of human herpesvirus 8 in Kaposi's sarcoma remains to be elucidated. The viral genome includes a number of open reading frames with sequence homology to genes encoding cellular proteins known to be important in growth control, cell signaling, and immunoregulation (see Fig. 7.12).

It is ironic that one of the first reports of AIDS in 1981 described seven patients with Kaposi's sarcoma. While HIV-1 was identified soon afterward, the discovery of human herpesvirus 8 did not occur until many years later even though both viruses were present and contributed to the pathology in these first patients.

B-Cell Lymphomas

B-cell lymphomas are 60 to 100 times more common in AIDS patients than in the general population. The incidence is especially high among patients whose survival has been prolonged by anti-HIV-1 drugs. Tumors can be found in many locations, including lymph nodes, the intestine, the central nervous system, and the liver. B-cell lymphomas in the peritoneal or other body cavities, called primary effusion lymphomas or body cavity-based lymphomas, are almost always associated with human herpesvirus 8. Both polyclonal and monoclonal B-cell lymphomas are found in the central nervous system, with monoclonal types being more common. Epstein-Barr virus is found in all AIDS-associated primary lymphomas in the brain. Why these two B-cell lymphoma-inducing herpesviruses show such site specificity remains unknown. On the other hand,

Figure 6.19 Induction of cancers in HIV-1 infection. Infection of macrophages, $CD4^+$ T cells, or other cells leads to the production of cytokines that can enhance the proliferation of certain other cells, such as B cells, endothelial cells, and epithelial cells. The enhanced replication of these cells, as a result of either cytokine production or subsequent viral infection, could lead to eventual development of the malignancies noted. In some cases, such as B-cell lymphomas and Kaposi's sarcoma, ongoing cytokine production by the tumor cells maintains the malignant state. Adapted from Fig. 12.12 of J. A. Levy, *HIV and the Pathogenesis of AIDS*, 3rd ed. (ASM Press, Washington, DC, 2007), with permission.



approximately 60% of the tumors outside the brain show no evidence of infection with either herpesvirus, indicating that B-cell transformation in HIV-1-infected individuals does not require infection with such viruses.

Lymphomas may arise because of the destruction of germinal centers in the lymphatic system. Lysis of antigen-presenting follicular dendritic cells would render B cells less sensitive to normal apoptotic processes, allowing them to live longer and to replicate. Epstein-Barr virus latent membrane protein 1 (see Table 7.8 and Fig. 7.15) also inhibits apoptosis. Proliferation of B cells as a result of production of cytokines by macrophages or $CD4^+$ T cells (Fig. 6.19), or even some viral proteins (e.g., SU or TM), may also play a role in this process. Uncontrolled proliferation, by whatever mechanism, could lead to the chromosomal changes required for cell transformation and malignancy.

Anogenital Carcinomas

Anogenital carcinomas are two to three times more frequent in HIV-1-infected individuals than in the general population. They are associated with human papillomavirus infections that are typically spread through sexual contact. The high-risk human serotypes 16 and 18 are associated with both cervical and anal carcinomas. Such cancers often arise in areas of squamous metaplasia near the glandular epithelium and reach more advanced stages in immunosuppressed individuals. The proposed mechanism of transformation is binding and inactivation of the tumor suppressor proteins p53 and Rb by the viral proteins E6 and E7, respectively (Chapter 7), or mutation in the p53 tumor suppressor gene in cases where a human papillomavirus is not involved.

Prospects for Treatment and Prevention

Antiviral Drugs and Therapies

Antiviral drugs and therapies are discussed in a broader context in Chapter 9. Some of the more clinically relevant details are summarized here. The first drug to be licensed in the United States for the treatment of AIDS was the nucleoside analog inhibitor of the viral reverse transcriptase zidovudine (AZT). AZT is converted to the triphosphate form after being taken up into cells. On the basis of laboratory experiments, it appears to act both as a competitor of normal nucleoside triphosphates and as a chain terminator when incorporated into viral DNA by reverse transcriptase. Effective treatment of HIV-infected patients with AZT was limited by the rapid appearance of resistant mutants. However, AZT, lamivudine (3TC, another nucleoside analog), or nevirapine (a nonnucleoside inhibitor) can be used as prophylactic treatment for pregnant woman to reduce infection of newborns. Indeed, a brief course of administration of such drugs has proven both effective and economical for this purpose in developing countries. Antiviral drugs have also been used after needle stick injuries and other types of acute exposure to the virus.

Highly Active Antiretroviral Therapy

The use of powerful antiviral drugs has had an enormous impact on AIDS in the developed world. As illustrated in Figure 6.9A, the incidence of AIDS in the United States has declined since its peak in the early 1990s, and the incidence of death has subsequently decreased fourfold. In addition, as treated patients experience a significant restoration of their immune cell function, it has been possible to withdraw prophylactic or suppressive therapies for a wide variety of opportunistic microorganisms. For maximal effectiveness, these antiviral drugs are given in combinations, as has proven advantageous in anticancer therapy. The most dramatic decrease in viral RNA levels in the blood of AIDS patients has been seen with triple-drug therapy in which two anti-reverse transcriptase drugs and one antiprotease drug are combined (often called HAART). As is often the case in science and medicine, the development of inhibitors for both enzymes benefited partly from serendipity. AZT had been developed initially as a potential anticancer drug, and the earliest protease inhibitors were derivatives of renin inhibitors being developed as antihypertensive drugs. In addition, the firm base of both structural and biochemical knowledge available from research with these enzymes was a great advantage in developing this first group of drugs and is still being exploited to design the next generations of improved drugs. Encouraging progress has been made recently with the introduction of inhibitors to the third retroviral enzyme, integrase.

Many patients treated with HAART sustain levels of fewer than 50 copies of HIV RNA per ml of plasma (the lower limit of detection) for years. But closer examination of tissues and blood shows that virus replication is not completely suppressed in such patients. Instead, they experience intermittent virus replication that is difficult to detect. In some patients, the emergence of drug-resistant virus hinders subsequent treatment. As drugs may have significant side effects, noncompliance is a large factor in treatment failure. Newly developed therapies and treatment regimens may help to minimize this problem in the future.

Other Approaches for Antiviral Agents

Many steps in the HIV-1 replication cycle offer opportunities for possible intervention (see Fig. 9.4); a number have been or are currently being exploited. One obvious candidate is the third retroviral enzyme, integrase. Structural data

and biochemical information about this protein are available (Volume I, Chapter 7), and potent inhibitors of its activity have been approved for clinical use. Drugs that disrupt the Zn^{2+} finger domain of the viral nucleocapsid protein, NC, may also have utility. Finally, inhibitors of HIV fusion that target either the viral envelope protein or the host cell chemokine coreceptors are available or in clinical trials.

Eventually, treatments must be found that can cope with proviruses in inaccessible tissues and in long-lived cells, including quiescent lymphocytes that are invisible to the host immune system because no viral products are made. For example, to accomplish a cure via drug therapy, it will be necessary to develop drugs that can penetrate the blood-brain barrier and enter the central nervous system to block replication of virus at this and other "privileged" sites. Development of drugs that promote the activation of latent proviruses, such as those in long-lived $CD4^+$ memory T cells, could help to eliminate this reservoir by exposing viral antigens on the surface of these cells so that they would now be recognized by the immune system. Activated memory T cells that harbor replication-competent proviruses would die (Fig. 6.4), and replication of progeny virus could be blocked with existing antiviral drugs. However, solving the problems associated with these inaccessible and long-lived reservoirs presents formidable challenges, and a "cure" for HIV infections seems far from the horizon.

Immune System-Based Therapies

Because immunization seems to be protective against subsequent SIV infection of rhesus macaques, treatment strategies that combine HAART with augmentation of HIV-specific immunity have been proposed. One approach, called strategic treatment interruption, is based on the idea that temporary interruption of HAART will allow some virus replication and the release of HIV antigens that can provide autoimmunization. The rationale is that several cycles of such interruption might augment antiviral immunity sufficiently to alter the course of the disease. Another approach is to deliver HIV immunogens while the patient is being maintained on HAART. Thus far, trials with strategic treatment interruptions have shown little benefit to chronically infected individuals, but studies with both approaches are continuing.

Prophylactic Vaccine Development To Prevent Infection

The most potent defense against viral infection is a vaccine, a topic that is discussed in more detail in Chapter 8. For HIV-1, vaccine development presents unique challenges, not the least of which is the fact that the virus infects the immune system. In addition, the correlates of protective immunity to HIV are still not well defined (Box 6.4). Other confounding issues are the large genetic diversity and high mutation rates of the virus and the danger of producing an inappropriate immune response that enhances infection. Finally, animal models of HIV that are faithful to the human disease are not available for testing vaccine candidates. The SIV-macaque model has been useful, and the discovery and cloning of HIV-1 coreceptors has made it possible to design promising transgenic mouse models. Ultimately, however, any protective vaccine must be tested in humans. Here there are many societal and ethical issues to be considered. At present, the best prophylaxis for people at risk is to adjust their behavior to avoid infection.

Despite the many problems, there is hope that an effective vaccine can be developed, and several new global initiatives have brought new resources, energy, and focus to this enterprise. Some encouragement can be drawn from the cross-protection against HIV-1 obtained in HIV-2-infected individuals and from other lentiviral systems, in which inoculation with killed cell-free viruses has been shown to induce protection against high-dose virus challenges. Some individuals may have better innate defenses that abrogate any primary infection. Other genetic traits that confer protection are mutations in specific chemokines or chemokine coreceptors. In addition, as established by a study of a

BOX 6.4 BACKGROUND

The correlates of protective immunity against HIV-1 have not been well defined

Vaccine development requires a deep understanding of the specific immune responses essential for protection against HIV-1.

Is the humoral response essential? Must the antibodies be neutralizing or should they be able to activate killer cells? Is mucosal immunity in regions such as the rectum and vagina essential? Are T cells essential, and, if so, which kind?

The answers to many of these questions will dictate not only the nature of the vaccine but also the manner and site of its administration.

Possible requirements for protective immunity against HIV-1 infection[a]

Cytotoxic lymphocyte activity ($CD4^+$ and $CD8^+$ cells)
Innate immune responses (plasmacytoid dendritic cells and $CD8^+$ cell noncytotoxic antiviral response)
NK cell activity
Antibody-dependent cellular cytotoxicity
Neutralizing antibodies

[a]Adapted from Table 15.2 of J. A. Levy, *HIV and the Pathogenesis of AIDS*, 3rd ed. (ASM Press, Washington, DC, 2007), with permission.

group of Kenyan and Gambian prostitutes who remained uninfected despite continuous exposure to HIV-1, particular MHC class I alleles are associated with protection. Such protection may signify that peptides presented by these MHC molecules specify strongly immunogenic regions of HIV-1 proteins. The identification of such peptides might present a fruitful approach for vaccine development. Another promising approach is based on detailed analysis of human neutralizing antibodies that target different features of the viral envelope, which can provide new rationales for vaccine design.

In some instances in which prior infection with HIV appears to offer protection, the infecting virus may be defective or the dose of infecting virus may be such that the immune system is activated and the infection is cleared. Long-term protection against SIV infection in adult macaques was achieved by immunization with a live attenuated virus carrying a deletion of the *nef* gene. Unfortunately, as discussed above, immunized female animals were found to bear infected infants that succumbed to the attenuated virus. Whether a live attenuated vaccine will ever be acceptable for humans is doubtful. However, it is important to appreciate that an AIDS vaccine need not be 100% effective to be useful. With such a deadly disease, even partial protection that might spare 20 to 40% of potential victims would extend millions of lives and reduce transmission significantly.

There are several AIDS Vaccine Evaluation Units in the United States that can carry out initial vaccine trials. However, these studies are limited by the relatively small number of potential participants in this country. To determine efficacy properly, vaccines have to be tested in large, high-incidence populations. Such trials will require international collaboration, extensive testing in developing countries where such populations exist, and preparation of vaccines that target the predominant clade in the region of study. Such efforts are under way, and it is hoped that some of them will be successful in the future.

Perspectives

> *Pneumocystis* Pneumonia—Los Angeles. In the period October 1980–May 1981, 5 young men, all active homosexuals, were treated for biopsy-confirmed *Pneumocystis carinii* pneumonia at three different hospitals in Los Angeles, California. Two of the patients died. . . .
>
> M. S. Gottlieb et al. (Centers for Disease Control)
> *Morb. Mortal. Wkly. Rep.* **30:**250–252, 1981

So began the first warning, soon echoed in large urban centers throughout the United States and Europe, where physicians were being confronted by a puzzling and ominous new disease that was killing young homosexual men. In a deceptively low-key editorial note with this report, it was observed that "*Pneumocystis* pneumonia is almost exclusively limited to severely immunosuppressed patients," that "the occurrence of the disease in these five previously healthy individuals is unusual," and that "the fact that these patients were all homosexuals suggests an association between some aspect of a homosexual lifestyle or disease acquired through sexual contacts . . ." Soon the disease was to ravage this group and also the hemophiliac community, whose lives depended on blood products. This state of distress led to unprecedented social activism that demanded significant public investment for AIDS research and put patient advocates on scientific review panels for the first time.

Initial progress in AIDS research was impressive. The etiological agent of the disease was identified within 2 years, and screening assays to safeguard the blood supply were developed shortly thereafter. As a broad base of knowledge about retroviruses already existed, it seemed that treatment and a vaccine should soon be available. Unfortunately, almost 3 decades later, AIDS is still an emerging epidemic with a death toll that rises every year, and about 16,000 people are newly infected each day. As is the case for most sexually transmitted diseases, efforts at prevention are fraught with difficulty and complicated by societal factors. Although a godsend to some, the treatments that have been developed to date are expensive, difficult to tolerate, and unavailable to most of the world's victims. And an effective vaccine is not yet on the horizon. Why has the initial optimism been so frustrated?

A major difficulty in combating HIV is the fact that the virus infects the immune system, destroying the delicate balance needed for that system to function properly. The pathology is complex, and the physician is forced to battle not only HIV but also many other infectious agents, some rarely seen in immunocompetent patients. Other problems include the enormous replicative capacity of the virus and the lack of fidelity of reverse transcriptase, which produces vast populations of mutants. Drug and vaccine developers therefore face an ever-changing target, with few good animal models to help them. Furthermore, HIV is much more complicated than was first imagined, with several auxiliary and structural proteins that are themselves toxic to certain cell types. Perhaps, in the end, this very complexity will provide a key to effective and affordable treatment. There are numerous steps in the virus replication cycle, and several additional viral and host proteins may be good targets for such intervention. Although we already know a great deal about the biology of HIV, much more knowledge will have to be acquired if we are to exploit these targets,

identify others, and learn what is needed to develop an effective vaccine.

> What is it that we expected from our shamans, millennia ago, and still require from the contemporary masters of the profession? To do something, that's what.
>
> Lewis Thomas, *The Fragile Species*

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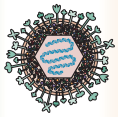


7

Transformation and Oncogenesis

Cause and effect, means and ends, seed and fruit, cannot be severed; for the effect already blooms in the cause, the end pre-exists in the means, the fruit in the seed.

RALPH WALDO EMERSON

Introduction

Cancer is a leading cause of death in developed countries: about 500,000 individuals succumb each year in the United States alone. Consequently, efforts to understand and control this deadly disease have long been high priorities for public health institutions. Our general understanding of the mechanisms of **oncogenesis**, the development of cancer, as well as of normal cell growth, has improved enormously in the past half century. Such progress can be traced in large part to efforts to elucidate how members of several virus families cause cancer in animals. In fact, as we discuss in this chapter, study of such oncogenic viruses has led to a detailed understanding of the molecular basis of oncogenesis.

It is now clear that cancer (defined in Box 7.1) is a genetic disease: it results from the growth of successive populations of cells in which mutations have accumulated (Box 7.2). These mutations affect various steps in the regulatory pathways that control cell communication, growth, and proliferation, and lead to uncontrolled growth, increasing tissue disorganization, and ultimately cancer. One or more mutations may be inherited (Box 7.2), or they may arise as a consequence of endogenous DNA damage as well as exposure to environmental carcinogens or infectious agents, including viruses. It is estimated that viruses are a contributing factor in approximately 20% of all human cancers. For some, such as liver and cervical cancer, they are the major cause. However, it is important to understand that the induction of malignancy is **not** a requirement for the propagation of any virus. Rather, this unfortunate outcome for the host is a side effect of either infection or the host's response to the presence of the virus. From this perspective, viruses can be thought of as cofactors, or unwitting initiators of oncogenesis.

Understanding the development of cancer ultimately depends on knowledge of how individual cells behave within an animal. As described in Chapters 1, 3, and 4, analysis of viral pathogenesis must encompass a consideration of the organism as a whole, especially the body's immune defenses. However, understanding how members of several virus families cause cancer in animals

BOX 7.1 TERMINOLOGY
Some cancer terms

Benign An adjective used to describe a growth that does not infiltrate into surrounding tissues; opposite of malignant

Cancer A malignant tumor; a growth that is not encapsulated and that infiltrates into surrounding tissues, replacing normal with abnormal cells; it is spread by the lymphatic vessels to other parts of the body; death is caused by destruction of organs to a degree incompatible with their function, by extreme debility and anemia, or by hemorrhage

Carcinogenesis The complex, multistage process by which a cancer develops

Carcinoma A cancer of epithelial tissue

Endothelioma A cancer characterized by overproduction of erythrocytes

Fibroblast A cell derived from connective tissue

Fibropapilloma A solid tumor of cells of the connective tissue

Hepatocellular carcinoma A cancer of liver epithelial cells

Leukemia A cancer of white blood cells

Lymphoma A cancer of lymphoid tissue

Malignant An adjective applied to any disease of a progressive and fatal nature; opposite of benign

Neoplasm An abnormal new growth, i.e., a cancer

Oncogenic Causing a tumor

Retinoblastoma A cancer of cells of the retina

Sarcoma A cancer of fibroblasts

Tumor A swelling, caused by abnormal growth of tissue, not resulting from inflammation; may be benign or malignant

began with studies of cultured cells in the laboratory. In particular, early investigators noticed that the growth properties and morphologies of some cultured cells could be changed upon infection with certain viruses. We therefore consider such cells **transformed**. The advantages of these cell culture systems are many. The molecular virologist can focus attention on particular cell types, can manipulate their behavior in a controlled manner, and can easily distinguish effects specific to the virus. In many cases, cells transformed by viruses in culture can form tumors when implanted in animals. But tumors do not always form, and it is important to realize that transformed cultures are **not** tumors. The major benefit of cell culture systems is that they allow researchers to study the molecular events that establish an oncogenic potential in virus-infected cells.

Properties of Transformed Cells

Cellular Transformation

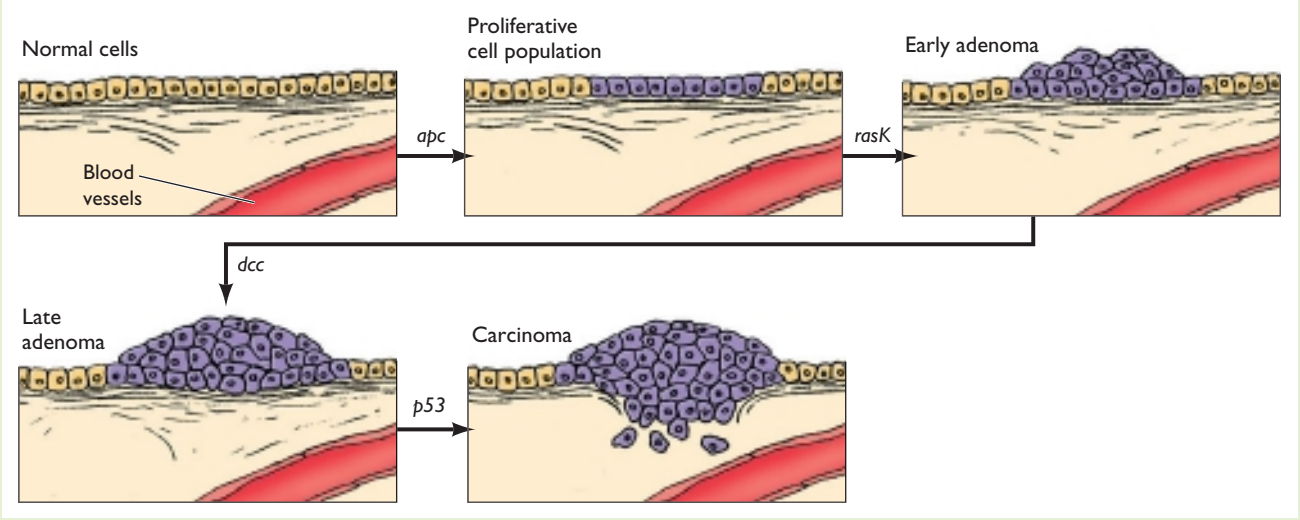
The proliferation of cells in the body is a strictly regulated process. In a young animal, total cell multiplication exceeds cell death as the animal grows to maturity. In an adult, the processes of cell multiplication and death are carefully balanced. For some cells, high rates of proliferation are required to maintain this balance. For example, human intestinal cells and white blood cells have half-lives of only a few days and need to be replaced rapidly. On the other hand, red blood cells live for over 100 days, and healthy neuronal cells rarely die. Occasionally, this carefully regulated process breaks down, and a particular cell begins to grow and divide even though the body has sufficient numbers of its type; such a cell behaves as if it were immortal. Acquisition of **immortality** is generally acknowledged to be an early step in oncogenesis. An immortalized cell may acquire one or more additional genetic changes to give rise to a clone of cells that is able to expand, ultimately forming a mass called a **tumor**. Some tumors are **benign**; they cease to grow in the body after reaching a certain size and generally do no great harm. Other tumor cells grow and divide indefinitely to form **malignant** tumors that damage and impair the normal function of organs and tissues. Occasionally, some cells in a tumor acquire additional mutations that confer the ability to escape the boundary of the mass, to invade surrounding tissue, and to be disseminated to other parts of the body, where the cells take up residence. There they continue to grow and divide, giving rise to secondary tumors called **metastases**. Such cells cause the most serious and life-threatening disease.

Many studies of the molecular biology of oncogenic animal viruses employed primary cultures of normal cells, for example, rat or mouse embryo fibroblasts. The advantage of these cells is that all the molecular changes necessary to convert them to the oncogenic state can be studied. Such primary cells, like their normal counterparts in the animal, have a finite capacity to grow and divide in culture. Cells from some animal species, such as rodents, undergo a spontaneous transformation when maintained in culture. Immortalized cells appear after a "crisis" period in which the great majority of the cells die (Fig. 7.1). As such cells are otherwise normal, and do not induce tumors when introduced into animals, they can be used to identify viral gene products needed for steps in transformation subsequent to immortalization. For reasons that are not fully understood, human and simian cells rarely undergo spontaneous transformation to immortality when passaged in culture. In fact, established lines of human cells generally can be derived only from tumors, or following exposure of primary cells to chemical carcinogens or to oncogenic

BOX 7.2 BACKGROUND

Genetic alterations associated with the development of colon carcinoma

Colorectal cancer is the fourth most common cancer worldwide, and the second most frequent in developed countries. The clinical stages in the development of this cancer are particularly well defined. Furthermore, as shown, several genes that are frequently mutated to allow progression from one stage to the next have been identified. The early adenomas or polyps that initially form are benign lesions. Their conversion to malignant metastatic carcinomas correlates with the acquisition of additional mutations in the *p53* and *dcc* ("deleted in colon carcinoma") genes. Inherited mutations in the genes listed can greatly increase the risk that an individual will develop colon carcinoma. For example, patients with familial adenomatous polyposis can inherit defects in the *apc* (adenomatous polyposis coli) gene that result in the development of hundreds of adenomatous polyps. The large increase in the **number** of these benign lesions increases the chance that some will progress to malignant carcinomas. In contrast, patients with hereditary nonpolyposis colorectal cancer develop polyps at the same rate as the general population. However, polyps develop to carcinomas more frequently in these patients, because defects in genes encoding proteins that correct mismatched bases in DNA lead to a higher mutation rate. Consequently, the **likelihood** that an individual polyp will develop into a malignant lesion increases from 5 to 70%.



RNA or DNA viruses (or their transforming genes). The realization that such transformed cells share a number of common properties, regardless of how they were obtained, provided a major impetus for the investigation of viral transformation.

Properties That Distinguish Transformed from Normal Cells

The definitive characteristic of transformed cells is their lack of response to the signals or conditions that normally control DNA replication and cell division. This property is illustrated by the list of growth parameters and behaviors provided in Table 7.1. As described above, transformed cells are immortal: they can grow and divide indefinitely, provided that they are diluted regularly into fresh medium. Production of **telomerase**, an enzyme that maintains telomeric DNA at the ends of chromosomes, has been implicated in immortalization. In addition, transformed cells typically exhibit a reduced requirement for the growth factors present in serum. Some transformed cells actually produce their own growth factors and the cognate receptors, providing themselves **autocrine growth stimulation**. Normal cells cease to grow and enter a quiescent state (called G_0, described in "Control of Cell Proliferation" below) when essential nutrient concentrations drop below a threshold value. Transformed cells are deficient in this capacity, and some may even kill themselves by trying to continue to grow in an inadequate environment.

Transformed cells grow to high densities. This characteristic is manifested by the cells piling up, over, or under each other. They also grow on top of untransformed cells, forming visually identifiable clumps called **foci** (Fig. 7.2). Transformed cells behave in this manner because they have lost **contact inhibition**, a response by which normal cells

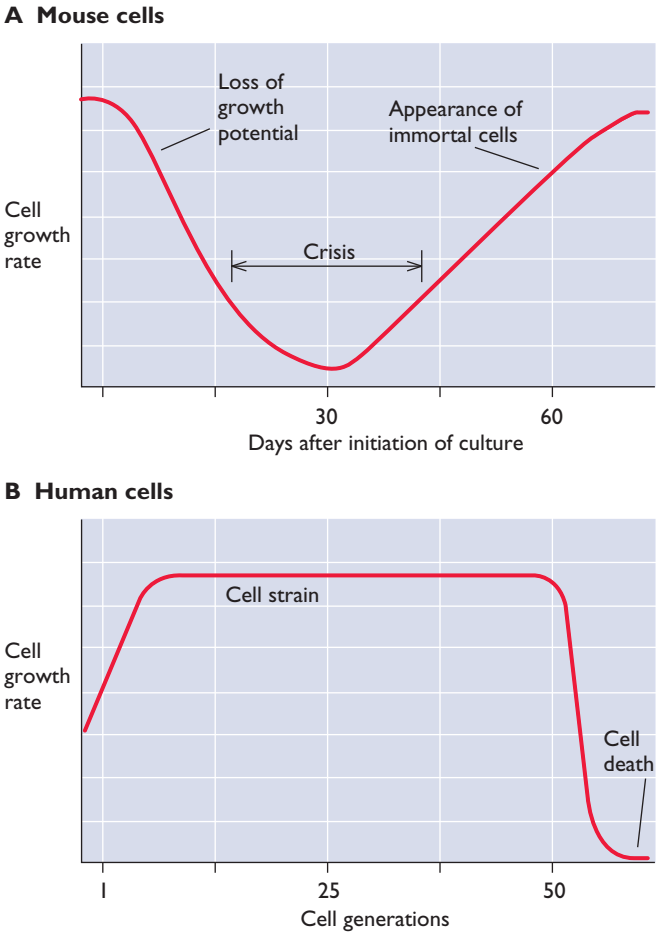


Figure 7.1 Stages in the establishment of a cell culture. **(A)** Mouse or other rodent cells. When mouse embryo cells are placed in culture, most cells die before healthy growing cells emerge. As these cells are maintained in culture, they begin to lose growth potential and most cells die (the culture goes into crisis). Very rarely cells do not die but continue growth and division until their progeny overgrow the culture. These cells constitute a cell line, which will grow indefinitely if it is appropriately diluted and fed with nutrients: the cells are immortal. **(B)** Human cells. When an initial explant is made (e.g., from foreskin), some cells die and others (mainly fibroblasts) start to grow; overall, the growth rate increases. If the surviving cells are diluted regularly, the cell strain grows at a constant rate for about 50 cell generations, after which growth begins to decrease. Eventually, all the cells die.

cease growth and movement when they sense the presence of their neighbors. Unlike normal cells, many transformed cells have also lost the need for a surface on which to adhere, and we describe them as being **anchorage independent**. Some anchorage-independent cells form isolated colonies in semisolid media (e.g., 0.6% agar). This property correlates well with the ability to form tumors in animals and often is used as an experimental surrogate for malignancy. Finally, transformed cells **look** different from normal cells; they are more rounded, with fewer processes, and as a result appear more refractile when observed under a microscope (Fig. 7.2).

Table 7.1 Growth parameters and behavior of transformed cells

Immortal: can grow indefinitely
Reduced requirement for serum growth factors
Loss of capacity for growth arrest upon nutrient deprivation
Growth to high saturation densities
Loss of contact inhibition (can grow over one another or normal cells)
Anchorage independence (can grow in soft agar)
Altered morphology (appear rounded and refractile)
Tumorigenic

There are other ways in which transformed cells can be distinguished from their normal counterparts. These properties include metabolic differences and characteristic changes in cell surface and cytoskeletal components. However, the list in Table 7.1 comprises the standard criteria used to judge whether cells have been transformed.

Control of Cell Proliferation

Sensing the Environment

As noted previously, proliferation of cells in an organism is strictly regulated to maintain tissue or organ integrity and normal physiology. Normal cells therefore possess elaborate pathways that receive and process growth-stimulatory or growth-inhibitory signals transmitted by other cells in the tissue or organism. Much of what we know about these pathways comes from study of the cellular genes transduced or activated by oncogenic retroviruses. Signaling often begins with the secretion of a growth factor by a specific type of cell. The growth factor may enter the circulatory system, as in the case of many hormones, or may simply diffuse through the spaces around cells in a tissue. Growth factors bind to the external portion of specific receptor molecules on the surface of the same or other types of cells. Alternatively, signaling can be initiated by binding of a receptor on one cell to a specific protein (or proteins) present on the surface of another cell, or to components of the extracellular matrix (Volume I, Chapter 5). The binding of the ligand triggers a change, often via oligomerization of receptor molecules, that is transmitted to the cytoplasmic portion of the receptor. In the case illustrated in Figure 7.3, the cytoplasmic domain of the receptor possesses protein tyrosine kinase activity, and interaction with the growth factor ligand triggers autophosphorylation. This modification sets off a **signal transduction cascade**, a chain of sequential physical interactions among,

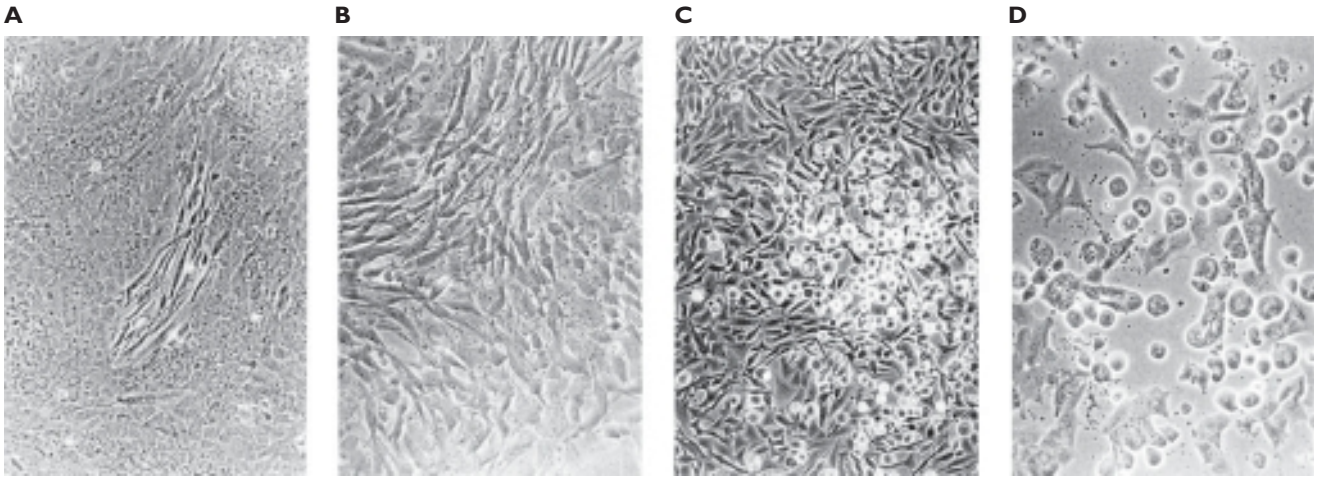


Figure 7.2 Foci formed by avian cells transformed with two strains of Rous sarcoma virus. Differences in morphology are due to genetic differences in the transduced *src* oncogene. **(A)** A focus of infected cells with fusiform morphology shown on a background of flattened, contact-inhibited, uninfected cells. **(B)** Higher magnification of a fusiform focus showing lack of contact inhibition of the transformed cells. **(C)** A focus of highly refractile infected cells with rounded morphology and reduced adherence. **(D)** Higher magnification of rounded infected cells, showing tightly adherent normal cells in the background. Courtesy of P. Vogt, The Scripps Research Institute.

and biochemical modifications of, membrane-bound and cytoplasmic proteins. Ultimately, the behavior of the cell is altered. Signaling proteins include small guanine nucleotide-binding proteins (G proteins), such as Ras, and protein kinases that phosphorylate serine or threonine residues. Signal transduction cascades can also include enzymes that produce small molecules (e.g., cyclic adenosine monophosphate AMP and certain lipids) that act as diffusible **second messengers** in the signal relay. Changes in ion flux across the plasma membrane, or in membranes of the endoplasmic reticulum, may also contribute to transmission of signals.

Relay of the signal can terminate at cytoplasmic sites to alter metabolism or cell morphology and adhesion. However, many signaling cascades culminate in the modification of transcriptional activators or repressors, and therefore alter the expression of specific cellular genes. The products of these genes either allow the cell to progress through another growth cycle or cause the cell to stop growing, to differentiate, or to die, whichever is appropriate to the situation. Errors in the signaling pathways that regulate these decisions can lead to cancer.

Regulation of the Cell Cycle

The capacity of cells to grow and divide is regulated by a molecular timer. The timer comprises an assembly of proteins that integrate stimulatory and inhibitory signals received by, or produced within, the cell. Eukaryotic cells do not attempt to divide until all their chromosomes have been duplicated and are precisely organized for segregation into daughter cells. Nor do they initiate DNA synthesis and chromosome duplication until the previous cell division is complete, or unless the extra- and intracellular environments are appropriate. Consequently, the molecular timer controls a tightly ordered **cell cycle** comprising intervals, or **phases**, devoted to specific processes.

The duration of the phases in the cell cycle shown in Fig. 7.4 is typical of those of many mammalian cells growing actively in culture. However, there is considerable variation in the length of the cell cycle, largely because of differences in the **gap phases** (G_1 and G_2). For example, early embryonic cells of animals dispense with G_1 and G_2, do not increase in mass, and move immediately from the **DNA synthesis phase (S)** to **mitosis (M)** and again from M to S. Consequently, they possess extremely short cycles of 10 to 60 min. At the other extreme are cells that have ceased growth and division and have withdrawn from the cell cycle. The variability in duration of this specialized **resting state**, termed G_0, accounts for the large differences in the rates at which cells in multicellular organisms proliferate. As discussed in Volume I, Chapter 9, many viruses replicate successfully in cells that spend all or most of their lives in G_0, a state that has been likened to "cell cycle sleep." In many cases, synthesis of viral proteins in such resting or slowly cycling cells induces them to reenter the cell cycle and grow and divide rapidly. To describe the mechanisms by which these viral proteins induce such abnormal activity and transform cells, we first introduce

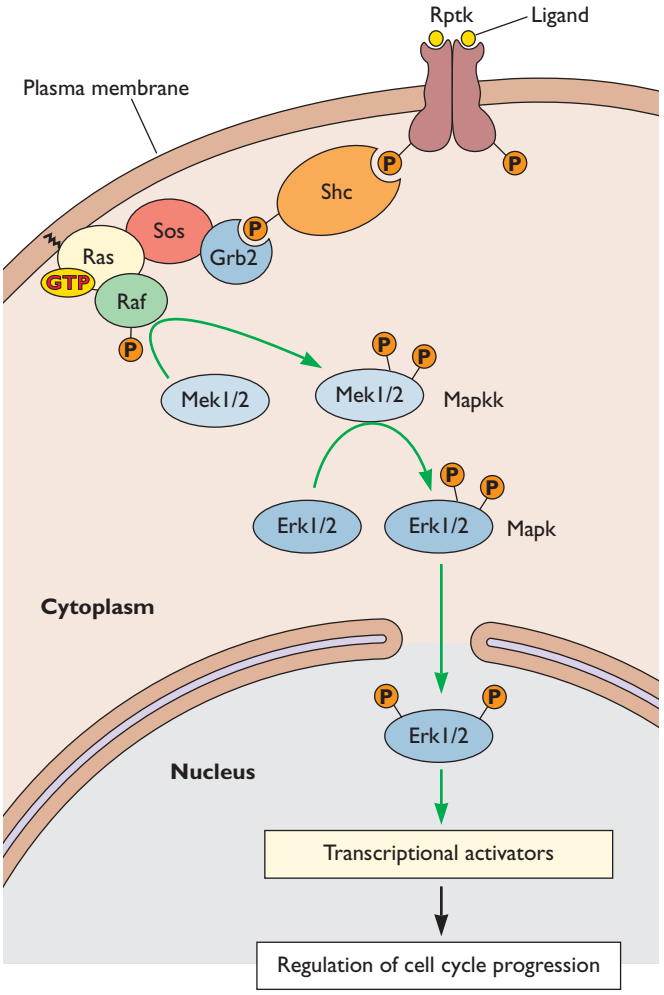


Figure 7.3 The mitogen-activated protein kinase signal transduction pathway. Signal transduction is initiated by binding of ligand to the extracellular domain of a receptor protein tyrosine kinase (Rptk), for example, the receptors for epidermal growth factor or platelet-derived growth factor. Binding of ligand (yellow circles) induces receptor dimerization and autophosphorylation of tyrosine residues in the cytoplasmic domain. Adapter proteins like Shc and the Grb2 component of the Grb2-Sos complex are recruited to the membrane by binding to these phosphotyrosine-containing sequences (or to a substrate phosphorylated by the activated receptor), along with Ras. Sos is the guanine nucleotide exchange protein for Ras and stimulates exchange of GDP for GTP bound to Ras by. The GTP-bound form of Ras binds to members of the Raf family of serine/threonine protein kinases. Raf then becomes autophosphorylated, and initiates the Map kinase (Mapk) cascade. The pathway shown contains Mek1 and Mek2 (Mek1/2) (Map kinase kinases [Mapkk]) and Erk1 and Erk2 (Erk1/2) (Map kinases). Phosphorylated Erk1/Erk2 can enter the nucleus, where they modify and activate transcriptional regulators. These kinases can also regulate transcription indirectly, by effects on other protein kinases.

the molecular mechanisms that control passage through the cell cycle.

The Cell Cycle Engine

The orderly progression of eukaryotic cells through periods of growth, chromosome duplication, and nuclear and cell division is driven by intricate regulatory circuits. The elucidation of these circuits must be considered a *tour de force* of contemporary biology. The first experimental hint that cells contain proteins that control transitions from one phase of the cell cycle to another came more than 35 years ago. Nuclei of slime mold (*Physarum polycephalum*) cells in early G_2 were found to enter mitosis immediately following fusion with cells in late G_2 or M. This crucial observation led to the conclusion that the latter cells must contain a **mitosis-promoting factor.** Subsequently, similar experiments with mammalian cells in culture identified an analogous S-phase-promoting factor. The convergence of many observations eventually led to the identification of the highly conserved components of the cell cycle engine (Fig. 7.5A).

Mitosis-promoting factor purified from amphibian oocytes proved to be an unusual protein kinase: its catalytic subunit is activated by the binding of an unstable regulatory subunit. Furthermore, the concentration of the regulatory subunit was found to oscillate reproducibly during each and every cell cycle. The regulatory subunit was therefore given the descriptive name **cyclin**, and the associated protein kinase was termed **cyclin-dependent kinase (Cdk)**. Similar proteins were implicated in cell cycle control in the yeast *Saccharomyces cerevisiae*, and it soon became clear that all eukaryotic cells contain multiple cyclins and Cdks, which operate in specific combinations to control progression through the cell cycle. The cyclins are related in sequence to one another, and they share such properties as activation of cyclin-dependent kinases and controlled destruction by the proteasome.

Figure 7.5A lists the various mammalian cyclin-Cdk complexes and shows the phases in the cell cycle in which they accumulate. The patterns of accumulation illustrate a critical feature of the cell cycle: individual cyclin-Cdks, the active protein kinases, accumulate in successive waves. The concentration of each increases gradually during a specific period in the cycle, but decreases abruptly as the cyclin subunit is degraded. In mammalian cells, proteolysis is important in resetting the concentrations of individual cyclins at specific points in the cycle, but production of cyclin mRNAs is also regulated. For example, the concentrations of mRNAs encoding cyclins E, A, and B, like those of the proteins, oscillate during the cell cycle. The orderly activation and inactivation of specific kinases govern passage through the cell cycle. For example, cyclin E synthesis is rate limiting for the transition from G_1 to S phase in

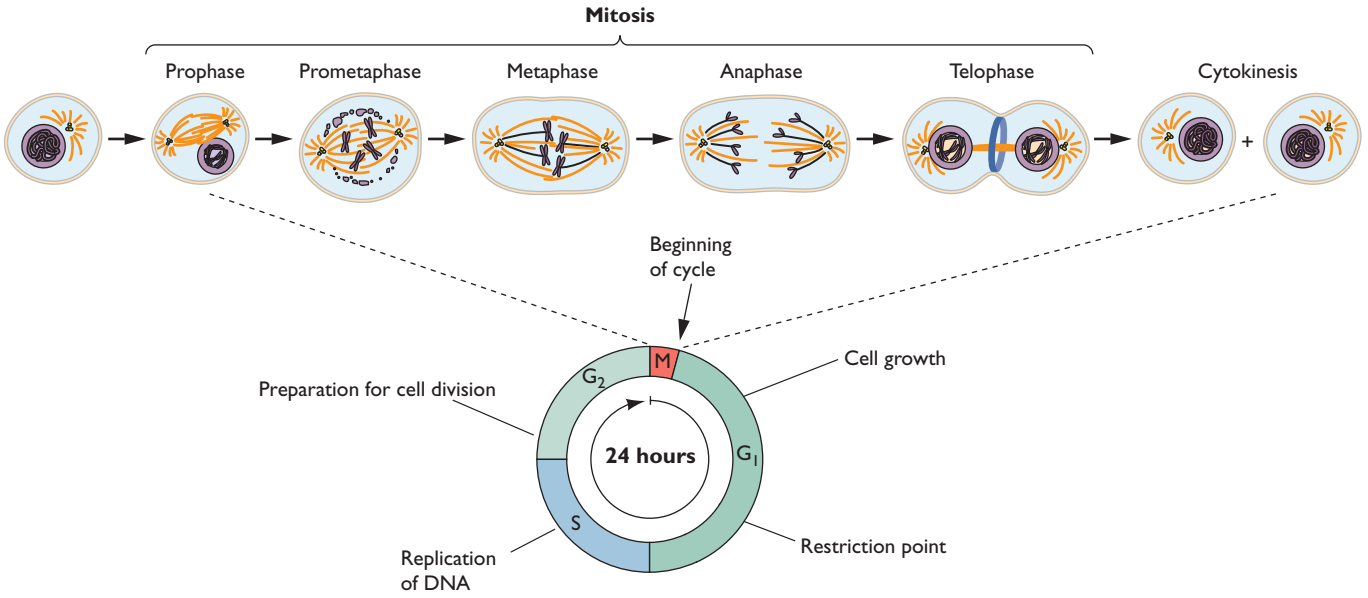


Figure 7.4 The phases of a eukaryotic cell cycle. The most obvious phase morphologically, and hence the first to be identified, is mitosis, or M phase, the process of nuclear division that precedes cell division. During this period, the nuclear envelope breaks down. Duplicated chromosomes become condensed and aligned on the mitotic spindle and are segregated to opposite poles of the cell, where nuclei reform upon chromosome decondensation (top). The end of M phase is marked by cytokinesis, the process by which the cell divides in two. Despite this remarkable reorganization and redistribution of cellular components, M phase occupies only a short period within the cell cycle. During the long interphase from one mitosis to the next, cells grow continuously. Interphase was divided into three parts with the recognition that DNA synthesis takes place only during a specific period, the synthetic or S phase, beginning about the middle of interphase. The other two periods, which appeared as "gaps" between defined processes, are designated the G_1 and G_2 (for gap) phases.

mammalian cells, and cyclin E-Cdk2 accumulates during late G_1. Soon after cells have entered S phase, cyclin E rapidly disappears from the cell; its task is completed until a new cycle begins.

While the oscillating waves of active Cdk accumulation and destruction are thought of as the ratchet that advances the cell cycle timer, it is important not to interpret this metaphor too literally. The orderly and reproducible sequence of DNA replication, chromosome segregation, and cell division is not determined solely by the oscillating concentrations of individual cyclin-Cdks. Rather, the cyclin-Cdk cycle serves as a device for integrating numerous signals from the exterior and interior of the cell into appropriate responses. The regulatory circuits that feed into and from the cycle are both many and complex (e.g., Fig. 7.5B). These regulatory signals ensure that the cell increases in mass and divides **only** when the environment is propitious or, in multicellular organisms, when the timing is correct. Many signal transduction pathways that convey information about the local environment or the global state of the organism therefore converge on the cyclin-Cdk integrators. In addition, various surveillance mechanisms monitor such internal parameters as DNA damage, problems with DNA replication, and proper assembly and function of the mitotic spindle. Such mechanisms protect cells against potentially disastrous consequences of continuing a cell division cycle that could not be completed correctly. As we shall see, it is primarily these signaling and surveillance (**checkpoint**) mechanisms that are compromised during transformation by oncogenic viruses.

Oncogenic Viruses

Oncogenic viruses cause cancer by inducing changes that affect the control of cell proliferation. Indeed, the study of the mechanisms of viral transformation and oncogenesis laid the foundation for our current understanding of cancer, for example, with the identification of oncogenes that are activated or captured by retroviruses (originally known as RNA tumor viruses) and viral proteins that inactivate tumor suppressor gene products (Fig. 7.6). Specific members of a number of different virus families, as well as an unusual, unclassified virus (Box 7.3), have been implicated in naturally occurring or experimentally induced cancers in animals (Table 7.2). It has been estimated that

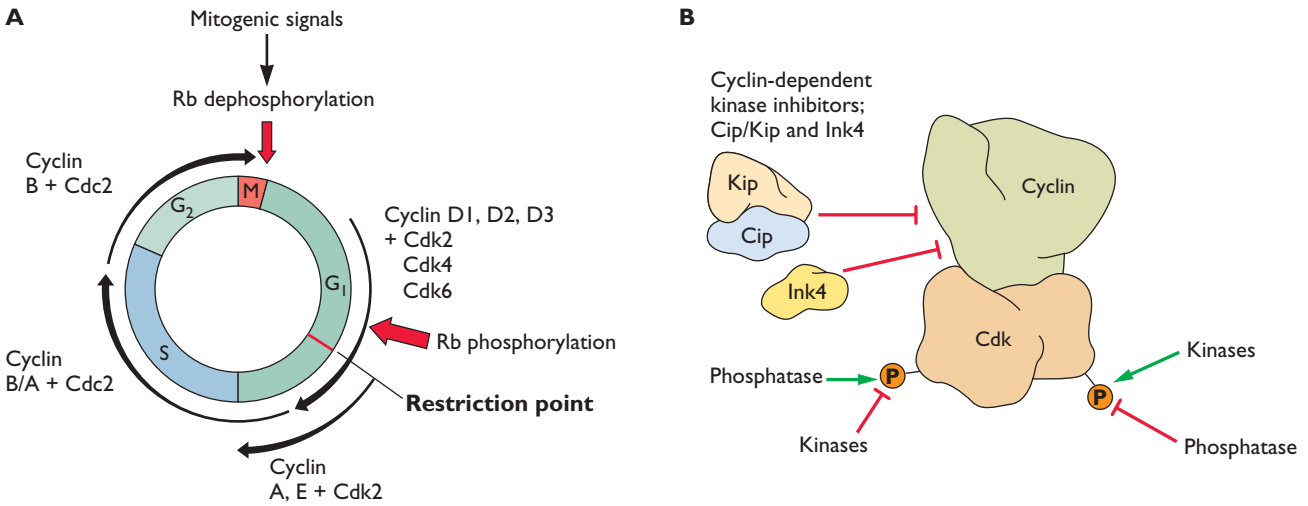
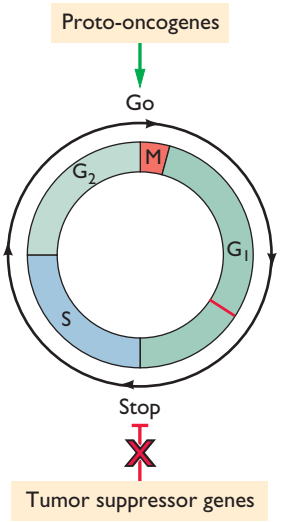


Figure 7.5 The mammalian cyclin-Cdk cell cycle engine. (A) The phases of the cell cycle are denoted on the circle. The progressive accumulation of specific cyclin-Cdks is represented by the broadening arrows, with the arrowheads marking the time of abrupt disappearance. **(B)** The production, accumulation, and activities of both cyclins and cyclin-dependent kinases are regulated by numerous mechanisms. Activating and inhibitory reactions are indicated by green arrows and red bars, respectively. Activation of the kinases can require not only binding to the appropriate cyclin, but also phosphorylation at specific sites and removal of phosphate groups at others. The activities of the kinases are also controlled by association with members of two families of cyclin-dependent kinase-inhibitory proteins, which control the activities of only G_1 (Ink4 proteins) or all (Cip/Kip proteins) cyclin-Cdks. Both types of inhibitor play crucial roles in cell cycle control. For example, the high concentration of $p27^{Kip1}$ characteristic of quiescent cells falls as they enter G_1, and inhibition of synthesis of this protein prevents cells from becoming quiescent.

Figure 7.6 A genetic paradigm for cancer. The pace of the cell cycle can be modulated both positively and negatively by different sets of gene products. Cancer arises from a combination of dominant, gain-of-function mutations in proto-oncogenes and recessive, loss-of-function mutations in tumor suppressor genes, which encode proteins that block cell cycle progression at various points. The function of either type of gene product can be affected by oncogenic viruses.



approximately 20% of all cases of human cancer are associated with infection by one of five viruses: Epstein-Barr virus, hepatitis B virus, hepatitis C virus, human T-lymphotropic virus type 1, and human papillomaviruses. In this section, we introduce oncogenic viruses and general features of their transforming interactions with host cells.

Discovery of Oncogenic Viruses

Retroviruses

Oncogenic viruses were discovered 100 years ago when Vilhelm Ellerman and Olaf Bang (1908) first showed that avian leukemia could be transmitted by filtered extracts (i.e., viruses) of leukemic cells or serum from infected birds. Because leukemia was not recognized as cancer in those days, the significance of this discovery was not generally appreciated. Shortly thereafter (in 1911), Peyton Rous demonstrated that solid tumors could be produced in chickens by using cell extracts from a transplantable sarcoma that had appeared spontaneously. Despite the viral etiology of this disease, the cancer viruses of chickens were thought to be oddities until similar murine malignancies, as well as mammary tumors, were found to be associated with infection by viruses. These oncogenic viruses all

BOX 7.3 EXPERIMENTS

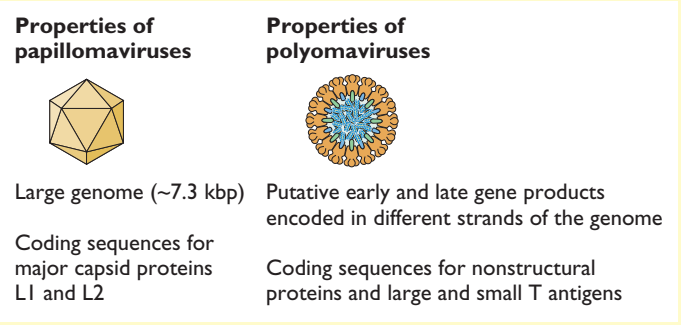
A cancer virus with genomic features of both papillomaviruses and polyomaviruses

Efforts are under way to prevent the extinction of the western barred bandicoot, an endangered marsupial now found only on two islands in the UNESCO World Heritage Area of Shark Bay, Western Australia. Unfortunately, conservation has been hindered by a debilitating syndrome, in which wild and captive animals develop papillomas and carcinomas in several areas of the skin. The histogical properties of the tumors suggested that a papillomavirus or a polyomavirus might contribute to development of the disease.

In fact, a previously unknown viral genome was discovered in tumor tissues from these animals by multiply primed amplification, cloning, and sequencing, and also by polymerase chain reaction (PCR) with degenerate primers specific for papillomavirus DNA. As summarized in the figure, this DNA genome exhibits features characteristic of both papillomaviruses and polyomaviruses and includes coding sequences related to those of both families. The papillomavirus-like and polyomavirus-like sequences were shown to be continuous with one another in the viral DNA genome. This property excludes the possibility that the tumor tissues were coinfected with a member of each family, as well as artifacts such as laboratory contamination of samples.

The origin of this unique virus, which was named bandicoot papillomatosis carcinomatosis virus type 1, is not known. The virus might have arisen as a result of a recombination event between the genomes of a papillomavirus and a polyomavirus. Alternatively, it might represent the first known member of a new virus family that evolved from a common ancestor of the *Papillomaviridae* and *Polyomaviridae*. Regardless, the viral genome was detected in 100% of bandicoots with papillomatosis and carcinomatosis syndrome, implicating the virus as a necessary factor in the development of this disease.

Woolford, L., A. Rector, M. Van Ranst, A. Ducki, M. D. Bennett, P. K. Nicholls, K. S. Warren, R. A. Swan, G. E. Wilcox, and A. J. O'Hara. 2007. A novel virus detected in papillomas and carcinomas of the endangered western barred bandicoot (*Perameles bougainville*) exhibits genomic features of both the *Papillomaviridae* and *Polyomaviridae*. *J. Virol.* **81:**13280–13290.



proved to be members of the retrovirus family, which do not kill their host cells. We now know that retroviruses are endemic in many species, including mice and chickens. For example, most chickens in a flock will have been infected by avian leukosis virus within a few months of hatching. In the vast majority of cases, chickens experience a transient viremia but disease is rare. Embryos can also be infected before their immune systems have been developed, and eventually become tolerant to the virus.

Early researchers classified the oncogenic retroviruses into two groups depending on the rapidity with which they caused cancer (Table 7.3). The first group comprises rare, rapidly transforming **transducing oncogenic retroviruses**. These are all highly carcinogenic agents that cause malignancies in nearly 100% of infected animals in a matter of days. They were later discovered to have the ability to transform susceptible cells in culture. The second class, **nontransducing oncogenic retroviruses**, includes less carcinogenic agents. Not all animals infected with these viruses develop tumors, which appear only weeks or months after infection. In the late 1980s, a third type of oncogenic retrovirus, a **long-latency virus** was identified in humans. Tumorigenesis is very rare, and occurs months or even years after infection.

Infection by each group of oncogenic retroviruses induces tumors by a distinct mechanism. As their name

Table 7.2 Oncogenic viruses and cancer

Family	Associated cancer(s)
RNA viruses	
Flaviviridae	
Hepatitis C virus	Hepatocellular carcinoma
Retroviridae	Hematopoietic cancers, sarcomas, and carcinomas
DNA viruses	
Adenoviridae	Various solid tumors
Hepadnaviridae	Hepatocellular carcinoma
Herpesviridae	Lymphomas, carcinomas, and sarcomas
Papillomaviridae	Papillomas and carcinomas
Polyomaviridae	Various solid tumors
Poxviridae	Myxomas and fibromas

Table 7.3 The oncogenic retroviruses

Property or characteristic	Transducing viruses	Nontransducing viruses	Nontransducing, long-latency viruses
Efficiency of tumor formation	High (ca. 100% of infected animals)	High to intermediate	Very low (<5%)
Tumor latency	Short (days)	Intermediate (weeks, months)	Long (months, years)
Infecting viral genome	Viral-cellular recombinant; normally replication defective	Intact; replication competent	Intact; replication competent
Oncogenic element	Cell-derived oncogene carried in viral genome	Cellular oncogene activated *in situ* by a provirus	Virus-encoded regulatory protein controlling transcription?
Mechanism	Oncogene transduction	*cis*-acting provirus	*trans*-acting protein?
Ability to transform cells in culture	Yes	No	No

implies, the genomes of transducing retroviruses contain cellular genes that become **oncogenes** (genes encoding proteins that cause transformation or tumorigenesis) when expressed in the viral context. The virally transduced versions of cellular genes are called **v-oncogenes**, and their normal cellular counterparts are called **c-oncogenes** or **proto-oncogenes**. The genomes of the nontransducing retroviruses do not encode cell-derived oncogenes. Rather, the transcription of proto-oncogenes is activated inappropriately as a consequence of the nearby integration of a provirus in the host cell genome. In either situation, the oncogene products play no role in the reproductive cycle of the retroviruses themselves. The oncogenic potential of these viruses is, in a sense, an accident of their lifestyle. Nevertheless, the study of v-oncogenes and proto-oncogenes that are affected by retroviruses has been of great importance in advancing our understanding of the origins of cancer.

Oncogenic DNA Viruses

The first DNA virus to be associated with oncogenesis was the papillomavirus that causes warts (papillomas) in cottontail rabbits; this virus was isolated by Richard Shope in 1933. The lack of cell culture systems for papillomaviruses initially precluded their use as experimental models for oncogenesis. Other viruses, in particular polyomaviruses such as simian virus 40 and human adenoviruses, proved much more tractable and soon dominated early studies of transformation and tumorigenesis by DNA viruses. It is important to note that neither simian virus 40 nor adenoviruses are associated with oncogenesis in their natural hosts. However, it was shown soon after their discovery that these viruses can induce tumors in rodents and transform cultured mammalian cells, and the possibility that simian virus 40 could have contributed to human cancers is currently under investigation (Box 7.4). Replication of these viruses destroys permissive primate host cells within a few days of infection. In contrast, rodent cells are nonpermissive for viral reproduction, or support only limited replication. Consequently, some infected cells survive infection and in rare cases become transformed.

The transforming genes of polyomavirus and adenoviruses **are** necessary for viral replication. However, transformation is not, but rather is a "collateral" consequence of the activities of the viral transforming proteins. These proteins contribute to transformation by altering the activities of cellular gene products. In some cases, such cellular proteins are encoded by the same proto-oncogenes transduced or otherwise affected by retroviruses. This important discovery, initially made in studies of the middle T protein (mT) of mouse polyomavirus in the early 1980s, provided the first indication that retroviruses and DNA viruses can transform cells by related mechanisms. Investigation of the biochemical properties of proteins encoded in other transforming genes of these DNA viruses led to equally important insights, notably the characterization of cellular proteins that can block cell cycle progression, the products of **tumor suppressor genes**.

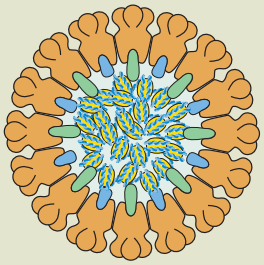
Since the discovery of Epstein-Barr virus in cells derived from Burkitt's lymphoma in 1966, it has been appreciated that herpesviruses can be associated with the development of tumors in humans and other animals. Infection of susceptible cells in culture by members of this family results in immortalization or induction of typical transformed phenotypes. Poxvirus infection can also induce cell proliferation, which may be prolonged or rapidly followed by cell death, depending on the virus. Indeed, some members of this family, such as Shope fibroma virus, are associated with tumors of the skin. However, poxviruses do not transform cells in culture, in part because they are highly cytotoxic. The large sizes of these genomes initially presented a major impediment to analysis of the transforming properties of these viruses. It is now clear that herpesviral and poxviral gene products generally alter cell growth and proliferation

BOX 7.4 DISCUSSION

A debate reopened: has simian virus 40 contributed to human cancer?

In 1960, simian virus 40 (SV40) was discovered in the African green monkey kidney cells used to produce poliovirus vaccines; within 2 years it was shown to be tumorigenic in hamsters. These were observations of great concern, because it was realized that many batches of the vaccines contained quite high concentrations of infectious SV40. It has been estimated that 98 million people in the United States, and many more worldwide, were exposed to potentially contaminated poliovirus vaccines before screening to ensure preparation of SV40-free vaccines was introduced in 1963. Ironically, monkey cells had been adopted for poliovirus vaccine production, because of the concern that human cells might contain then unknown human cancer viruses!

Epidemiological studies initiated in the 1960s and 1970s monitored thousands of vaccine recipients for up to 20 years, with no evidence for increased cancer risk in this population. The initial alarm raised by the tumorigenicity of SV40 in rodents therefore appeared to be laid to rest. However, during the past decade, reports that the DNA of this virus is present in human tumors, analogous to those induced by SV40 in hamsters, have led some researchers to reconsider the contribution of this monkey virus to human cancer. Others remain skeptical. Some of the pros and cons of the current debate are listed below.



It is important to note that even if the debate is settled with a consensus that SV40 DNA **is** present in human tumors, the question of whether it is a causal agent of cancer will remain to be addressed.

Garcea, R. L., and M. J. Imperiale. 2003. Simian virus 40 infection of humans. *J. Virol.* **77:**5039–5045.

Jasani, B, A. Cristaudo, S. A. Emri, A. F. Gazdar, A. Gibbs, B. Krynska, C. Miller, L. Mutti, C. Radu, M. Tognon, and A. Procopio. 2001. Association of SV40 with human tumours. *Semin. Cancer Biol.* **11:**49–61.

López-Ríos, F., P. B. Illei, V. Rusch, and M. Ladanyi. 2004. Evidence against a role for SV40 infection in human mesotheliomas and high risk of false positive PCR results owing to presence of SV40 sequences in common laboratory plasmids. *L ancet* **364**:1157–1166.

Pro	Con
SV40 DNA has been detected in several human tumors, including osteosarcoma, mesothelioma, and non-Hodgkin's lymphoma; the virus induces similar tumors in hamsters.	SV40 DNA is not present in all samples of a particular cancer, and in some studies (e.g., of mesotheliomas) it has not been detected in any.
A poliovirus vaccine produced in 1954 was found to be contaminated with a variant of SV40 that can be distinguished from common laboratory strains; the same variant has now been found in three non-Hodgkin's lymphoma patients.	This viral DNA has been detected in tumors of individuals who could not have received potentially contaminated poliovirus vaccine.
	In a comparison of mesotheliomas and normal samples, SV40 DNA was detected just as frequently in the normal tissues as in the tumors.
	Careful analysis of the SV40 sequences detected in mesotheliomas by PCR established that the viral DNA detected arose from common laboratory plasmids: it contained an engineered gap that is not present in the natural viral genome.

by mechanisms related to those responsible for transformation by the smaller DNA viruses or retroviruses. However, some may also encode micro-RNAs that contribute transformation (Box 7.5).

Contemporary Identification of Oncogenic Viruses

Oncogenic viruses associated with human disease continue to be isolated with some regularity. One discovered in 1994 was a previously unknown member of the family *Herpesviridae,* human herpesvirus 8, which was isolated from tumor cells from patients with Kaposi's sarcoma. Its genome, like those of transducing retroviruses, contains homologs of cellular proto-oncogenes. Most recently (in 2008), it was reported that a polyomavirus may be associated with a rare form of skin cancer (Box 7.6). Perhaps an even greater surprise was the realization that RNA viruses other than retroviruses can be associated with cancer: hepatitis C virus, a (+) strand RNA virus belonging to the family *Flaviviridae* identified in 1989, is associated with a high risk for hepatocellular carcinoma (Appendix A, Fig. 6). Humans are the only known natural hosts, and the virus has only recently been propagated in tissue culture. It appears that neither oncogenes nor tumor suppressor genes contribute to hepatitis C virus oncogenesis. This virus cannot, therefore, be considered a classic tumor virus.

BOX 7.5 EXPERIMENTS

A human herpesvirus 8 micro-RNA that may contribute to transformation of host cells

Micro-RNAs (miRNAs) are small (21- to 23-nucleotide) RNAs that base pair with complementary sequences in mRNAs to inhibit translation of the mRNA, or induce its degradation (see Volume I, Chapter 10). Viral miRNAs are made in cells infected by members of several families of DNA viruses, included herpesviruses. The genome of human herpesvirus 8, which is associated with Kaposi's sarcoma and certain B-cell lymphomas, encodes at least 10 miRNAs.

One of the human herpesvirus 8 miRNAs, miR-K-11-12 has sequence homology to the human mi-RNA miR-155, particularly in the region critical for base pairing with target mRNAs, the so-called seed region (see the figure). The viral miR-K-11-12 miRNA was shown to target a set of cellular mRNAs that are also affected by miR-155.

- Cellular mRNAs with at least one match to the sequence complementary to the seed sequence of miR-155 were highly enriched in the 150 mRNAs that decreased in concentration to the greatest degree when the viral miRNA was made in human B cells in the absence of any other viral gene products
- The cellular miR-155 reduced the concentrations of a set of target mRNAs to the same degree as the viral miRNA, and both reduced the expression of reporter genes carrying 12 different mRNA 3′ untranslated regions that were predicted to be complementary to the common miRNA seed sequence
- Reduction of the concentrations of 6 target mRNAs by miR-K-11-12 was prevented by mutation of the seed sequence
- The cellular and viral miRNAs both induced decreases in the concentrations of the proteins encoded by two predicted target mRNAs (encoding Fos and Bach1).

The sequences of human miR-155 and human herpesvirus type 8 K-miR-11-12 are shown aligned, with conserved nucleotides indicated in blue. The "seed" region important for base pairing of miRNAs with target mRNA is outlined in red.

These observations strongly suggest that K-miR-11-12 is a functional analog of cellular miR-155. The latter is the product of the *bic* gene, which is overexpressed in several types of human B-cell lymphoma. When introduced into transgenic mouse, *bic* induced B-cell lymphomas, indicating that it is an oncogene. These properties suggest that the viral miRNA may contribute to the development of B-cell tumors associated with human herpesvirus 8.

Gottwein, E., N. Mukherjee, C. Sachse, C. Funsel, W. H. Majoros, J.-T. A. Chi, R. Braich, M. Manoharan, J. Soutschek, V. Ohler, and B. R. Cullen. 2007. A viral microRNA functions as an orthologue of cellular mi-R-155. *Nature* **450:**1096–1101.

Common Properties of Oncogenic Viruses

Although they are members of different families (Table 7.2), the majority of oncogenic viruses share several general features. In all cases that have been analyzed, transformation is observed to be a single-hit process (defined in Volume I, Chapter 2), in the sense that infection of a susceptible cell with a single virus particle is sufficient to cause transformation. In addition, all or part of the viral genome is usually retained in the transformed cell. With few exceptions, cellular transformation is accompanied by the continuous expression of specific viral genes. On the other hand, while specific viral genes are present and expressed, transformed cells need not and (except in the case of some retroviruses) **do not** produce infectious virus particles. Most importantly, viral transforming proteins alter cell proliferation by a limited repertoire of molecular mechanisms.

Viral Genetic Information in Transformed Cells

State of Viral DNA

Cells transformed by oncogenic viruses generally retain viral DNA in their nuclei. These DNA sequences correspond to all or part of the infecting DNA genome, or the proviral DNA made in retrovirus-infected cells. Viral DNA sequences are maintained by one of two mechanisms: they can be integrated into the cellular genome (Box 7.7) or persist as autonomously replicating episomes.

As discussed in Volume I, Chapter 7, integration of retroviral DNA by the viral enzyme integrase is an essential step in the viral life cycle. Although there are some virus-specific biases, integration can occur at essentially any site in cellular DNA, but the reaction preserves a fixed order of viral genes and control sequences in the provirus (see Volume I, Fig. 7.15). When the provirus carries a v-oncogene, the site at which it is integrated into the cellular genome is of no importance (provided that viral transcription is unimpeded). In contrast, integration of proviral DNA within specific regions of the cellular genome is a hallmark of the induction of tumors by nontransducing retroviruses.

The proviral sequences present in every cell of a tumor induced by nontransducing retroviruses are found in the same chromosomal location, an indication that all arose from a single transformed cell. Such tumors are, therefore, **monoclonal**. Although infection is initiated with a nondefective retrovirus, the proviruses in the resulting tumor cells

BOX 7.6 DISCUSSION

Does a polyomavirus contribute to development of Merkel cell carcinoma in humans?

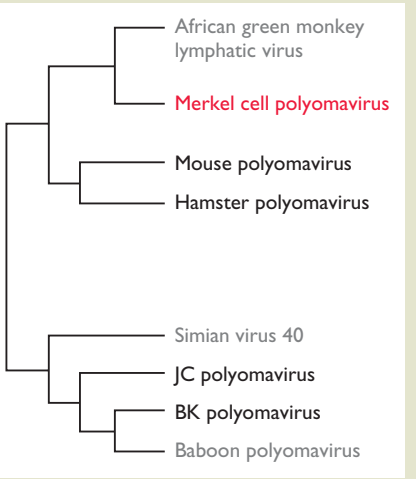
Mouse polyomavirus and simian virus 40 have been important models for studies of oncogenesis and transformation (see the text). Two human members of this family, BK and JC polyomaviruses, were discovered in 1971. These viruses commonly infect the urinary tract, and can be pathogenic in immunosuppressed patients (Appendix A, Fig. 20). A distantly related polyomavirus genome was detected recently in tumors from patients with Merkel cell carcinoma, a rare but rapidly metastasizing skin cancer.

Viral DNA sequences initially were identified in tumor tissue by a method based on high-throughput sequencing. Complementary DNA (cDNA) libraries were prepared from a single tumor and from a pool of three tumors. A total of 395,734 cDNAs were then sequenced. The vast majority (99.4%) were of human chromosomal or mitochondrial origin. Among the unassigned sequences, one from the single tumor exhibited significant homology to African green monkey lymphotropic polyomavirus and BK polyomavirus T antigen coding sequences. The 3′ end of this cDNA was shown to include sequences of the human receptor tyrosine phosphatase type G, suggesting that viral DNA sequences were integrated in the genome of tumor cells. Integration of the viral genome was subsequently confirmed by several methods, including Southern blotting (see Box 7.7). The organization of the viral genome is that typical of polyomaviruses, and includes sequences homologous to the early transforming gene products, large and small T antigens, of animal members of the family.

The genome of this virus, which was called Merkel cell polyomavirus, was detected in tumor tissue from 8 of 10 patients with Merkel cell carcinoma, but was present only at lower concentrations in 9 of 84 control samples. Furthermore, the pattern of viral DNA integration in the tissues examined indicated that the tumors were monoclonal in origin, implying that viral DNA integration proceeded proliferation of the cells. These observations suggest a causal association between virus infection and the development of Merkel cell carcinoma. More extensive epidemiological studies will be required to assess such a role. Furthermore, it is not yet known whether the predicted Merkel cell polyomavirus large and small T antigens are present in tumor cells, or possess transforming activity.

Feng, H., M. Shuda, Y. Chang, and P. S. Moore. 2008. Clonal integration of a polyomavirus in Merkel cell carcinoma. *Science* **319:**1096–1100.

The evolutionary relationship of Merkel cell carcinoma polyomavirus to some other mammalian polyomaviruses is shown schematically. Adapted from R. P. Viscidi and K. V. Slak, *Science* **319:**1049–1050, 2008, with permission.



have usually lost some or most of the proviral sequences. Nevertheless, at least one long terminal repeat (LTR) containing the transcriptional control region is always present. Viral transcription signals, but not protein-coding sequences, are therefore required for transformation by nontransducing retroviruses. The significance of these properties became apparent when it was discovered that in several tumors proviruses were integrated in the vicinity of some of the same cellular oncogenes that are captured by transducing retroviruses. The study of nontransducing oncogenic retroviruses has also led to the identification of some additional proto-oncogenes, as illustrated for two murine retroviruses in Table 7.4. As integration of retroviral DNA into the host genome is essentially random, there is a limited probability that integration will occur in the vicinity of an oncogene. The long latency for tumor induction by these viruses can be explained by the need for multiple cycle of replication and integration for such an event to occur.

Integration of viral DNA sequences is not a prerequisite for successful propagation of **any** oncogenic DNA viruses. Nevertheless, integration is the rule in adenovirus- or polyomavirus-transformed cells. Such integration is the result of rare recombination reactions (catalyzed by cellular enzymes) between generally unrelated DNA sequences. Integration can therefore occur at many sites in the cellular genome. The great majority of cells transformed by these viruses retain only partial copies of the viral genome. The genomic sequences integrated can vary considerably among independent lines of cells transformed by the same virus, but a common, minimal set of genes is always present. The low probability that viral DNA will become integrated into the cellular genome, and the fact that only a fraction of these recombination reactions will maintain the integrity of viral transforming genes, are major factors contributing to the low efficiencies of transformation by these viruses. In the case of simian virus 40, infection of cultures with 10^2 to 10^4 plaque-forming units (PFU) is required to obtain one focus of transformed cells, while the ratio is even higher for human adenoviruses.

BOX 7.7 METHODS

Detection and characterization of integrated viral DNA sequences in transformed cells

Integration of viral DNA into the cellular genome was initially demonstrated by the cosedimentation of viral DNA with high-molecular-mass cellular DNA under strongly denaturing conditions. The development of restriction endonucleases as molecular tools and of the Southern hybridization assay allowed direct proof of such integration, as well as characterization of integrated viral DNA. These approaches are illustrated for a circular, double-stranded viral DNA genome.

Cleavage of genomic DNA isolated from transformed cells with restriction endonuclease A (no sites in the viral genome) yields one high-molecular-mass fragment of lower mobility than free viral DNA (F) for each site of integrated viral DNA (I). The number of integration sites can therefore be counted. When used with appropriate standards, the number of copies of viral sequences at each such site can also be estimated. Restriction endonuclease B (one site in the viral genome) generates two fragments differing in mobility from the free, linear viral DNA. The results obtained with enzymes A and B demonstrate that viral DNA must be covalently attached to cellular DNA. Restriction endonuclease C, with two cleavage sites in the viral genome, produces three fragments from transformed cell DNA. The comigration of one fragment with the smaller product of digestion of free viral DNA indicates that this segment of the viral genome is intact in the integrated viral DNA.

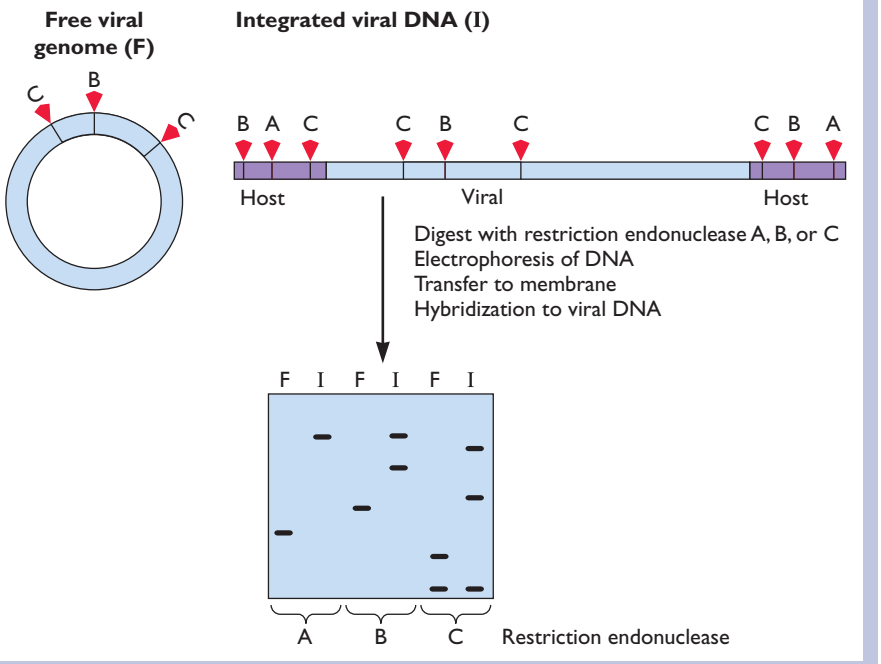


Table 7.4 New proto-oncogenes targeted by two nontransducing, simple retroviruses[a]

Virus	Neoplasia	Cellular gene	Normal function
Moloney murine leukemia virus	Pre-B lymphoma	*Ahi1*	Adapter protein
	T-cell lymphoma	*cyclin D2*	G_1 cyclin
		Lck	Nonreceptor protein tyrosine kinase
		Notch-1	Transmembrane receptor that functions in development
		Pim1	Serine/threonine kinase
		Tpl2	Serine/threonine kinase
	B-cell lymphoma	*Pim2*	Serine/threonine kinase
Mouse mammary tumor virus	Mammary tumors	*Int-1* (*wnt-1*)	Secreted glycoprotein important in pattern formation in early embryonic development
		Int-2 (*hst*)	Secreted proteins that may act as a growth factor
		Int-3	Protein presumed to function in development (*notch* family)
		IntH/Int5	Protein that converts androgens to estrogens

[a]Adapted from J. M. Coffin, S. H. Hughes, and H. E. Varmus (ed.), p. 482–484, *Retroviruses*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1997, with permission.

A second mechanism by which viral DNA can persist in transformed cells is as a stable, extrachromosomal episome (Volume I, Box 1.4). Such episomal viral genomes are a characteristic feature of B cells immortalized by Epstein-Barr virus, and they can also be found in cells transformed by papillomaviruses. The viral episomes are maintained at concentrations of tens to hundreds of copies per cell, by both replication of the viral genome in concert with cellular DNA synthesis and orderly segregation of viral DNA to daughter cells (see Volume I, Chapter 9). Consequently, transformation depends on the viral proteins necessary for the survival of viral episomes, as well as those that modulate cell growth and proliferation directly.

Identification and Properties of Viral Transforming Genes

Transforming genes of oncogenic viruses have been identified by classical genetic methods, characterization of the viral genes present and expressed in transformed cell lines, and analysis of the transforming activity of viral DNA fragments directly introduced into cells (Box 7.8). For example, analysis of transformation by temperature-sensitive mutants of mouse polyomavirus established as early as 1965 that the viral early transcription unit is necessary and sufficient to initiate and maintain transformation. Of even greater value were mutants of retroviruses, in particular two mutants of Rous sarcoma virus isolated in the early 1970s. The genome of one mutant carried a spontaneous deletion of approximately 20% of the viral genome; this mutant could no longer transform the cells it infected, but it could still replicate. The second mutant was temperature sensitive for transformation, but the virus could replicate at both temperatures. These properties of the mutants therefore showed unequivocally that cellular transformation and viral replication are distinct processes. More importantly, the deletion mutant allowed preparation of the first nucleic acid probe specific for a v-oncogene, v-*src* (Box 7.9). This *src*-specific probe was found to hybridize to cellular DNA, providing the first conclusive evidence that v-oncogenes are of cellular and not viral origin. This finding, for which J. Michael Bishop and Harold Varmus received the 1989 Nobel Prize in physiology or medicine, had far-reaching significance, because it immediately suggested that such cellular genes might become oncogenes by means other than viral transduction.

The presence of cellular oncogenes in their genomes turned out to be the definitive characteristic of transducing retroviruses, as illustrated in Fig. 7.7. As noted earlier, the acquisition of these cellular sequences is a very rare event. In addition, with the exception of Rous sarcoma virus, the transducing retroviruses are replication defective, having lost all or most of the viral coding sequences during oncogene capture. Such defective transducing viruses can, however, be propagated in mixed infections with replication-competent "helper" viruses, which provide all the

BOX 7.8 DISCUSSION

Multiple lines of evidence identified the transforming proteins of the polyomavirus simian virus 40

Early-gene products are necessary and sufficient to initiate transformation.

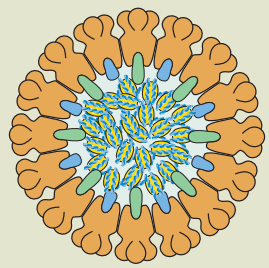
1. Viruses carrying temperature-sensitive mutations in the early transcription unit (*ts*A mutants), but no other region of the genome, fail to transform at a nonpermissive temperature.
2. Simian virus 40 DNA fragments containing only the early transcription unit transform cells in culture; DNA fragments containing other regions of the genome exhibit no activity.

Early-gene products are necessary to maintain expression of the transformed phenotype.

1. Many lines of cells transformed by simian virus 40 *ts*A mutants at a permissive temperature revert to a normal phenotype when shifted to a nonpermissive temperature.
2. Integration of viral DNA sequences disrupts the late region of the viral genome but not the early transcription unit, and early-gene products are synthesized in all transformed cell lines.

Both LT and sT contribute to transformation.

1. Simian virus 40 mutants carrying deletions of sequences expressed only in sT fail to transform rat cells to anchorage-independent growth.
2. Introduction and expression of LT complementary DNA are sufficient for induction of transformation, but expression of sT can stimulate transformation (especially at low LT concentrations), is necessary for expression of specific phenotypes in specific cells, and is required for transformation of resting cells.



BOX 7.9 METHODS

Preparation of the first oncogene probe

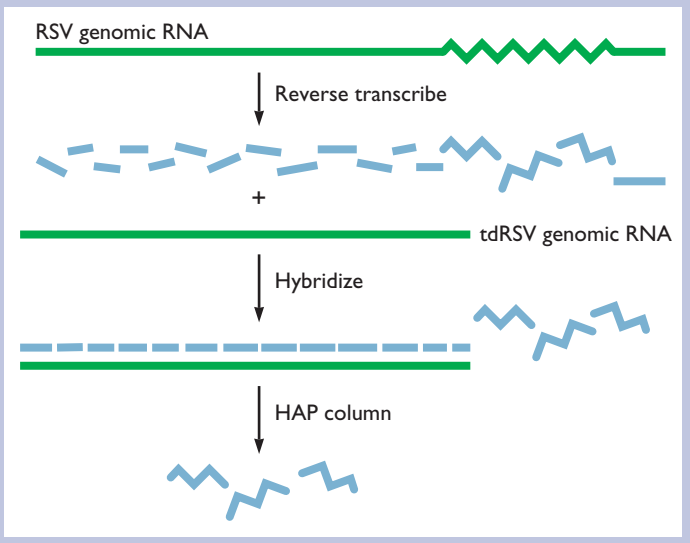
In the early 1970s, modern molecular biology was already in full bloom, but some techniques that are currently commonplace, such as PCR amplification of specific genes and molecular cloning, had not yet been invented. It was, however, possible at that time to make cDNA copies of RNA with retroviral reverse transcriptase, and to separate double-stranded (hybridized) from single-stranded (nonhybridized) nucleic acids. The existence of two genetically related viral genomes, one that contained a transforming gene (Rous sarcoma virus [RSV]) and the deletion mutant (tdRSV), which was replication competent but nontransforming, made it possible to isolate a radioactively labeled probe for the transforming gene, *src*, by exploiting these available techniques, using a strategy known as **subtractive hybridization**.

Complementary (−) strand DNA was prepared by reverse transcription of the (+) strand RSV genome and then hybridized to genomic RNA of the td-RSV mutant. The nonhybridizing DNA was then separated from the double-stranded hybrids, by hydoxylapatite chromatography. This radioactive DNA was then used as a probe to search for corresponding genetic material in a variety of cells.

Hybridization to chicken genomic DNA and the DNA of other avian species immediately suggested that the *src* sequences and, by inference, other retroviral oncogenes had been captured from the host cells infected by the virus. The observation that *src*-related sequences are conserved among cells from widely different species in the animal kingdom suggested that the proteins they encode play a central role in cell growth and division and that their malfunction could explain the origin of cancers that arise independently of retroviral infection. This important insight contributed to our understanding of the genetic basis of this disease and led to a Nobel Prize for Harold Varmus and Michael Bishop.

Spector, D. H., H. E. Varmus, and J. M. Bishop. 1978. Nucleotide sequences related to the transforming gene of avian sarcoma viruses are present in DNA of uninfected vertebrates. *Proc. Natl. Acad. Sci. USA* **75:**4102–4106.

Stehelin, D., H. E. Varmus, and J. M. Bishop. 1976. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature* **260:**170–173.



necessary virion proteins. Such mixed viral stocks have been the source of many transducing oncogenic viruses. The majority carry a single v-oncogene in their genomes, but some include more than one oncogene (e.g., *erb*A and *erb*B in avian erythroblastosis virus ES4). In such cases, one is sufficient for transformation; the second enhances oncogenicity.

Viral and cellular protein-coding sequences are fused in many v-oncogenes (Fig. 7.7). The presence of viral sequences can enhance the efficiency of translation of the oncogene mRNA, stabilize the protein, or determine its location in the cell. Unregulated expression or overexpression of the cellular sequence from the viral promoter is sufficient to cause transformation by some v-oncogenes (e.g., *myc* and *mos*). However, in most cases, the captured oncogenes have undergone additional changes that contribute to their transforming potential. These alterations, which include nucleotide changes, truncations at one or both ends, or other rearrangements, affect the normal function of the gene product.

Transformation of primary cells by DNA viruses requires the products of two or more viral genes, as illustrated in Table 7.5. The majority of these genes exhibit some ability to alter the properties of the cells in which they are expressed in the absence of other viral proteins. However, some are required only for the induction of specific transformed phenotypes or only under certain conditions (e.g., simian virus 40 small T antigen), and several exhibit no activity on their own (Table 7.5). A classic example of the latter phenomenon is provided by the adenoviral E1B gene: this gene, together with the E1A gene, was initially shown to be essential for transformation of rodent cells in culture, but it possesses no intrinsic ability to induce **any** transformed phenotype. This apparent paradox has been resolved with elucidation of the molecular functions of the viral gene products: E1A gene products induce apoptosis,

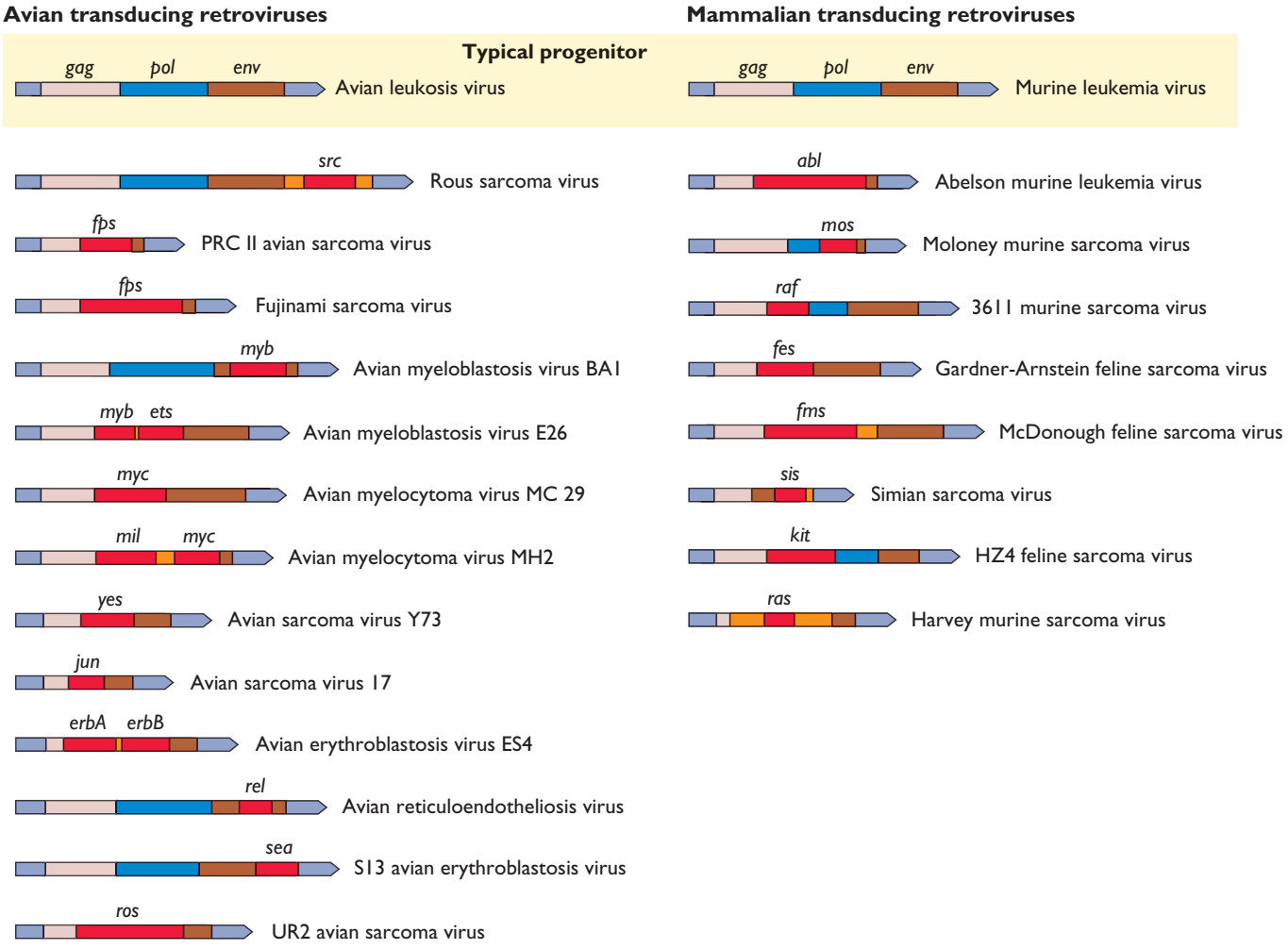


Figure 7.7 Genome maps of avian and mammalian transducing retroviruses. Avian leukosis virus (e.g., Rous-associated virus) and murine leukimia virus are prototypical retroviruses. Their genomes contain the three major coding regions, *gag* (pink), *pol* (blue), and *env* (brown). In Rous sarcoma virus, the oncogene *src* is added to the complete viral genome. In all other avian and mammalian transducing retroviruses, some of the viral coding information is replaced by cell-derived oncogene sequences (red). Consequently, such transducing viruses are defective in replication. In some cases, additional cellular DNA sequences (orange) were also captured in the viral genome. Adapted from T. Benjamin and P. Vogt, p. 317–367, *in* B. N. Fields et al. (ed.), *Fields Virology*, 2nd ed. (Raven Press, New York, NY, 1990), with permission.

but E1B proteins suppress this response. E1B proteins therefore allow cells that synthesize E1A proteins to survive and display transformed phenotypes. A requirement for multiple viral genes is also the rule for transformation by the larger DNA viruses.

The Origin and Nature of Viral Transforming Genes

Two classes of viral oncogenes can be distinguished on the basis of their similarity to cellular sequences. The oncogenes of transducing retroviruses and certain herpesviruses (e.g., human herpesvirus 8) are so closely related to cellular genes that it is clear that they were captured relatively recently from the genomes of cells in which these viruses replicate. Such acquisition of cellular genetic information must be a result of recombination between viral and cellular nucleic acids, a process that has been documented for transducing retroviruses. Retrovirus particles contain some cellular RNAs, and rare recombination reactions during reverse transcription can give rise to transducing retroviruses. The limiting factor appears to be the frequency with which cellular mRNA molecules are encapsidated into virions. Two mechanisms that can increase the likelihood of such encapsidation, and consequently increase the frequency of gene capture, have been described (Fig. 7.8). Both mechanisms depend on integration of a provirus in

Table 7.5 Some transforming gene products of adenoviruses, papillomaviruses and polyomaviruses

Virus	Gene product	Activities
Adenoviridae		
Human adenovirus type 2	E1A: 243R and 289R	Essential for transformation of established and primary rodent cells; cooperate with E1B products to transform primary cells; not sufficient for establishment of transformed cell lines
	E1B: 55 kDa and 19 kDa	Necessary for E1A-dependent transformation of primary and established cells; when expressed alone, cannot alter cell growth or proliferation; counter apoptosis by different mechanisms
	E4: ORF1	Transforms established cells; the adenovirus type 9 protein is necessary for induction of estrogen-dependent mammary tumors by this virus
Papillomaviridae		
Human papillomavirus types 16 and 18	E6	Required for efficient immortalization of primary human fibroblasts and keratinocytes
	E7	Transforms established rodent cells; cooperates with E6 to transform primary rodent cells; required for efficient immortalization of primary human fibroblasts or keratinocytes
Bovine papillomavirus type 1	E5	Transforms bovine and rodent fibroblasts in culture; major viral protein synthesized in transformed cells
	E6	Required for expression of typical transformed phenotypes in primary mouse cells; can transform established cells
Polyomaviridae		
Polyomavirus	LT	Immortalizes primary cells; required to induce but not to maintain transformation of primary cells
	mT	Transforms established cell lines; required to both induce and maintain transformation of primary cells
Simian virus 40	LT	Immortalizes primary cells; required to induce and maintain transformation of primary and established cells
	sT	Required under many conditions, depending on LT concentration, genetic background of recipient cells, and transformation assay; necessary for transformation of resting cells

or near a cellular gene and final recombination reaction(s) between largely nonhomologous sequences. Model systems have provided experimental support for these more efficient mechanisms.

Many of the cellular proto-oncogenes from which v-oncogenes are derived have been highly conserved throughout evolution; many vertebrate examples have homologs in yeast. The products of such genes must therefore fulfill functions that are indispensable for a wide variety of eukaryotic cells. Furthermore, as single copies of v-oncogenes are sufficient to transform cells, their functions must override those of the resident, cognate proto-oncogenes. Accordingly, v-oncogenes function as **dominant** transforming genes.

Members of the second class of viral oncogenes, such as adenovirus E1A and polyomavirus LT, are not obviously related to cellular genes. However, the products of these genes may contain short amino acid sequences also present in cellular proteins (for example, see Fig. 7.19). The precise origins of such oncogenes, like those of viruses themselves, therefore remain shrouded in mystery (see Chapter 10).

Functions of Viral Transforming Proteins

Many approaches have been employed to determine the functions of viral oncogene products. In some cases, the sequence of a viral transforming gene can immediately suggest the function of its protein product. For example, the genomes of certain herpesviruses and poxviruses include coding sequences that are closely related to cellular genes that encode growth factors, cytokines, and their receptors. Or the protein may contain predicted amino acid motifs characteristic of particular biochemical activities, such as tyrosine phosphorylation or sequence-specific DNA binding. In other cases, notably many retroviral v-oncogene products, it has been possible to identify important biochemical properties, such as enzymatic activity, binding to a hormone or growth factor, or sequence-specific binding to nucleic acids. Table 7.6 lists some examples of these

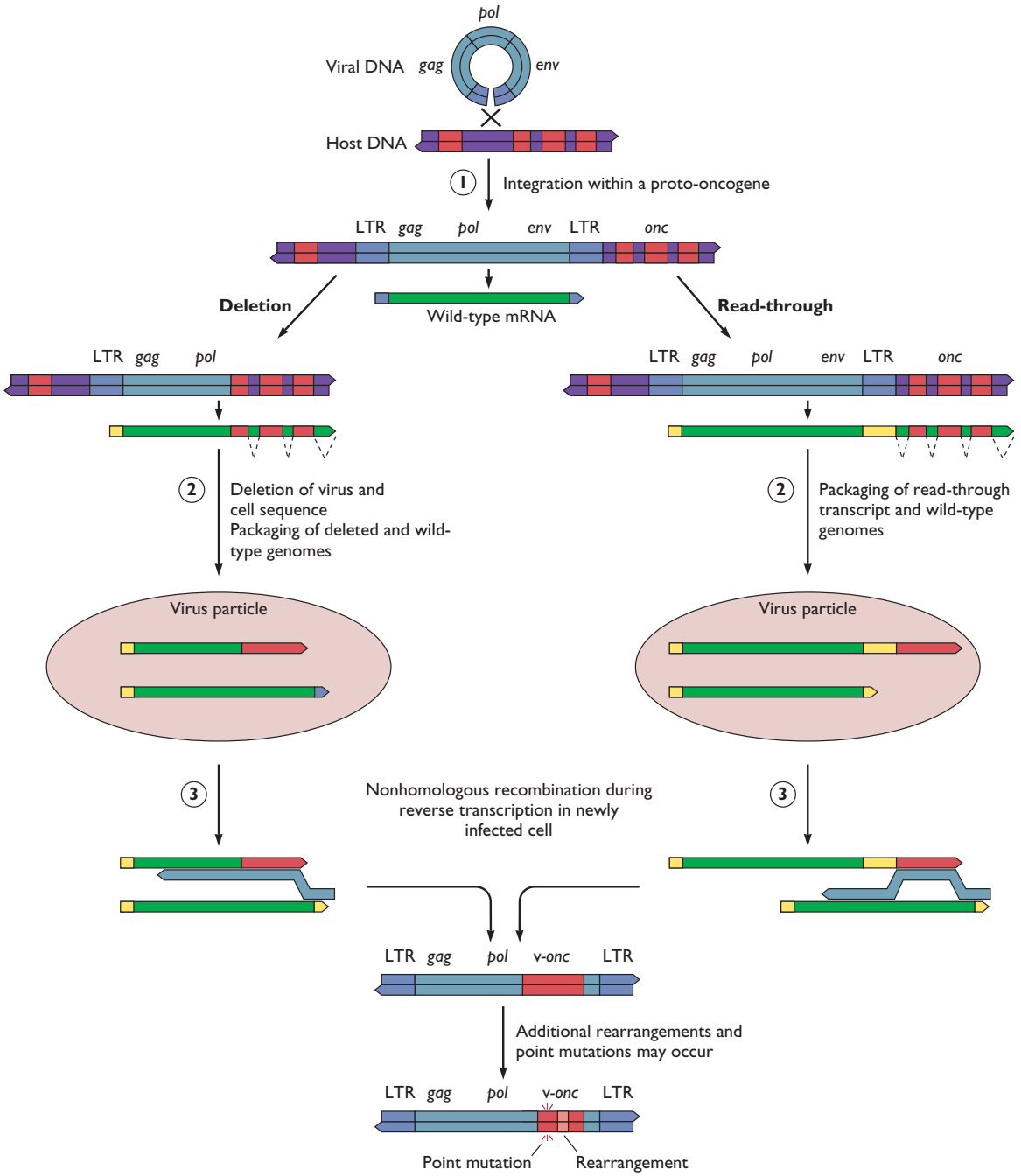


Figure 7.8 Possible mechanisms for oncogene capture by retroviruses. The first step in each of two mechanisms shown is integration of a provirus in or near a cellular gene (e.g., *onc*). The deletion mechanism (left) requires removal of the right end of the provirus, thereby linking cellular sequences to the strong viral transcriptional control region in the left LTR. The first recombination step in this mechanism therefore takes place at the DNA level. It leads to synthesis of a chimeric RNA, in which viral sequences from the left end of the provirus are joined to cellular sequences. Chimeric RNA molecules that include the viral packaging signal can be incorporated efficiently into virions as a heterozygote with a wild-type genome produced from another provirus in the same cell. A second recombination reaction, during reverse transcription (as described in Volume I, Chapter 7), is then required to add right-end viral sequences to the recombinant. At a minimum, these right-end sequences must include signals for subsequent integration of the recombinant viral DNA into the genome of the newly infected host cell, from which the transduced gene is then expressed. The read-through mechanism (right) does not require a chromosomal deletion. Viral transcription does not always terminate at the 3′ end of the proviral DNA, but continues to produce transcripts containing cellular sequences. Such chimeric transcripts can then be incorporated into virions together with the normal viral transcript. The cellular sequences can then be captured by recombination during reverse transcription, as indicated. Important additional mutations and rearrangements probably occur during subsequent virus replication. Adapted from J. M. Coffin et al. (ed.), *Retroviruses* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1997), with permission.

Table 7.6 Functional classes of oncogenes transduced by retroviruses[a]

Transduced oncogene[b]	Function of cellular homolog
Growth factors	
sis	Platelet-derived growth factor
Tyrosine kinase growth factor receptors	
erbB	Epithelial growth factor receptor
kit	Hematopoietic receptor; product of the mouse W locus
Hormone receptors	
erbA	Thyroid hormone receptor
G proteins	
H-*ras*, K-*ras*	GTPases
Adapter protein	
crk	Signal transduction
Nonreceptor tyrosine kinases	
src, abl	Signal transduction
Serine/threonine kinases	
mos	Required for germ cell maturation
akt	Signal transduction
Nuclear proteins	
jun, fos	Transcriptional regulator (AP-1 complex)
myc	Transcriptional regulator

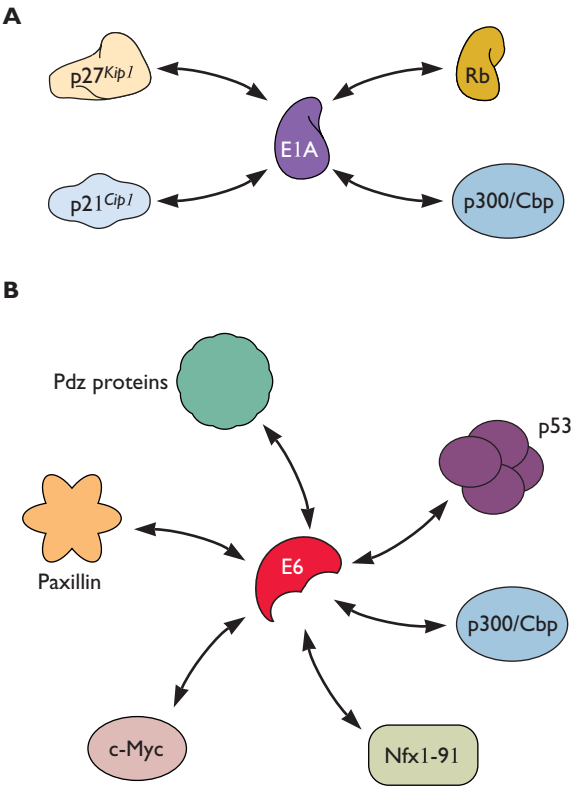
[a]Adapted from J. Benjamin and P. Vogt, *in* D. M. Knipe and P. M. Howley (ed.), *Fields Virology* (Lippincott-Raven Publishers, Philadelphia, PA, 1996).
[b]Only some representative examples are listed.

retroviral v-oncogenes and the proteins they encode, organized according to the known or deduced functions of their normal cellular homologs.

The breakthrough to understanding transformation by small oncogenic DNA viruses came with mutational analyses that correlated the transforming activities of viral gene products with binding to specific cellular proteins, notably tumor suppressors. The first such interaction identified was between sequences of adenoviral E1A proteins that are necessary for transformation and the cellular retinoblastoma tumor suppressor protein, Rb. The similar relationship between Rb binding and the transforming activities of simian virus 40 large T antigen (LT) and the E7 protein of oncogenic human papillomaviruses rapidly established the general importance of interaction with Rb in transformation. Interactions of transforming proteins of these virus with a second cellular tumor suppressor, the p53 protein, is also required. These observations established the importance of tumor suppressors (Fig. 7.6), which become inactivated (see below), in transformation by these DNA viruses. A second common feature is that their transforming proteins affect multiple cellular proteins and pathways (Fig. 7.9).

Viral transforming proteins exhibit great diversity in all their properties, from primary amino acid sequence to biochemical activity. They also differ in the number and

Figure 7.9 Schematic illustration of interactions of DNA virus transforming proteins with multiple cellular proteins. **(A)** The association of adenoviral E1A proteins with Rb family tumor suppressors, the histone acetyltransferases p300/Cbp, and the cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p21^{Cip1}$ has been implicated in tranformation: E1A protein substitutions that impair these interactions reduce or eliminate transforming activity. **(B)** The human papillomavirus type 16 or 18 E6 protein also interacts with p300/Cbp and tumor suppressors, in this case the p53 protein, and several that contain the Pdz domain and are localized at cell junctions. In addition, it associates with the transcriptional regulators c-Myc and Nfx1-91, which represses transcription of the gene encoding the protein component of telomerase. These interactions have been implicated in increased production of telomerase in cells synthesizing the E6 protein. In some cases, including p53 and Nfx1-91, the complex includes the cellular ubiquitin ligase E6-Ap, and the other cellular proteins are targeted for proteosomal degradation. Degradation of Pdz domain-containing proteins is also induced by the viral E6 protein, but E6-Ap does not appear to be required.



nature of the cellular pathways they alter. Despite such variation, viral proteins induce continuous cell proliferation, the definitive characteristic of transformation, by related mechanisms. Indeed, the best characterized fall into one of only two classes, permanent activation of cellular signal transduction cascades, or disruption of the circuits that regulate cell cycle progression.

Activation of Cellular Signal Transduction Pathways by Viral Oncogene Products

The products of transforming genes of both RNA and DNA viruses can alter cellular signal transduction cascades. The consequence is permanent activation of pathways that promote cell growth and proliferation. However, as discussed in subsequent sections, these viral proteins can intervene at various points in signaling cascades, and they operate in several different ways.

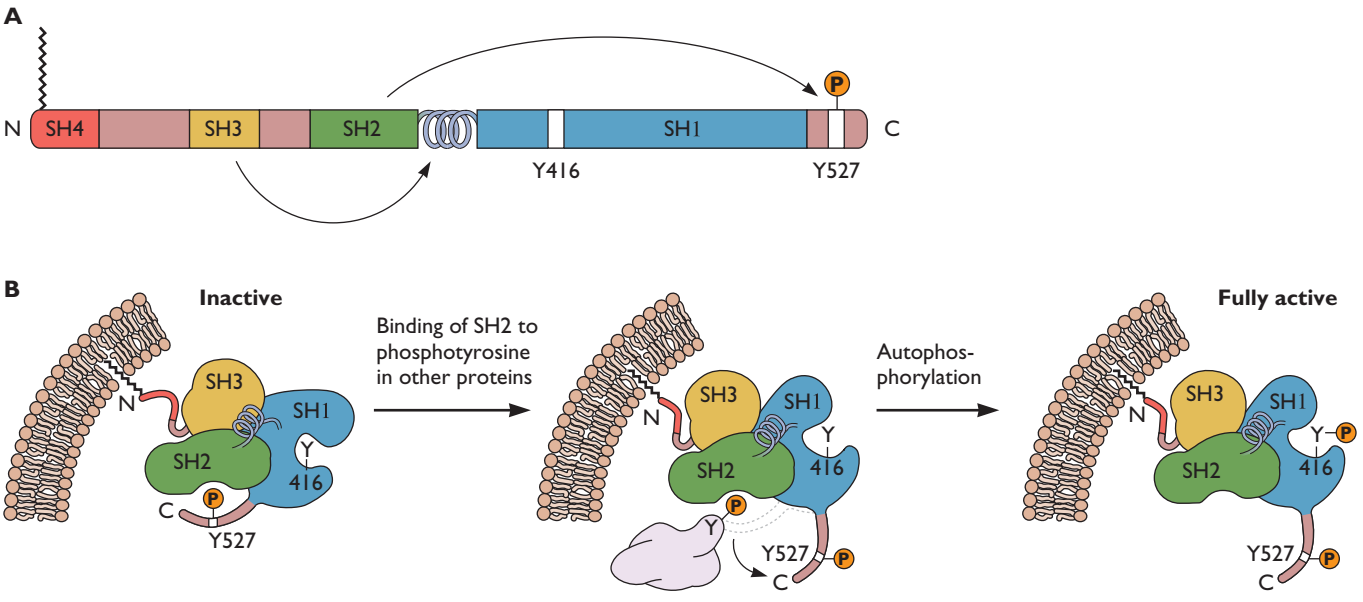
Viral Mimics of Cellular Signaling Molecules

The Transduced Cellular Genes of Acutely Transforming Retroviruses

The v-*src* paradigm. The protein product of v-*src* was the first retroviral transforming protein to be identified, when serum from rabbits bearing tumors induced by Rous sarcoma virus was shown to immunoprecipitate a 60-kDa phosphoprotein (pp60^{src}). The v-Src protein was soon found to possess protein tyrosine kinase activity, a property that provided the first clue that phosphorylation of cellular proteins can be critical to oncogenesis. The discovery of this protein tyrosine kinase led to the identification of a large number of other proteins with similar enzymatic activity and important roles in cellular signaling.

The Src protein contains a tyrosine kinase domain (SH1, for Src homology region 1) and two domains that mediate protein-protein interactions (Fig. 7.10A). The

Figure 7.10 Organization and regulation of the c-Src tyrosine kinase. (A) The functional domains of the protein. The SH4 domain contains the site for addition of the myristate chain that anchors the protein in the cell membrane. The SH2 and SH3 domains mediate protein-protein interactions by binding to phosphotyrosine-containing and proline-rich sequences, respectively. Arrows represent intramolecular interactions observed in the repressed-state crystal structures of Src. **(B)** The interactions and their reversal. When Y527 is phosphorylated, the C-terminal region of c-Src in which this residue lies is bound to the SH2 domain. This interaction brings a polyproline helix located between the SH2 and SH1 domains into contact with the SH3 domain, as illustrated at the top. Binding of SH3 to the helix deforms the kinase domain, accounting for the inactivity of the Y527-phosphorylated form of the protein. Such intramolecular associations maintain the kinase domain (SH1) in an inactive conformation. A conformational change that activates the kinase can be induced as shown, as well as by binding of the SH3 domain to proline-rich sequences in other proteins and probably by dephosphorylation of Y527 (see Fig. 7.15). Once released from the autoinhibited state in this way, Y416 in the kinase domain is autophosphorylated, a modification that stabilizes the active conformation of the SH1 domain. The v-Src protein is not subject to such autoinhibition, because the sequence encoding the C-terminal regulatory region of c-Src was deleted during transduction of the cellular gene.



first, the SH2 domain, binds to phosphotyrosine-containing sequences, whereas the SH3 domain has affinity for proline-rich sequences. Both domains are found in other proteins that participate in signal transduction pathways. One transforming protein, Crk, is made up of only an SH2 and an SH3 domain: it functions as an **adapter**, bringing other proteins in a signal transduction pathway together (Table 7.6). A fourth Src domain (SH4) includes the N-terminal myristoylation signal that directs Src to the plasma membrane. All four domains are required for transforming activity.

The Src kinase phosphorylates itself at specific tyrosine residues; these modifications regulate its enzymatic activity. For example, phosphorylation of Y416 in the kinase domain activates the enzyme, whereas phosphorylation of Y527 in the C-terminal segment inhibits activity. The crystal structures of cellular Src and another member of the Src family revealed the importance of the SH2 and SH3 domains in such regulation. As illustrated in Figure 7.10B, exchange of the intramolecular interaction of SH2 with Y527 for binding of the SH2 (or SH3) domains to phosphotyrosine- or polyproline-containing motifs, respectively, in **other** proteins initiates conformational changes that activate the kinase. This autoregulatory mechanism explains earlier findings that transduction and overproduction of the normal Src protein does not lead to cellular transformation, and that the constitutive oncogenic activity of v-*src* requires loss or mutation of the Y527 codon.

Soon after its kinase activity was first discovered, v-Src was shown to localize to focal adhesions, the areas where cells make contact with the extracellular matrix. This observation led to identification of a second protein tyrosine kinase enriched in these areas as a protein exhibiting increased tyrosine phosphorylation in v-Src-transformed cells. This focal adhesion kinase (Fak) and Src family proteins turned out to be crucial components of a signal transduction cascade normally controlled by cell adhesion. This pathway modulates the properties of the actin cytoskeleton, and hence cell shape and adhesion, and also signals to the Ras-Map kinase pathway that controls cell proliferation (Fig. 7.11). The constitutive activity of v-Src can therefore account for the morphological and growth properties of cells transformed by this oncogene product.

Other transduced oncogenes. The transduced oncogenes of retroviruses are homologs of cellular genes that encode many components of signal transduction cascades, from the external signaling molecules (e.g., v-Sis) and their receptors (v-ErbB and v-Kit) to the nuclear proteins at the end of the relay (v-Fos and v-Myc) (Table 7.6). It therefore seems likely that any positively acting protein in such a cascade has the potential to act as a transforming protein.

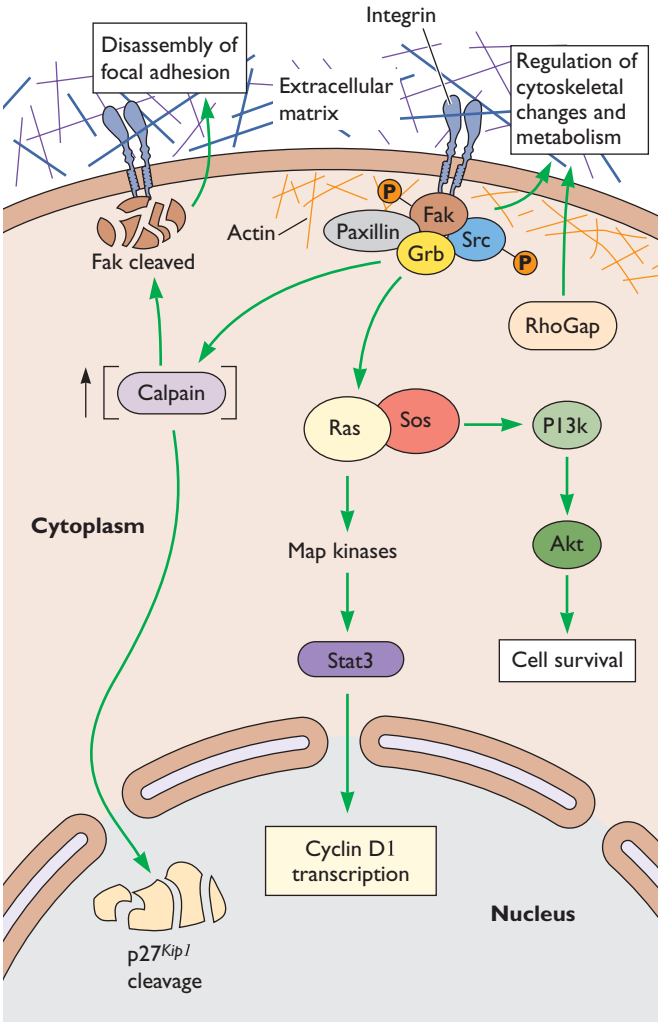


Figure 7.11 Regulation of cell proliferation and adhesion by Src. Both c-Src and v-Src are localized to focal adhesions, where they are associated with focal adhesion kinase (Fak) and adapter proteins, such as Grb2 and paxillin. These protein assemblies normally maintain contacts between the extracellular matrix via integrins and the actin cytoskeleton of the cell. When the Src tyrosine kinase is active, Fak is phosphorylated at specific sites and cleaved into several fragments by the protease calpain. These changes result in disruption of focal adhesions, and account for the changes in morphology and motility of v-Src transformed cells. Calpain-mediated proteolysis of Fak results at least in part, from increased translation of calpain mRNA induced by v-Src. Another substrate of this protease is the cyclin-dependent kinase inhibitor $p27^{Kip1}$. As shown, v-Src also induces transcription of the cyclin D1 gene via Ras, the Map kinase cascade, and the transcriptional activator Stat3. These responses to v-Src result in cell proliferation.

The oncogenic potential of such transduced oncogenes is realized by two nonexclusive mechanisms, genetic alterations, which lead to constitutive protein activity, and inappropriate production, or overproduction of the protein. The former mechanism applies to most of the retroviral

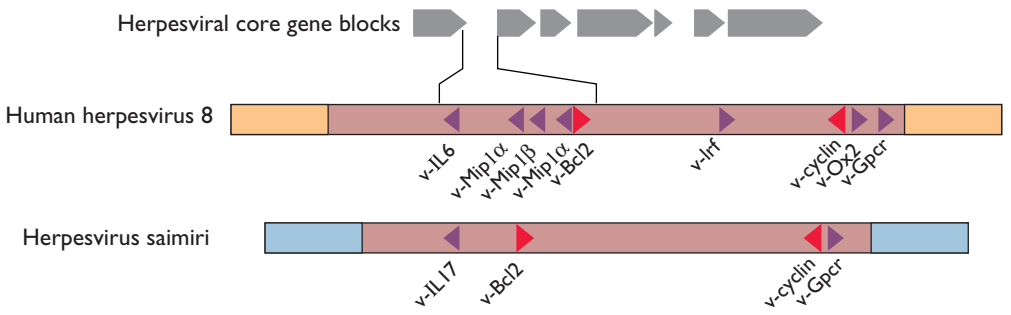
oncogenes listed in Table 7.6. For example, like other small guanine nucleotide-binding proteins, Ras normally cycles between an active, guanosine triphosphate (GTP)-bound and an inactive, guanosine diphosphate (GDP)-bound conformation. Such cycling is under the control of GTPase-activating and guanine nucleotide exchange proteins. The latter proteins (e.g., Sos [Fig. 7.3]) stimulate the release of GDP once bound GTP has been hydrolyzed. However, v-Ras proteins fail to hydrolyze GTP efficiently and therefore persist in the active, GTP-bound conformation that relays signals to downstream pathways, such as the Map kinase cascade (Fig. 7.3). Such constitutive activity is the result of mutations that lead to substitution of specific, single amino acids in the protein (at residues 12, 13, or 61) and render the protein refractory to the GTPase-activating protein. Analogous mutations are common in certain human tumors, such as colorectal cancers (Box 7.2), and were the first discrete genetic changes in a proto-oncogene linked to neoplastic disease in humans.

Less commonly, over- or misexpression of the transduced oncogene is sufficient to disrupt normal cell behavior. This type of mechanism is best characterized for *myc*. In normal cells, the expression of this gene is tightly regulated, such that the c-Myc protein is made only during a short period in the G_1 phase of the cell cycle, and is not synthesized when cells withdraw from the cycle, or differentiate. The production of even small quantities of Myc or Myc-fusion proteins specified by retroviruses, such as avian myelocytoma virus MH2 (Fig. 7.7), at an inappropriate time results in cellular transformation.

Other Viral Homologs of Cellular Genes

The genomes of some larger DNA viruses also contain coding sequences that are clearly related to cellular genes encoding signal transduction molecules. Human herpesvirus 8, a gammaherpesvirus related to Epstein-Barr virus, has been strongly implicated in the etiology of Kaposi's sarcoma, a malignancy common in acquired immunodeficiency syndrome (AIDS) patients, and primary effusion lymphoma (see Chapter 6). Its structural proteins and viral enzymes are closely related to those of other herpesviruses. The genome also contains several homologs of cellular genes that encode signaling proteins, which are clustered in regions interspersed among blocks of genes common to all herpesviruses (Fig. 7.12). Among the best characterized is the gene (v-*gpcr*) specifying a guanine nucleotide-binding protein-coupled receptor that is most closely related to a cellular receptor for CXC chemokines. The v-*gpcr* gene induces morphological transformation when introduced into mouse fibroblasts or endothelial cells in culture, and formation of tumors resembling Kaposi's sarcoma in transgenic mice. Cellular chemokine receptors bind chemokines released at sites of inflammation to activate signal transduction. In contrast, v-Gpcr is fully active in the absence of any ligand, and can trigger specific cellular signal transduction pathways, for example, a mitogen-activated protein (Map) kinase cascade, and activation of transcription of cellular and viral genes. The latter include genes encoding other viral proteins closely related to the cellular signaling protein vascular endothelial growth factor (v-Veg) and interleukin-6 (v-IL-6).

Figure 7.12 Some genes of human herpesvirus 8 and herpesvirus saimiri that are homol ogous to cellular genes. The two viral genomes are shown in orientations that align genes conserved among herpesviruses, the core gene blocks shown at the top. The conserved genes encode proteins needed for virus reproduction and assembly. These genomes carry homologs of cellular genes (arrowheads) interspersed among the core gene blocks. Those shown in purple are related to cellular chemokines (v-IL-6, v-IL-17, and macrophage inflammatory factor [v-Mip1α or 1β]), chemokine receptors (v-Gpcr; see the text), or other signaling molecules (interferon-responsive protein [v-Irf], and an N-Cam family transmembrane protein that participates in intercellular signaling [v-Ox2]). The human herpesvirus 8 v-IL-6 protein blocks the action of interferon (Table 3.8) and can also induce proliferation of B cells. Viral genes shown in red are related to cellular genes that encode proteins that regulate cell proliferation or apoptosis, cyclin D [v-cyclin; see the text] and Bcl-2 [v-Bcl2].



A characteristic feature of Kaposi's sarcoma is extensive **angiogenesis**, the proliferation of new blood vessels, which is essential for tumor progression. Angiogenesis appears to be the result of the production of large quantities of v-Veg, for example, by spindle cells that proliferate in the tumor. As discussed in Chapter 4, IL-6 is an important modulator of the adaptive immune response, and a B-cell mitogen. Like its human counterpart, v-IL-6 can maintain proliferation of those cultured B-cell lines that depend on this cytokine. It has been implicated in autocrine stimulation of proliferation of human herpesvirus type 8-infected B cells in primary effusion lymphoma. These properties suggest that the v-*gpcr* gene plays a critical role in human herpesvirus 8 oncogenesis.

Alteration of the Production or Activity of Cellular Signal Transduction Proteins

Insertional Activation by Nontransducing Retroviruses

Most tumors induced by nontransducing retroviruses arise as a result of increased transcription of cellular genes located in the vicinity of integrated proviruses. This mechanism of oncogenesis is known as **insertional activation**. It has been implicated in a leukemia-like disease developed by patients participating in a gene transfer trial (Box 7.10). As in the case of the transducing retroviruses, Rous sarcoma-derived avian viruses played a seminal role in delineating the mechanisms of insertional activation. The original stocks of viruses isolated by Rous included replication-competent leukosis viruses, called Rous-associated viruses 1 and 2. These viruses do not carry an oncogene, but in young chickens they induce B-cell tumors that originate in the bursa of Fabricius, the major lymphoid organ of these birds. A provirus was found integrated in the vicinity of the cellular *myc* gene in each of these tumors. Although the exact integration site varies from tumor to tumor, many integration sites lie in the intron between exon 1 (a noncoding exon) and exon 2 (Fig. 7.13). However, in some tumors proviruses are located upstream or downstream of the cellular *myc* gene. In this avian system, inappropriate synthesis of the cellular Myc protein is associated with lymphomagenesis; no changes in the protein are required. Analysis of the sites of proviral DNA integration and the gene products formed in these tumors provided the first

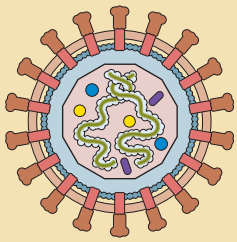
BOX 7.10

WARNING

Inadvertent insertional activation of a cellular gene during gene transfer

Retroviruses have long been considered likely to be valuable vectors for gene therapy. One reason is that integration of the retroviral vector into the host genome results in permanent delivery of the potentially therapeutic gene to all infected cells and their descendents. However, an outcome detected in one clinical trial indicates that this property is a double-edged sword.

A French trial was examining the potential of gene therapy using a vector based on mouse Moloney leukemia virus to treat children with severe combined immunodeficiency (SCID). This disease is caused by mutation in a single gene on the X chromosome, and the only therapies available are associated with severe, often fatal side effects. A trial with 10 children with the disease, who were given gene transfer as early as possible, initially appeared to be very successful: in most cases, the immune system was restored without side effects. However, early in 2002, one patient was found to have developed a T-cell leukemia-like disease. The overproliferating T cells were monoclonal: all carried a provirus integrated into the



same site on chromosome 11, near a gene (*Imo2*) that is expressed abnormally in a form of childhood acute lymphoblastic leukemia.

The monoclonal origin of the T cells that proliferated in this child indicates that proviral insertion contributed to the development of the disease. It initially seemed likely that other factors also did so: a predisposition to childhood cancers was evident in other members of the child's family. Sadly, however, two other children participating in the same trial and one in a similar trial in the United Kingdom have since been diagnosed with leukemia associated with insertion of the provirus in the same chromosome 11 site.

This outcome temporarily halted this and numerous other clinical trials of gene transfer using retroviral vectors in the United States and Europe. The National Institutes of Health then recommended that retroviral therapy for SCID be used only as a last resort. Further study of the preferences for integration site selection by retroviral vectors and study of the transduced progenitor cells of patients in the SCID trials is providing additional insights into the factors that affect the *in vivo* fate of transduced progenitor cells.

Deichmann, A., S. Hacein-Bey-Abina, M. Schmidt, A. Garrigue, M. H. Brugman, J. Hu, H. Glimm, G. Gyapay, B. Prum, C. C. Fraser, N. Fischer, K. Schwarzwaelder, M.-L. Siegler, D. de Ridder, K. Pike-Overzet, S. J. Howe, A. J. Thrasher, G. Wagemaker, U. Abel, F. J. T. Staal, E. Delabesse, J.-L. Villeval, B. Aronow, C. Hue, C. Prinz, M. Wissler, C. Klanke, J. Weissenbach, I. Alexander, A. Fischer, C. von Kalle, and M. Cavazzana-Calvo. 2007. Vector integration is nonrandom and clustered and influences the fate of lymphomopoiesis in SCID-XI gene therapy. *J. Clin. Investig.* **117:**2225–2232.

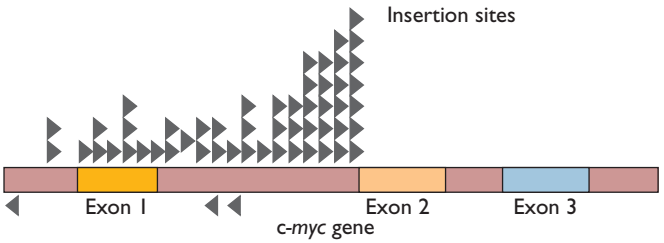


Figure 7.13 Insertional activation of c-*myc* by avian leukosis viruses. In avian cells derived from avian leukosis virus-induced B-cell lymphomas, individual proviral integration sites are clustered as shown (arrowheads) within noncoding exon 1 and intron 1 of the *myc* gene. Most integrated proviruses are oriented in the direction of *myc* transcription (arrowheads pointing to the right). Adapted from J. Nevins and P. Vogt, p. 301–343, *in* B. N. Fields et al. (ed.), *Fields Virology* (Lippincott-Raven Publishers, Philadelphia, PA, 1996), with permission.

evidence for two types of insertional activation, promoter insertion and enhancer insertion (Fig. 7.14).

The first mechanism, **promoter insertion**, results in production of a chimeric RNA in which sequences transcribed from the proviral LTR are linked to cellular proto-oncogene sequences. If transcription originates from the left-end LTR, some viral coding sequences may be included. However, transcription from the right-end LTR seems to be more common, and in these cases the proviral left-end LTR has usually been deleted. Such deletion is probably important, because transcriptional read-through from the left-end promoter reduces transcription from the right-end LTR. Proviral integration often occurs within the cellular proto-oncogene, truncating cellular coding sequences and eliminating noncoding domains that may include negative regulatory sequences. Some chimeric transcripts formed by promoter insertion are analogous to the intermediates required for oncogene capture by the transducing retroviruses (compare Fig. 7.14 and 7.8). Indeed, it has been possible to isolate newly generated, oncogene-transducing retroviruses from tumors arising as a result of promoter insertion.

In the second type of insertional activation, **enhancer insertion**, viral and cellular transcripts are not fused. Instead, activation of the cellular gene is mediated by the strong viral enhancers, which increase transcription from the cellular promoter (Fig. 7.14). Because enhancer activity is independent of orientation and can be exerted over long distances, the provirus need not be oriented in the same direction as the proto-oncogene, and may even lie downstream of it.

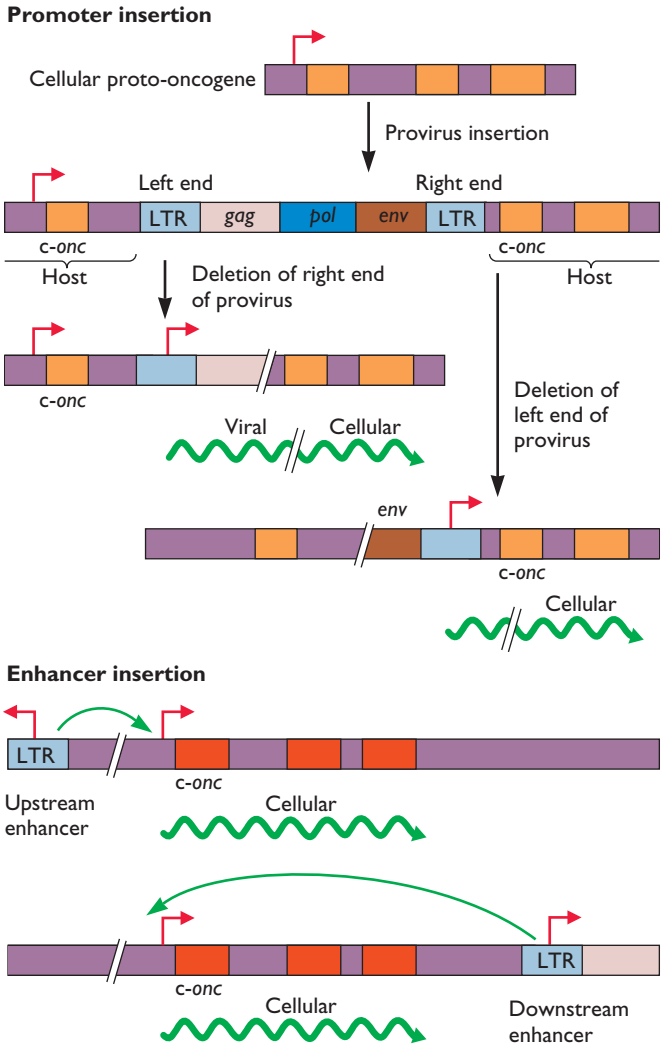


Figure 7.14 Mechanisms for insertional activation by nontransducing oncogenic retroviruses.

Viral Proteins That Alter Cellular Signaling Pathways

Some viruses alter the growth and proliferation of infected cells by the action of viral signal transduction proteins that are not obviously related in sequence to cellular proteins. Some of these viral proteins operate by mechanisms well established in studies of cellular signaling cascades, but others appear to modulate such cascades in virus-specific ways.

Constitutively active viral "receptors." The genomes of several gammaherpesviruses encode membrane proteins that initiate signal transduction. The best-understood example of this mechanism is provided by Epstein-Barr virus latent membrane protein 1 (LMP-1), one of several viral gene products implicated in immortalization of human B lymphocytes (Table 5.4). LMP-1 is the only one of this set of viral proteins that exhibits transforming activity: it induces typical transformed phenotypes when synthesized

in fibroblasts or epithelial cells in culture, as well as lymphomas in transgenic mice. LMP-1 is an integral plasma membrane protein that functions as a constitutively active receptor. In the absence of any ligand, LMP-1 oligomerizes to form patches in the cellular membrane and binds to the same intracellular adapter proteins as the active, ligand-bound form of members of the tumor necrosis factor receptor family (Fig. 7.15). When localized to the plasma membrane, the C-terminal segment of LMP-1 to which these proteins bind, is sufficient for both immortalization of B cells and activation of cellular transcriptional regulators. This viral protein activates the kinase cascade that normally releases Nf-κb from association with cytoplasmic inhibitors, activates the transcriptional regulator Ap-1, and activates signaling via the lipid kinase phosphatidylinositol 3-kinase and protein kinase B (Akt) (Fig. 7.15). Activation of these pathways accounts for the increased expression of most of the cellular genes that is observed in LMP-1-producing cells, and consequent alteration in the properties of these cells. These changes include increased production of certain cell adhesion molecules and cell proliferation.

Viral adapter proteins. Members of both the *Polyomaviridae* and the *Herpesviridae* encode proteins that permanently activate cellular signal transduction pathways as a result of binding to Src family tyrosine kinases (Table 7.7). This mechanism was first encountered in studies of the mouse polyomavirus middle T protein (mT), a viral early-gene product with no counterpart in the genome of the related polyomavirus simian virus 40 (Fig. 7.16A). This protein can transform established rodent cell lines (Table 7.5) and induce endotheliomas (Box 7.1) when overexpressed in transgenic animals.

mT protein is located in the plasma membrane of transformed or infected cells, where it associates with c-Src (or the related tyrosine kinases, c-Yes and c-Fyn). No increase in c-Src concentration is observed in mT-transformed cells; when c-Src is bound to mT, its catalytic activity is increased by an order of magnitude. This change in tyrosine kinase activity is the result of inhibition of the autoregulatory mechanism, in which binding of the SH2 domain of c-Src to phosphorylated Y527 inhibits kinase activity (Fig. 7.9). mT appears to be able to sequester the Y527-containing segment of c-Src and induce its dephosphorylation (see below) to stabilize the active conformation of the kinase. However, it is unlikely that mT can reverse the interaction of these c-Src domains in the inactive kinase, for only about 10% of the c-Src molecules, and a similar fraction of mT molecules, are bound to each other in transformed or infected cells. Such nonstoichiometric association suggests that mT can bind to c-Src molecules only during their transient activation by normal cellular mechanisms.

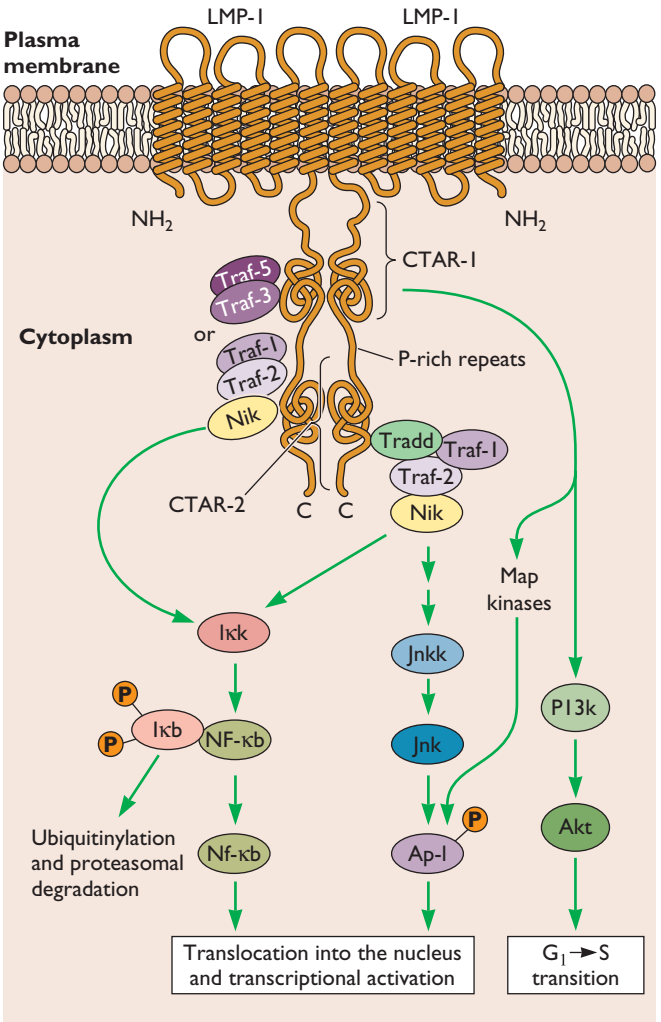


Figure 7.15 Constitutive signaling by Epstein-Barr virus latent membrane protein 1. LMP-1, which possesses six membrane-spanning segments but no large extracellular domain, oligomerizes in the absence of ligand, a property represented by the LMP-1 dimer depicted. The long cytoplasmic C-terminal domain of the viral protein contains three segments implicated in the activation of signaling, designated C-terminal activation regions (CTAR) 1 and 2, and proline (P)-rich repeats. As shown, multiple members of the tumor necrosis factor receptor-associated protein family (Trafs) bind to CTAR-1. Binding of Trafs leads to activation of the protein kinase Nik and Iκ-kinase (Iκk), and ultimately of Nf-κb, via induction of release of Nf-κb from association with the cytoplasmic inhibitors. The same pathway is activated in uninfected cells by binding of tumor necrosis factor to its receptor. The CTAR-2 domain of LMP-1 is responsible for activation of Ap-1 via the Jun N-terminal kinase (Jnk) pathway. The first reaction appears to be indirect association of this region of LMP-1 with Trafs via a second cellular protein (Tradd), a reaction that may lead to activation of both Nf-κb and Ap-1, as shown. The Traf-binding domain of CTAR-1 also induces activation of signaling via phosphatidylinositol 3-kinase (PI-3k) and the protein kinase Akt, and of the Map kinase cascade. These responses to LMP-1 are required for transformation of rat fibroblasts.

Table 7.7 Viral proteins that activate Src family tyrosine kinases

Viral protein	Cellular tyrosine kinase(s) bound	Consequences of interaction
Herpesvirus saimiri 484 tyrosine kinase-interacting protein (Tip)	Lck	Activation of the cellular kinase and signal transduction; required for viral transformation of primary T cells in culture and oncogenic disease *in vivo*
Mouse polyomavirus mT	Src, Fyn, Yes	Activation of mT-bound kinase; tyrosine phosphorylation of mT and adapter proteins bound to it; transformation of established cell lines

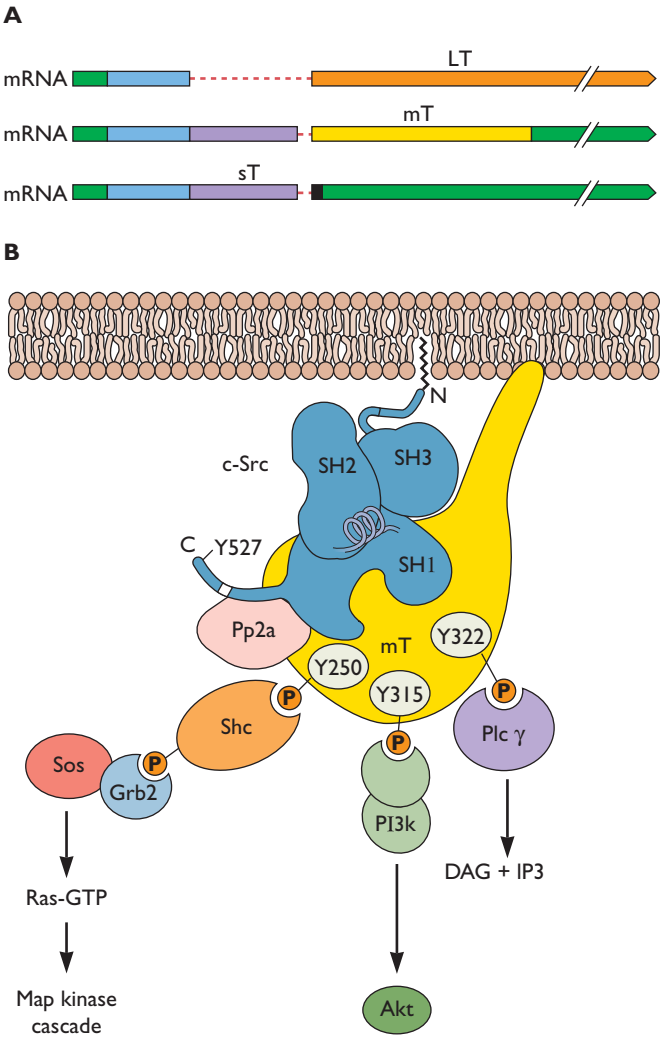


Figure 7.16 Polyomavirus mT protein, a virus-specific adapter. **(A)** The mouse polyomavirus early protein-coding sequences are shown as boxes within the mRNAs from which the proteins are synthesized. The mRNAs are drawn as arrows, in which the arrowheads indicate the site of polyadenylation and the dashed pink lines indicate the introns removed during RNA splicing. The three proteins produced from these mRNAs, LT, mT, and sT, share an N-terminal sequence but carry unique C-terminal sequences as a result of alternative splicing of early transcripts. **(B)** mT binds to c-Src at the plasma membrane and to protein phosphatase 2A (Pp2A). Presumably as a result of formation of the ternary complex, c-Src is trapped in the active conformation and Y527 is unphosphorylated. Consequently, mT-bound Src is catalytically active and phosphorylates specific tyrosines in mT. These are then bound by cellular proteins that contain phosphotyrosine-binding motifs, such as Shc, phospholipase C-γ (PlC-γ), and phosphoinositol 3-kinase (P_i3k). These proteins can then be phosphorylated by Src and activated. The lipids produced upon activation of Plc-γ act as second messengers, relaying signals to various pathways, while P_i3k activates signaling via the protein kinase Akt.

While it remains a mystery why so little of the mT protein is associated with cellular tyrosine kinases, the permanent alteration of cell proliferation induced via such a small fraction of these cellular enzymes is remarkable.

The mT protein also binds to cellular protein phosphatase 2A via an N-terminal sequence that is also present in sT (Fig. 7.16A). Binding of mT to this cellular enzyme appears to be necessary for association of the viral protein with c-Src. This requirement ensures that the phosphatase is brought into close physical association with c-Src, and accounts for the fact that Y527 is dephosphorylated in mT-bound c-Src molecules.

It was initially surprising that mT-transformed cells do not contain elevated levels of phosphotyrosine, despite activation of c-Src family kinases. It is now clear that mT itself is a critical substrate of the cellular enzyme: phosphorylation of specific mT tyrosine residues by activated c-Src allows a number of cellular proteins that contain phosphotyrosine-binding domains to bind to mT (Fig. 7.16B). These proteins include the adapter Shc and phospholipase Cγ, an enzyme responsible for production of lipid second messengers. In all cases, substitutions that disrupt binding of the cellular protein to mT impair the transforming activity of the viral protein. When bound to mT, such signaling proteins are phosphorylated by the activated c-Src kinase to trigger signal transduction, for example by activation of Ras and the Map kinase pathway (Fig. 7.16B). Consequently, mT both bypasses the normal mechanism by which the kinase activity of Src is regulated, and also serves as a virus-specific adapter, bringing together cellular signal transduction proteins when they would not normally be associated.

Alteration of the Activities of Cellular Signal Transduction Molecules

Alteration of cellular plasma membrane receptors. Many signal transduction cascades are initiated by binding of external growth factors to the extracellular portions of cell surface receptor tyrosine kinases. Ligand-bound receptors are internalized rapidly (within 10 to 15 min) by endocytosis. Following acidification of the endosomes, the ligand is released and all but a small fraction of the receptor molecules are usually degraded. As a result, the initial signal is short-lived. The E5 protein of bovine papillomaviruses is of interest in this context as it interferes with the mechanisms that control the function of this class of receptors.

The E5 protein of bovine papillomavirus type 1, a hydrophobic molecule of only 44 amino acids, is the major transforming protein of this virus (Table 7.5). E5 contributes to transformation of fibroblasts by binding to the platelet-derived growth factor receptor. The E5 protein is a dimer that accumulates in internal compartments of the host cell membranes, where it induces ligand-independent dimerization of the receptor, and hence activation of its tyrosine kinase and the downstream signaling relay (Fig. 7.3). The E5 protein binds stably to the transmembrane domain and an adjacent internal segment of the cellular receptor, in contrast to the natural ligand, which binds to the extracellular domain. Synthesis of the E5 protein has also been reported to increase the activity of phosphoinositol-3 kinase and c-Src, independently of effects on signaling from the platelet-derived growth factor receptor. These mechanisms are likely to be important in the oncogenicity of the virus in its natural hosts, in which it induces fibropapillomas (Box 7.1).

Other papillomaviruses, including many human serotypes, cause epithelial tumors by transforming cells that lack the platelet-derived growth factor receptor. It has been reported that the E5 protein of human papillomavirus type 16, which is mitogenic in primary keratinocytes, can bind to and activate the epidermal growth receptor. However, whether this activity of E5 contributes to transformation remains a matter of debate (Box 7.11).

Nontransducing retroviruses can also activate cell surface receptors, because these cellular gene products may be altered by provirus integration. In certain chicken lines, Rous-associated virus 1 induces erythroblastosis instead of lymphoma (Box 7.1). These tumors contain intact, nondefective proviruses integrated in the cellular *erb*B gene, which encodes the cell surface receptor for epidermal growth factor. The proviral integrations are clustered in a region that encodes the extracellular portion of this receptor, and read-through transcription produces chimeric RNAs (Fig. 7.14). The proteins synthesized from these RNAs are truncated growth factor receptors that lack the ligand-binding domain. Consequently, they produce a constitutive mitogenic signal. The v-*erb*B gene captured by transducing retroviruses encodes a protein with a similar truncation.

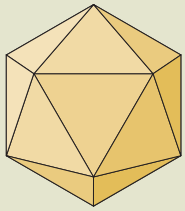
Inhibition of cellular protein phosphatase 2A. In preceding sections, we have discussed transformation by viral gene products as a result of permanent or prolonged

BOX 7.11 DISCUSSION

Similarities and differences between the E5 proteins of bovine and high-risk human papillomaviruses

The human and bovine papillomavirus E5 proteins share several properties, including their hydrophobic nature, localization to membranes of internal compartments, and the ability to transform fibroblasts in culture to anchorage-independent growth. Like the bovine papillomavirus (BPV) E5 protein, that of the high-risk human papillomavirus type 16 (HPV-16) has also been reported to bind to growth factor receptors, such as the epidermal growth factor and platelet-derived gowth factor receptors. However, whether these activities mediate transformation by the HPV-16 E5 protein was not clear; indeed, it is controversial.

In contrast to the BPV protein, the HPV-16 E5 protein is synthesized in only small quantities in mammalian cells in



culture, because its coding sequence contains a high frequency (40%) of rarely used codons. This property hampered studies of the HPV-16 protein, in particular direct comparison of its activities to those of the BPV protein.

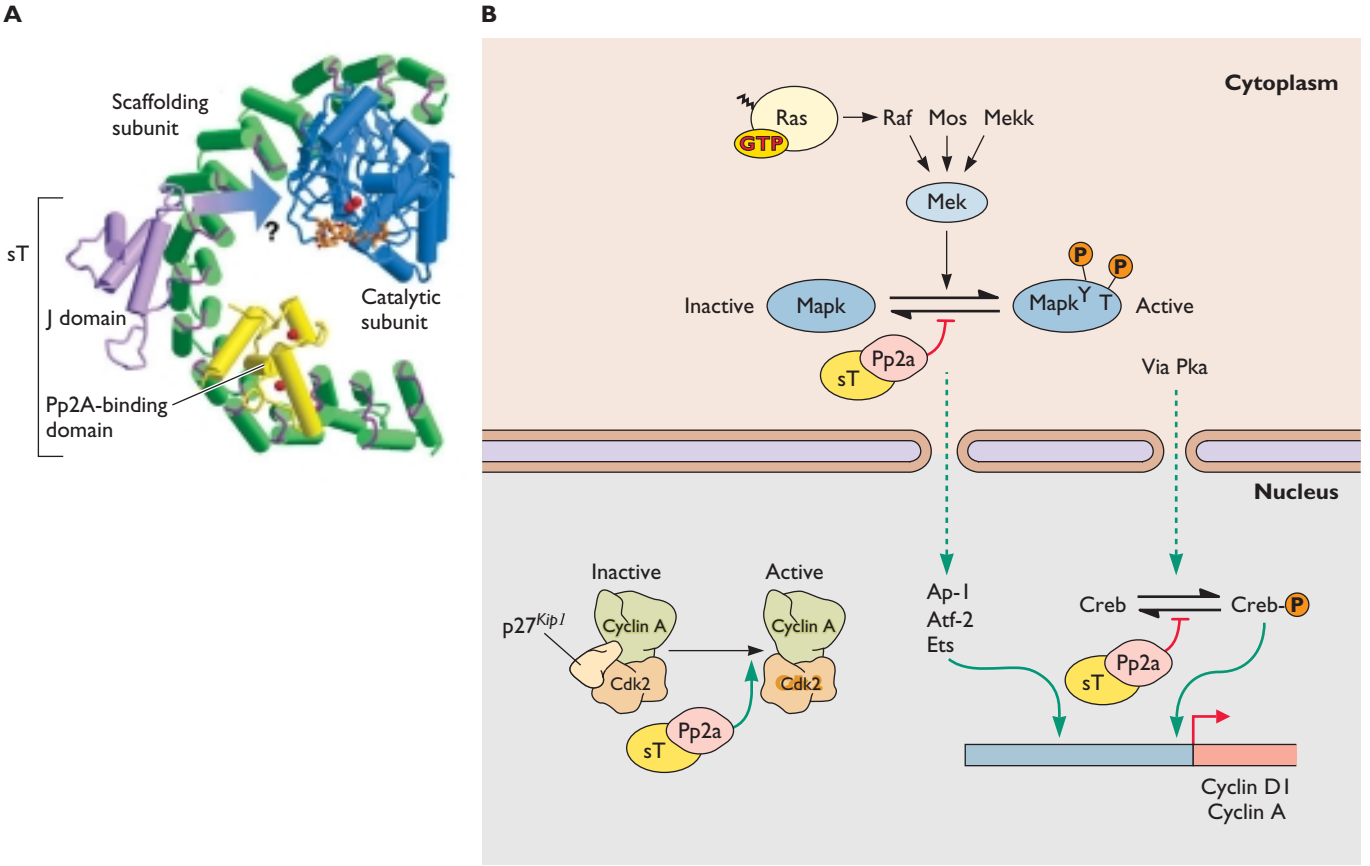
To overcome this problem, an HPV-16 E5 gene containing codons commonly used in mammalian cells was constructed. This codon-optimized gene was expressed stably and as efficiently as the BPV E5 gene in mouse NIH 3T3 cells. Under these conditions, the BPV E5 protein was observed to activate growth factor receptors, phosphoinositide 3-kinase, and c-Src, as expected from previous studies. In contrast, the HPV-16 protein did **not** activate any of these signaling molecules. Consequently, it can be concluded that the human and bovine papillomavirus E5 proteins operate by different mechanisms.

Suprynowicz, F. A., G. L. Disbrow, V. Simic, and R. Schlegel. 2005. Are transforming properties of the bovine papillomavirus E5 protein shared by E5 from high-risk human papillomavirus 16? *Virology* **332:**102–113.

activation of signal transduction pathways that control cell proliferation. In normal cells, such signaling is a transient process, for the molecular components are reset to the ground state once they have transmitted the signal. Inhibition of the reactions that terminate signaling therefore can also contribute to transformation, a mechanism exemplified by the sT protein of polyomaviruses such as simian virus 40.

Small T (sT) protein is not necessary for transformation of many cell types, but can stimulate transformation by simian virus 40 LT. sT is required for the transformation of resting cells and stimulates them to proliferate. In both infected and transformed cells, the sT protein binds to protein phosphatase 2A, a widespread, abundant serine/threonine protein phosphatase. This protein is a heterotrimer, composed of a two-subunit core enzyme bound to one of a substantial number of regulatory subunits. Structural and biochemical studies indicate that sT binds via two domains to the scaffolding subunit of the core enzyme (Fig. 7.17A). This interaction inhibits phosphatase activity *in vitro*, probably by blocking access of substrates to the active site

Figure 7.17 Inhibition of protein phosphatase 2A by simian virus 40 small T antigen. (A) Model of small T antigen bound to the core enzyme, which comprises a catalytic (blue) and a scaffolding (green) subunit. The sT domain shown in yellow is that required for binding to protein phosphatase 2A. The structure of this segment of sT bound to the core enzyme was determined by X-ray crystallography. Interactions with the N-terminal J domain, which increases the affinity with which sT binds to the phosphate, was modeled based on the structure of this domain when it is present in LT. In addition to inhibiting phosphatase activity, binding of sT may prevent association of a regulatory subunit: the binding sites of such subunits and sT overlap one another in the scaffolding subunit. Adapted from Y. Chan et al., *Nat. Struct. Mol. Biol.* **14:**527, 2007, with permission. Courtesy of Y. Shi, Princeton University. **(B)** Inhibition of the activity of protein phosphatase 2A (Pp2A) by sT results in activation of cellular transcriptional regulators, via the Map kinase pathway (e.g., activator protein 1 [Ap-1] and activating transcription factor 2 [Atf-2]). In addition, dephosphorylation of activated cyclic AMP response element-binding protein (Creb) within the nucleus is inhibited. Production of sT within cells stimulates cyclin D1 and cyclin A transcription. Binding of sT to protein phosphatase 2A also induces a large increase in the activity of cyclin A-dependent Cdk2, concomitant with inhibition of dephosphorylation of the cyclin-Cdk inhibitor $p27^{Kip1}$ (Fig. 7.5) and degradation of this protein.



in the catalytic subunit. As the viral protein binds with lower affinity, it cannot displace the regulatory subunit efficiently and acts primarily by inhibiting the activity of the core enzyme. One important consequence is failure to inactivate Map kinases, a process normally accomplished by the dephosphorylation of serine threonine or tyrosine residues (Fig. 7.17B). Consequently, sT increases the activity of sequence-specific transcriptional activators that are substrates of Map kinases, such as activator protein 1. The viral protein also prevents dephosphorylation and inactivation of the cyclic AMP-responsive element-binding protein. The increased activities of these transcriptional stimulators lead to synthesis of a G_1-phase and an S-phase cyclin, cyclins D1 and A, respectively, thereby circumventing the need for growth factors or other mitogens during transformation by simian virus 40.

Disruption of Cell Cycle Control Pathways by Viral Oncogene Products

One end point of many signal transduction pathways is the transcription of genes that encode cell cycle regulatory proteins. Consequently, **permanent activation** of such pathways by viral proteins, via any of the mechanisms described in the previous section, can result in an increased rate of cell growth and division or in proliferation of cells that would normally be in the resting state. Other viral proteins intervene directly in the intricate circuits by which cell cycle progression is mediated and regulated.

Abrogation of Restriction Point Control Exerted by the Rb Protein

The Restriction Point in Mammalian Cells

In mammalian cells, passage through G_1 into S and reentry into the cell cycle from G_0 depend on extracellular signals that regulate growth, termed **mitogens.** Late in G_1, cells responding to such external cues become committed to enter S, and to divide and complete the cell cycle; during this period, they are refractory to mitogens. Cells that have entered this state are said to have passed the G_1 **restriction point** (Fig. 7.5). Normal cells respond to mitogenic signals by mobilization of the G_1 Cdks that contain D-type cyclins. Expression of genes that encode one or more of these cyclins is induced by such signals via the Ras/Map kinase pathway (Fig.7.18A). The activation of Cdks by assembly with a cyclin D protein also depends on mitogenic stimulation, which suppresses production, and induces degradation, of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ by signaling via Akt (Fig. 7.18A). When such stimulation is continuous, Cdk activity appears at mid-G_1 and increases to a maximum near the G_1-to-S phase transition (Fig. 7.5). Such activity must be maintained until the restriction point has been passed, but then becomes dispensable. This property implies that the kinase activity of the cyclin D-dependent Cdks is necessary for exit from G_1. The best-characterized substrates of these kinases are the retinoblastoma (Rb) and the related p107 and p130 proteins. Many lines of evidence indicate that cyclin D-dependent Cdks initiate the transition through the restriction point by phosphorylation of Rb. For example, inhibition of cyclin D synthesis or function prevents entry into S phase in Rb-containing cells, but this cyclin is not required in Rb-negative cells. As discussed in Volume I , Chapter 9, the Rb protein controls the activity of members of the E2f family of sequence-specific transcriptional regulators.

Hypophosphorylated Rb present at the beginning of G_1 binds to specific members of the E2f family. Because Rb is a transcriptional repressor, these complexes inhibit transcription of E2f-responsive genes (Fig. 7.18B). The Rb protein is phosphorylated at numerous sites by G_1 cyclin-Cdks. Phosphorylated Rb can no longer bind to E2f, which therefore becomes available to activate transcription from E2f-responsive promoters (Fig. 7.18B). Such promoters include those of the genes encoding the kinase Cdk2, the cyclins that associate with this kinase, and E2f proteins themselves. The initial release of E2fs from association with Rb therefore triggers a positive feedback loop that augments both Rb phosphorylation and release of E2fs. The result is a rapid increase in the concentrations of E2fs and cyclin E-Cdk2. In this way, cell cycle progression becomes independent of the mitogens necessary for the production of cyclin D-Cdks. While these regulatory circuits account well for passage through the restriction point, there is accumulating evidence for functional redundancy among the Rb family proteins and cyclin D-independent mechanisms for detecting mitogenic stimuli.

The E2f proteins that accumulate upon Rb phosphorylation also stimulate transcription of genes that encode proteins needed for DNA synthesis (Volume I, Chapter 9), allowing genome replication to take place in S phase. The cyclin A-Cdk2 produced in response to E2f phosphorylates and inhibits the ubiquitin ligase that marks cyclin B for proteasomal degradation throughout much of the cell cycle (Fig. 7.18B). Consequently, cyclin B, which is required for entry into mitosis accumulates as S phase progresses. Phosphorylation of the Rb protein therefore ensures not only passage through the restriction point and entry into S phase, but also the coordination of these processes with later events in the cell cycle. Indeed, the results of genome-wide approaches to identify genes regulated by E2f family members suggest that E2fs contribute much more broadly than originally appreciated to orderly progression through the cell cycle.

Although E2f proteins are the best-characterized targets of Rb, the latter protein can also bind to numerous other

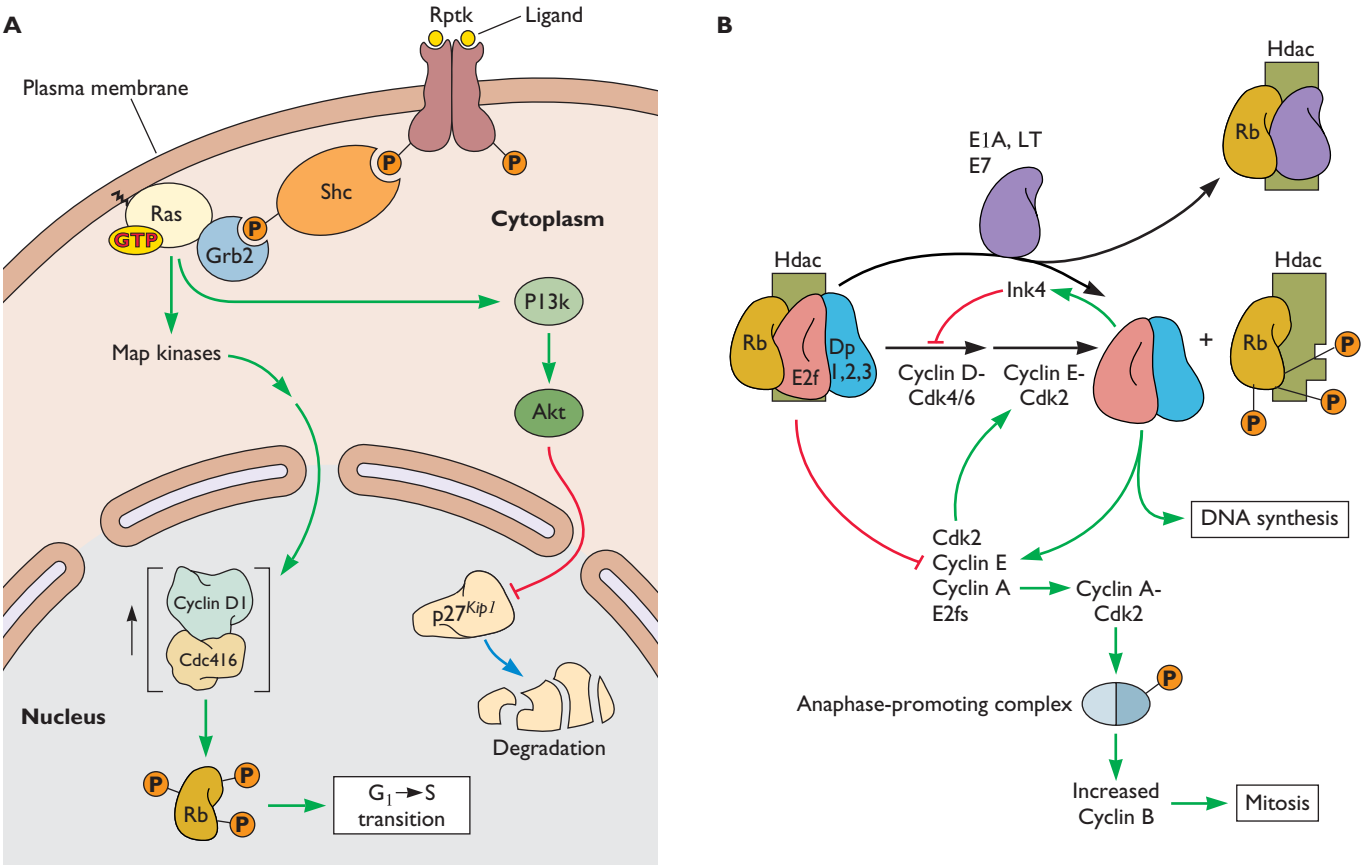


Figure 7.18 Passage through the restriction point in mammalian cells. (A) Mitogenic activation of cell cycle progression is initiated by binding of a growth factor to its cognate receptor protein (Rptk). Signaling via Ras and the Map kinase cascades leads to increased transcription of the cyclin D1 gene, and accummulation of cyclin D1-Cdc4/6 in the nucleus. Activation of this G_1 cyclin is facilitated by the degradation of its inhibitor $p27^{Kip1}$, which is induced by signaling via phosphoinositide 3-kinase (PI3k) and the protein kinase Akt. The active G1 cyclin phosphorylates the negative regulator of cell cycle progression, Rb (and the related p107 and p130 proteins). **(B)** When cells enter G_1, hypophosphorylated Rb is bound to transcriptional regulators of the E2f family, which are heterodimers of an E2f and a Dp protein. The E2f-Rb complex represses transcription when bound to E2f recognition sites in promoters of E2f-responsive genes. Rb is the transcriptional repressor, and this function requires the binding of histone deacetylases (Hdac). Phosphorylation of Rb by cyclin D- and cyclin E-dependent Cdks disrupts the binding of Rb to E2fs. Rb is phosphorylated at many sites by both cyclin D-Cdk4/6 and cyclin E-Cdk2. The latter cyclin, which appears in mid to late G_1 (Fig. 7.5A), is required for entry into S phase. Its modification of Rb depends on the prior action of cyclin D-Cdk4/6. Free E2f-DP heterodimers activate transcription from E2f-responsive promoters, including those of the genes encoding cyclins E and A, Cdk2, and E2f proteins themselves, to establish a positive autoregulatory loop. The positive feedback loop for activation of cyclin E-dependent kinases and E2fs late in G_1 is subject to several checks and balances imposed by inhibitory proteins (Fig. 7.5B). These inhibitory proteins therefore must be inactivated or destroyed to allow progression into S phase (see panel A). The synthesis of at least one member of the Ink4 family of cyclin-Cdk inhibitors is also induced in response to free E2f. It is therefore thought that the accumulation of this inhibitor establishes a feedback loop that blocks the activity of the cyclin D-Cdks and hence the ability of cells to respond to mitogens, a characteristic property of cells that have passed the G_1 restriction point.

proteins that mediate or stimulate transcription, as well as to regulatory proteins such as the Abl tyrosine kinase (Table 7.6). These interactions can lead to activation or repression of transcription and, at least in some cases, have been implicated in inhibition of cell cycle progression by Rb.

Inhibition of Rb Function by Viral Proteins

The products of transforming genes of several DNA viruses bypass the sophisticated circuits that impose restriction point control, and hence the dependence on environmental cues for passage into S phase. The adenoviral E1A

proteins, simian virus 40 LT, and the E7 proteins of oncogenic human papillomavirus (types 16 and 18) can induce DNA synthesis and cell proliferation. Such mitogenic activity requires regions of the viral proteins that are necessary for their binding to hypophosphorylated Rb. All three viral proteins make contacts with two noncontiguous regions by which Rb associates with E2f family members (regions A and B in Fig. 7.19A). In this way, they sequester the inhibitory form of Rb and disrupt Rb-E2f complexes. As a result, they induce transcription of E2f-dependent genes. The relief of Rb-mediated repression leads to inappropriate entry of cells into S phase (Fig. 7.18B).

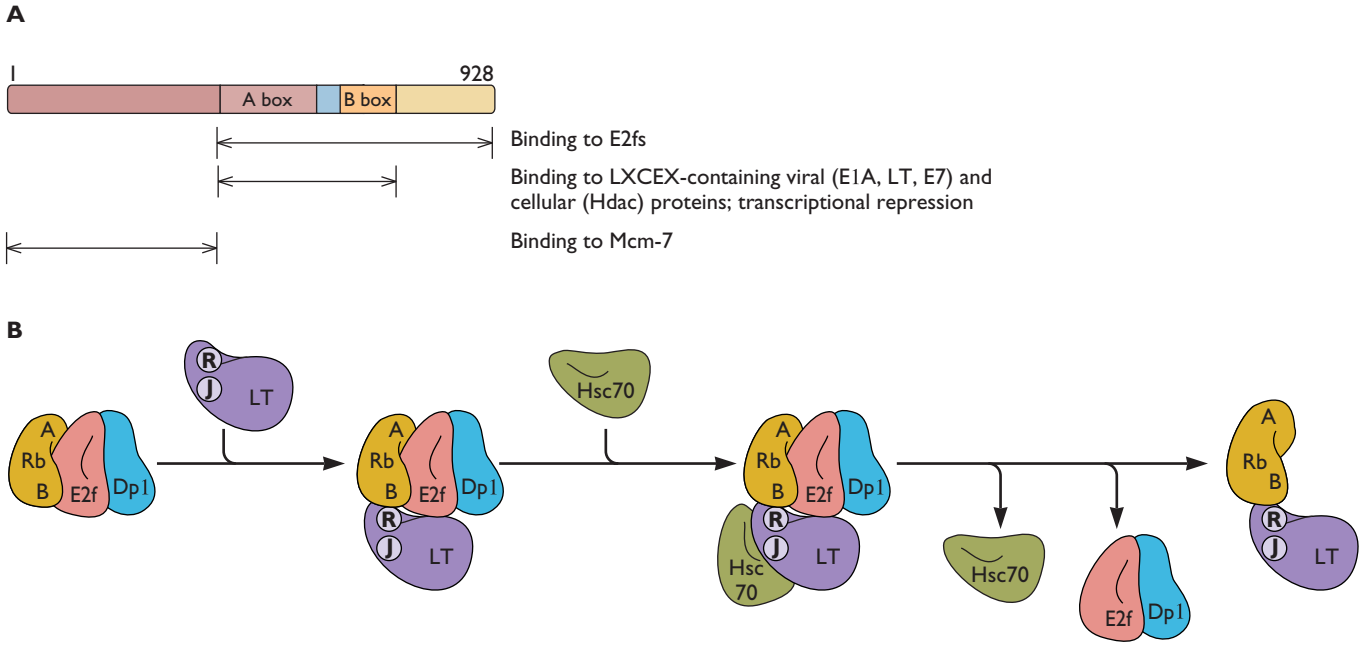
Disruption of E2f-Rb complexes by the viral proteins appears to be an active process, rather than simply the result of passive competition for binding to Rb. Induction of cell cycle progression by simian virus 40 LT requires not only the Rb-binding site, but also the N-terminal J domain located nearby (Fig. 7.19B). Because the J domain functions as a molecular chaperone (see Volume I, Chapter 9), it has been suggested that LT actively dismantles the Rb-E2f complex. The CR1 sequence of the adenoviral E1A proteins fulfills a similar function. Exactly how these viral proteins disrupt the association of Rb with E2f remains to be determined, but conformational alteration of Rb seems likely to be important.

In some cases the viral proteins also alter Rb metabolism. For example, the human papillomavirus type 16 and 18 E7 proteins can bind directly to one subunit of the proteasome and induce polyubiquitinylation and proteasome-mediated degradation of Rb in transformed epithelial cells.

Inhibition of Negative Regulation by Rb-Related Proteins

The Rb protein is the founding member of a small family of related gene products, which also includes the proteins p107 and p130. The latter two proteins were discovered

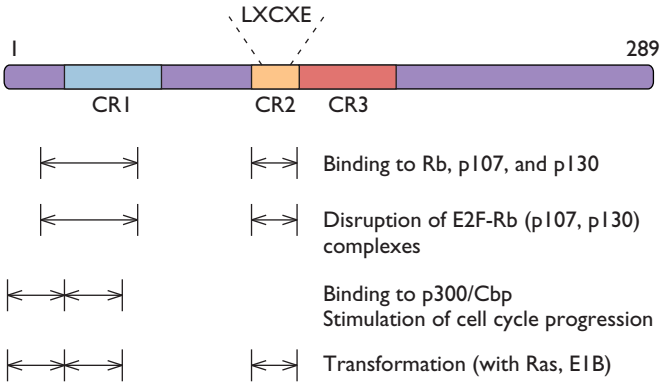
Figure 7.19 Model for active dismantling of the Rb-E2f complex by simian virus 40 LT. (A) Functional domains of the human Rb protein are shown to scale. The A- and B-box regions form the so-called pocket domain, which is necessary for binding of Rb to both E2fs and the viral proteins described in the text. This segment is also sufficient to repress transcription when fused to a heterologous DNA-binding domain, and it is required for binding to histone deacetylases (Hdacs). Like the viral Rb-binding proteins, Hdacs contain the motif LXCXE within the region that binds to Rb. The N-terminal segment of the protein, which is also important for suppression of cell proliferation, binds to human Mcm-7, a component of a chromatin-bound complex required for DNA replication and control of initiation of DNA synthesis. **(B)** The LT protein binds to the Rb A- and B-box domains via the sequence that contains the LXCXE motif, designated R. The adjacent, N-terminal J domain of LT is not necessary for binding to Rb, but is required for induction of cell cycle progression. It has been proposed that the J domain recruits the cellular chaperone Hsc70. The chaperone then acts to release E2f–Dp-1 heterodimers from their association with Rb, by a mechanism that is thought to depend on ATP-dependent conformational change.



by virtue of their interaction with adenoviral E1A proteins (Fig. 7.20), but they also bind to both simian virus 40 LT and human papillomavirus type 16 and 18 E7 proteins. The similarity of p107 and p130 to Rb is most pronounced in the A and B sequences needed for binding of Rb to both E2f and the viral transforming proteins (Fig. 7.19A). Indeed, the residues by which Rb contacts the common Rb-binding motif of the viral proteins (LXCXE) are invariant among the other family members. Binding of the viral proteins to p107 and p130 can make important contributions to transforming activities. For example, the LXCXE sequence of simian virus 40 LT is required for transformation of fibroblasts derived from *Rb*-null mice. The J domain of LT, which induces hypophosphorylation of p107 and p130, concomitant with their increased degradation, is also necessary.

The Rb, p107, and p130 proteins bind preferentially to different members of the E2f family during different phases of the cell cycle. Hypophosphorylated Rb binds primarily to E2f-1, E2f-2, or E2f-3 during the G_0 and G_1 phases. In contrast, p107 is largely associated with E2f-4 and E2f-5 during S phase, whereas p130 binds these same two E2fs in G_0. These properties, and the targeting of p107 and p130 by transforming proteins of the smaller DNA viruses, indicate that inhibition (or more subtle regulation) of the activity of E2f-4 and E2f-5 must be important for orderly progression through the cell cycle. Binding of p130 to these E2f family members appears to be critical for maintaining cells in the quiescent state, and such complexes predominate in mammalian cells in G_0. Their disruption by adenoviral, papillomaviral, or polyomaviral transforming proteins is thought to allow such cells to reenter the cycle, in part via stimulation of the transcription of genes encoding both the E2f proteins and the cyclin-dependent kinase (Cdk2) needed for entry into S phase. For example, when quiescent monkey cells are productively infected by simian virus 40, LT disrupts the association of p130 with E2fs.

Figure 7.20 Organization of the larger adenoviral E1A protein. Regions of the protein are shown to scale. Those designated CR1 to CR3 are conserved in the E1A proteins of human adenoviruses. The CR3 region, most of which is absent from the smaller E1A protein because of alternative splicing, is not necessary for transformation. The locations of the Rb-binding motif and of the regions required for binding to the other cellular proteins discussed in the text are indicated.



Production of Virus-Specific Cyclins

Human herpesvirus 8 and its close relative herpesvirus saimiri encode functional cyclins. The cyclin gene of human herpesvirus 8, designated v-*cyclin*, has 31% identity and 53% similarity to the human gene that encodes cyclin D2, and its product binds predominantly to Cdk6. Like its cellular counterpart, v-cyclin activates this protein kinase, which then phosphorylates the Rb protein. The viral cyclin also alters the substrate specificity of the kinase: the v-*cyclin*–Cdk6 complex phosphorylates proteins normally recognized by cyclin-bound Cdk2, but not by cyclin D-Cdk6. These targets include the cyclin-dependent kinase inhibitor p27 and the replication proteins Cdc6 and Orc1 (see Volume I, Fig 9.23). Normally, synthesis of the Cip/Kip and Ink4 proteins that inhibit cellular cyclin-Cdks (Fig. 7.5) blocks cell cycle progression. However, neither Cip/Kip nor Ink4 family members bind well to the viral cyclin. Synthesis of the viral cyclin can therefore overcome the G_1 arrest imposed when either type of inhibitory protein is made in human cells, and can induce cell cycle progression in quiescent cells and initiation of DNA replication. The specific advantages conferred by production of the viral cyclin during the infectious cycle have not been identified. However, it would be surprising if v-cyclin does not contribute to the oncogenicity of these herpesviruses in their natural hosts, as synthesis of v-cyclin in B cells of transgenic mice results in B-cell lymphoma (when the mice are p53-null [see below]).

Inactivation of Cyclin-Dependent Kinase Inhibitors

The production of viral cyclins in infected cells appears to be a unique property of certain herpesviruses, but other DNA viruses encode proteins that inactivate specific inhibitors of Cdks. For example, the E7 protein of human papillomavirus type 16 binds to the $p21^{Cip1}$ protein and inactivates it. This member of the cellular Cip/Kip family inhibits G_1 cyclin-Cdk complexes (Fig. 7.5). The increase in intranuclear concentrations of p53 triggered by unscheduled inactivation of the Rb protein (see next section) results in accumulation of $p21^{Cip1}$. The ability of the papillomaviral E7 protein to inactivate both Rb and $p21^{Cip1}$ would therefore appear to ensure entry of cells into S phase. Indeed, both these functions of the E7 protein are necessary to induce differentiated human epithelial cells to enter S phase.

The adenoviral E1A proteins can also overcome G_1 arrest induced by $p21^{Cip1}$ or $p27^{Kip1}$, probably by binding to the region of the inhibitors that mediates association with cyclin-dependent kinases. In addition, binding of E1A proteins to the transcriptional coactivators p300 and Cbp (Fig. 7.20) blocks activation of transcription of the $p21^{Cip1}$ gene in response to DNA damage or differentiation.

Transformed Cells Must Also Grow and Survive

Integration of Mitogenic and Growth-Promoting Signals

Prior to division, cells must increase in size and mass, as they duplicate their components for division between the two daughter cells produced by cytokinesis (Fig. 7.4). Consequently, signals that induce cell proliferation also lead to the metabolic changes required to promote and sustain cell growth. Not surprisingly, the mechanisms that regulate growth of normal cells are integrated with those that lead to cell proliferation in response to mitogenic signals (Fig. 7.21A). The small G protein Ras and the protein kinase Akt are important components of the networks that achieve such integration: their activation leads to not only increased production of active D-type cyclins, but also stimulation of translation and regulation of many metabolic enzymes.

The rapid proliferation of cells transformed by viral proteins depends on high rates of metabolism and growth during each cell cycle. It seems likely that any viral oncogene product that results in activation of Ras (or Akt) promotes cell growth, as well as proliferation. How viral transforming proteins that impinge directly on the nuclear circuits that govern cell cycle progression induce altered cell growth is less clear. However, the actions of many of these proteins lead to changes in the transcription of numerous cellular genes, responses that might increase the concentrations of biosynthetic and other metabolic enzymes.

Mechanisms That Permit Survival of Transformed Cells

As discussed in Chapter 3, metazoan cells can undergo programmed cell death (apoptosis). This program is essential during development and serves as a powerful antiviral defense of last resort. Apoptosis can be activated not only by external cues, but also by intracellular events, notably damage to the genome or unscheduled DNA synthesis. Consequently, viral transforming proteins that induce cells to enter S phase and proliferate when they would not normally do so will also promote the apoptotic response. This potentially fatal side effect is foiled by a variety of mechanisms that allow survival of infected, and under appropriate circumstances, transformed cells.

Viral Inhibitors of the Apoptotic Cascade

Many viral genomes encode mimics of cellular proteins that hold apoptosis in check (see Table 3.9). Such viral inhibitors of apoptosis can contribute to transformation. For example, the human adenovirus E1B 19-kDa protein, one of the first viral homologs of cellular antiapoptotic proteins to be identified, allows survival, and hence transformation, of rodent cells that also contain the viral growth proliferation-promoting E1A protein (Table 7.5).

Integration of Inhibition of Apoptosis with Stimulation of Proliferation

Cells must continually interpret the numerous internal and external signals that impinge upon them to execute an appropriate response. Not all the mechanisms that integrate the many types of information that cells receive have been elucidated. However, it is well established that signal transduction cascades that induce cell proliferation in response to external signals can simultaneously promote cell survival by blocking the apoptotic response. For example, signaling via the small G protein Ras results in activation of the cyclin-dependent kinases that drive the G_1-to-S-phase transition (Fig. 7.18A). In addition, such signaling activates phosphoinositide-3-kinase and the protein kinase Akt. Akt induces transcriptional and posttranscriptional mechanisms that inhibit the production and activity of proapoptotic proteins, such as Bad and Bim, and stimulate synthesis of inhibitors of apoptosis, including Bcl-2 (Fig. 7.21B). The various retroviral transforming proteins that function in the receptor tyrosine kinase pathway that activates Ras (Fig. 7.3; Table 7.6) therefore also induce inhibition of apoptosis. Signaling via Src proteins also activates phosphoinositide-3-kinase (Fig. 7.11). It therefore seems likely that v-Src and viral proteins that operate via c-Src, such as polyomavirus mT (Table 7.7; Fig. 7.16), block apoptosis in the same way.

Inactivation of the Cellular Tumor Suppressor p53

Transformation by several DNA viruses requires inactivation of a second cellular tumor suppressor, the p53 protein. This transcriptional regulator, first identified by virtue of its binding to simian virus 40 LT, is a critical component of regulatory circuits that determine the response of cells to damage to their genomes, as well as to low concentrations of nucleic acid precursors or hypoxia. The importance of this protein in the appropriate response to such damage or stress is emphasized by the fact that *p53* is the most frequently mutated gene in human tumors.

The accumulation and activity of p53 are tightly regulated. The intracellular concentration of p53 is

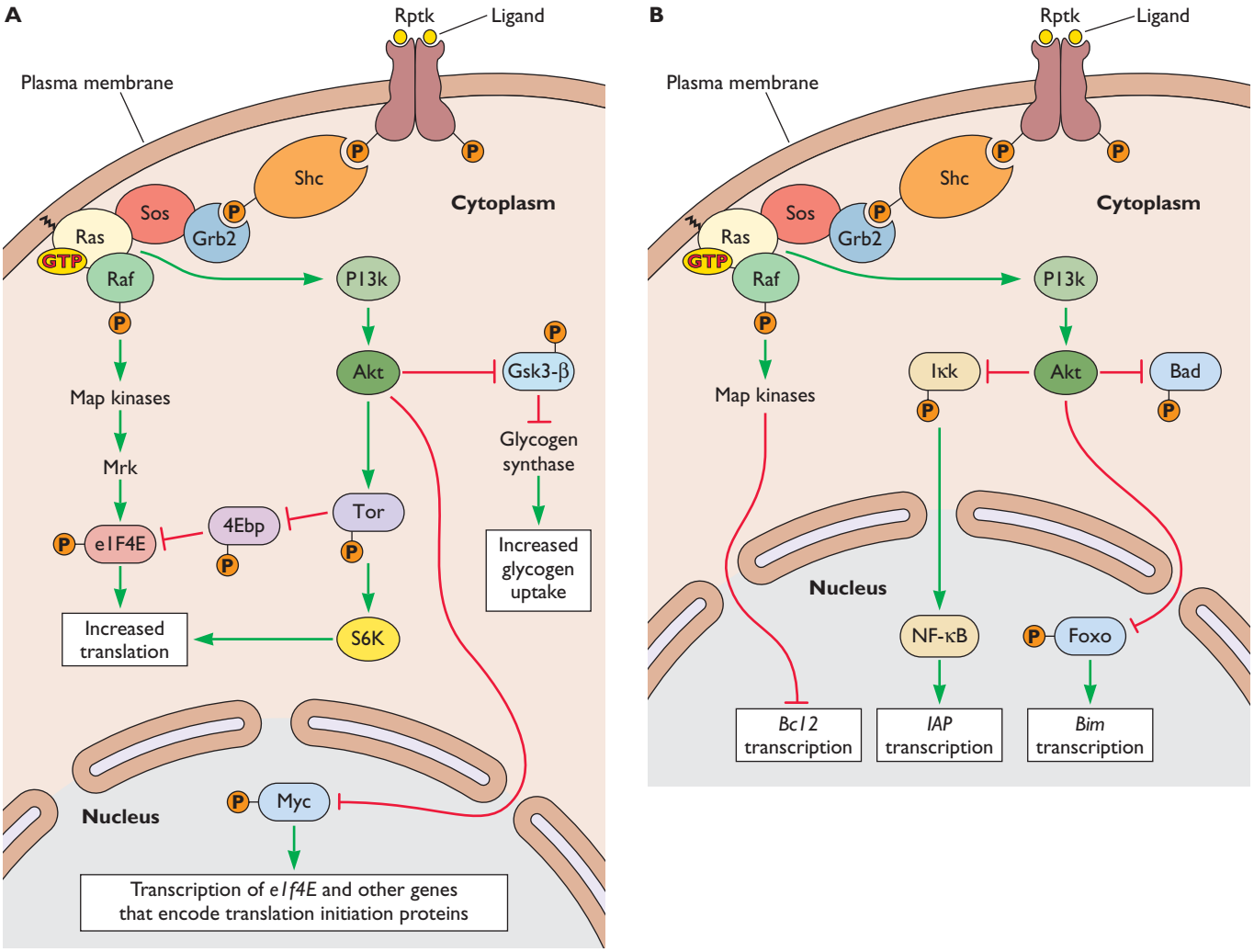


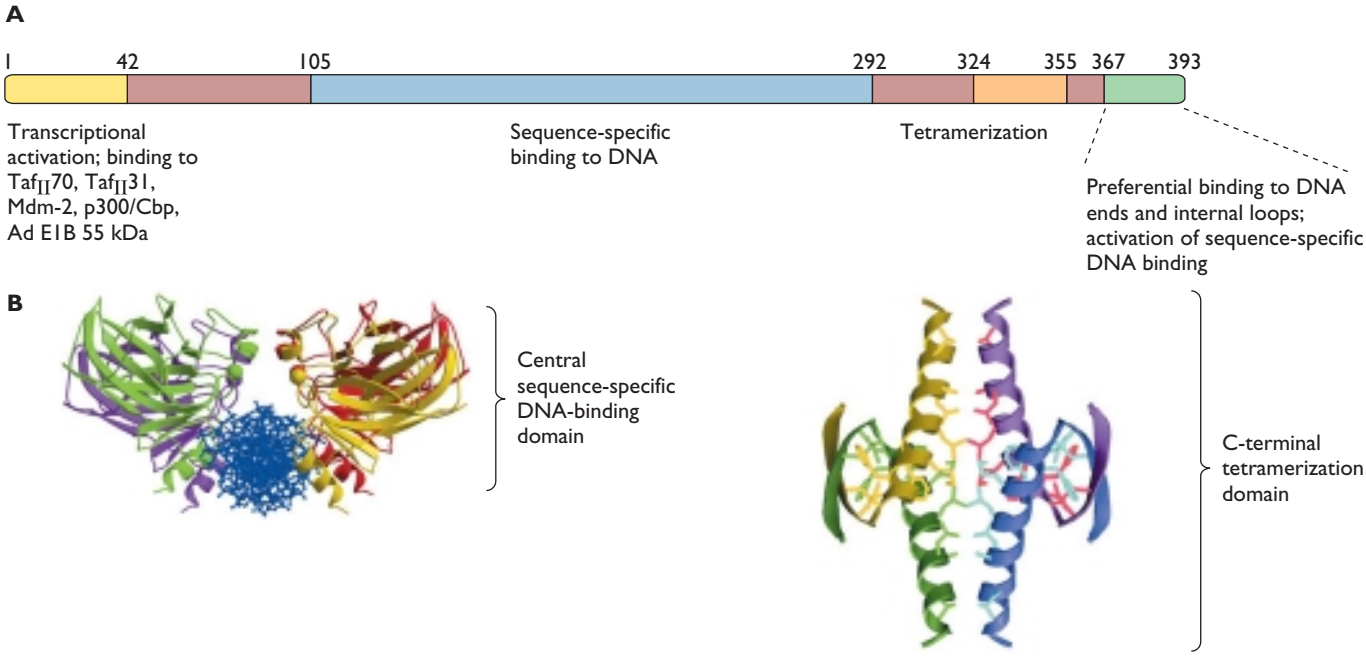
Figure 7.21 Some signaling pathways that promote cell growth and survival. (A) Cell growth. Upon activation, in this example by signaling initiated by binding of its ligand to a receptor protein tyrosine kinase (Rptk), signaling via Ras and the Map kinase cascade activates a Map kinase-dependent kinase (Mak), which phosphorylates and activates the translation initiation protein eIF4E. The activity of this initiation protein is also increased when signaling from Ras via phosphatidylinositol 3-kinase (PI3k) stimulates the protein kinase Akt. Phosphorylation of the inihibitory eIF4E-binding protein (4Ebp) by Akt suppresses its ability to inactivate eIF4E. The transcription of the genes encoding eIF4E and other translation initiation proteins is stimulated via effects on the transcription activator Myc. Akt-dependent phosphorylation of ribosomal protein S6 kinase increases the rate of translation elongation. The mechanisms increase the availability and activity of proteins crucial for protein synthesis, and allow cells to provide proteins at a rate that sustains cells growth. Signaling from Akt also regulates metabolism via phosphorylation and inactivation of glycogen synthase kinase (Gsk3-β). As a result, glycogen synthase cannot be phosphorylated and inactivated. **(B)** Cell survival. Activation of Ras also promotes cell survival by inhibition of synthesis or activity of proapoptotic proteins and by stimulation of production of inhibitors of programmed cell death. Substrates of activated Akt include the pro-apoptotic protein Bad and the transcriptional regulator Foxo, which are inactivated by phosphorylation. Akt also phosphorylates the inhibitor of Nf-κb (Iκk) to promote transcription of genes that encode other inhibitors of apoptosis. As shown in Fig. 7.18A, signaling via Ras and the Map kinase cascade induces cell proliferation. The PI3k-Akt pathway also promotes proliferation, for example by phosphorylation and inactivation of cyclin-dependent kinase inhibitors. Consequently, these (and other) signaling networks integrate cell proliferation and survival.

normally very low, because the protein is targeted for nuclear export and proteasomal degradation by binding of the Mdm-2 protein to an N-terminal sequence (Fig. 7.22 and 7.23). However, DNA damage, such as double-strand breaks produced by γ-irradiation or the accumulation of DNA repair intermediates following ultraviolet (UV) irradiation, leads to the stabilization of p53 and a substantial increase in its concentration (Fig. 7.23). The rate of translation of the protein may also increase. Various proteins that appear to be important for stabilization of p53 have been identified, including the product of a human gene called *Atm* (ataxia telangiectasia mutated), which recognizes potentially genotoxic DNA damage. The genetic disease caused by mutation in *Atm* is associated with a broad spectrum of defects, including hypersensitivity to X rays and ionizing radiation. Cells lacking the Atm protein do not accumulate the p53 protein, and fail to arrest at the G_1/S boundary in response to DNA damage.

The p53 protein is a sequence-specific transcriptional regulator, containing an N-terminal activation domain and a central DNA-binding domain (Fig. 7.22). Its ability to stimulate transcription of p53-responsive genes is tightly regulated. For example, the DNA-binding activity of p53 is stimulated by association of the C-terminal domain with double-stranded DNA ends or sites of excision repair damage, and binding of the Mdm-2 protein to the activation domain inhibits p53-dependent transcription. The many mechanisms by which the accumulation or activity of p53 can be regulated (Fig. 7.23) provide the means to integrate the multiple signals that are monitored to ensure that this potent protein alters cell physiology only under extreme conditions.

In response to damage to the genome, or other inducing conditions, p53 can promote one of two major responses, leading to either G_1/S arrest or apoptosis. One important component of the former pathway is the p53-dependent stimulation of transcription of the gene that encodes $p21^{Cip1}$, the G_1 cyclin-dependent kinase inhibitor. The p53 protein also stimulates transcription of a number of genes encoding proteins that participate in apoptosis, such as Apaf-1, Bax, and Fas, as well as of genes encoding proteins that inhibit signaling pathways that promote cell survival. For example, increased production of the protein Pten leads to impaired signaling via phosphoinositide 3-kinase to Akt (Fig. 7.21B), as Pten is a phosphatase that dephosphorylates phosphoinositides. The ability of p53 to repress

Figure 7.22 The human p53 protein. (A) The functional domains of the protein are shown to scale. TafII70 and TafII31 are Tbp-associated proteins present in TfIId (Volume I, Chapter 8); other proteins are defined in the text. **(B)** Structures of the central, DNA-binding domain bound to DNA (left) and of the C-terminal domain that mediates tetramer formation (right) were determined by X-ray crystallography. From Y. Cho et al., *Science* **265:**346–355, 1994, and P. D. Jeffrey et al., *Science* **267:**1498–1502, 1996, with permission. Courtesy of P. Jeffrey and N. Pavletich, Memorial Sloan-Kettering Cancer Center.



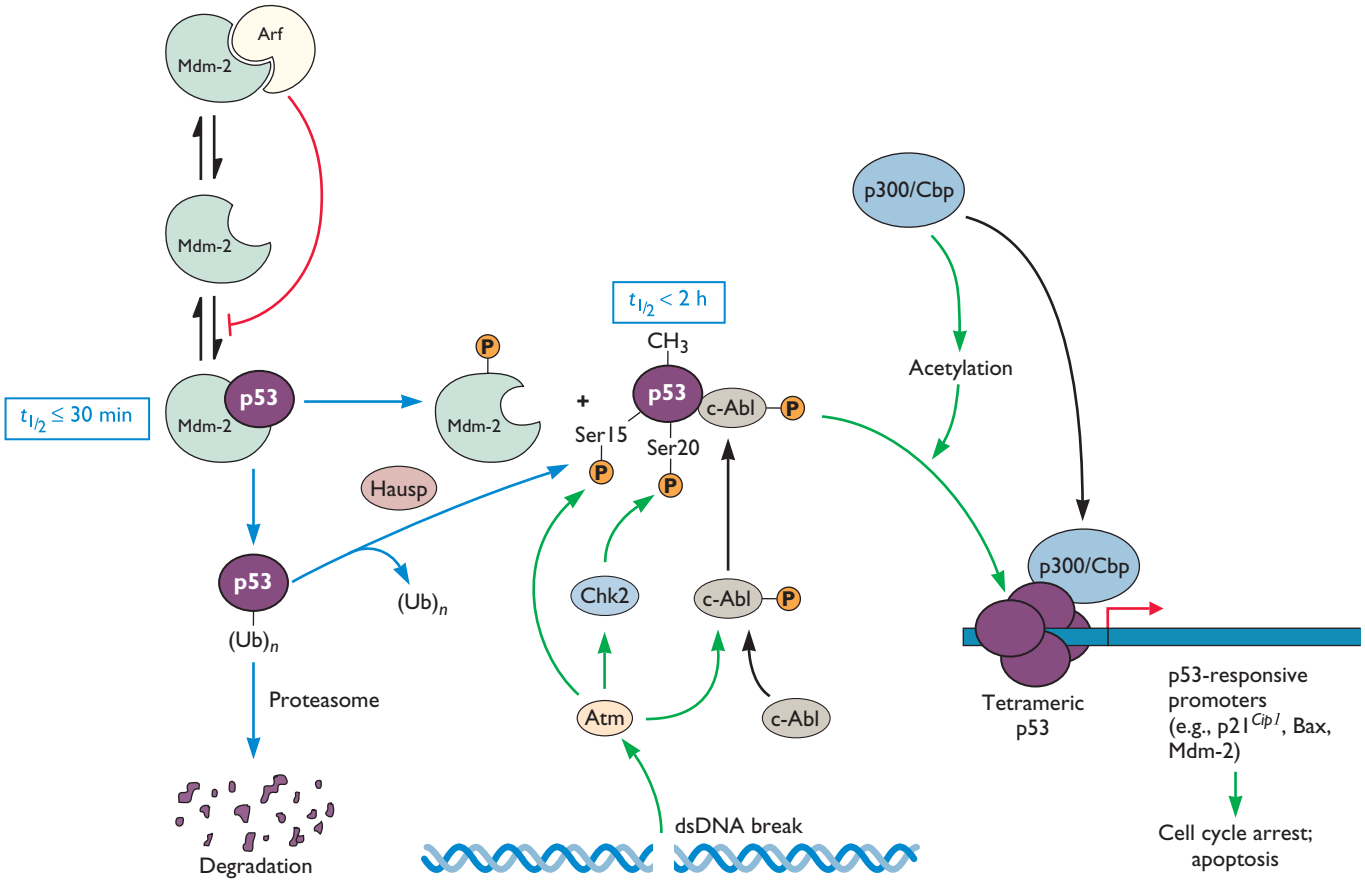


Figure 7.23 Regulation of the stability and activity of the p53 protein. Under normal conditions (left), cells contain only low concentrations of p53. This protein is unstable, turning over with a half-life of minutes, because it is targeted for proteasomal degradation by the Mdm-2 protein. Mdm-2 is a p53-specific E3 ubiquitin ligase that catalyzes polyubiquitinylation of p53, the signal that allows recognition by the proteasome. The Mdm-2 protein therefore maintains inactive p53 at low concentrations. The availability and activity of Mdm-2 are also regulated, for example, by Arf proteins encoded by the *ink4a/arf* tumor suppressor gene, and by stimulation of Mdm-2 transcription by the p53 protein itself. Signaling pathways initiated in response to damage to the genome or other forms of stress lead to stabilization of p53. Such posttranscriptional regulation is thought to allow a very rapid response to conditions that could be lethal to the cell. As illustrated with pathways operating in response to DNA damage (double-strand [ds] breaks) caused by ionizing radiation, p53 is stabilized in multiple ways. These mechanisms include phosphorylation of p53 at specific serines by Atm (see the text) and checkpoint kinase 2 (Chk2), binding to the c-Abl tyrosine kinase, sequestration of the Mdm-2 protein by Arf, and deubiquitinylation of p53 (in the presence of Mdm-2) by the herpesvirus-associated ubiquitin-specific protease (Hausp). Multiple mechanisms, including various modifications within the C-terminal domain (e.g., acetylation) also stimulate the sequence-specific DNA-binding activity of p53 or its association with the transcriptional coactivators p300/Cbp, and hence transcription from p53-responsive promoters.

transcription of genes for antiapoptotic proteins, such as *survivin*, may also be important. Whether p53 promotes cell cycle arrest or apoptosis is determined by numerous parameters, including the cell type, the nature of extracellular stimuli, and the concentration of the p53 protein itself. However, the apoptotic response prevails in many cell types under many circumstances: following DNA damage, in the absence of a hormone or growth factor necessary for cell survival, and following expression of viral oncogenes that induce entry into S phase.

Inactivation of p53 by binding to viral proteins. The genomes of many viruses encode proteins that have been reported to interact with p53. However, the mechanisms by which the functions of this critical cellular regulator can be circumvented are best understood for the small DNA tumor

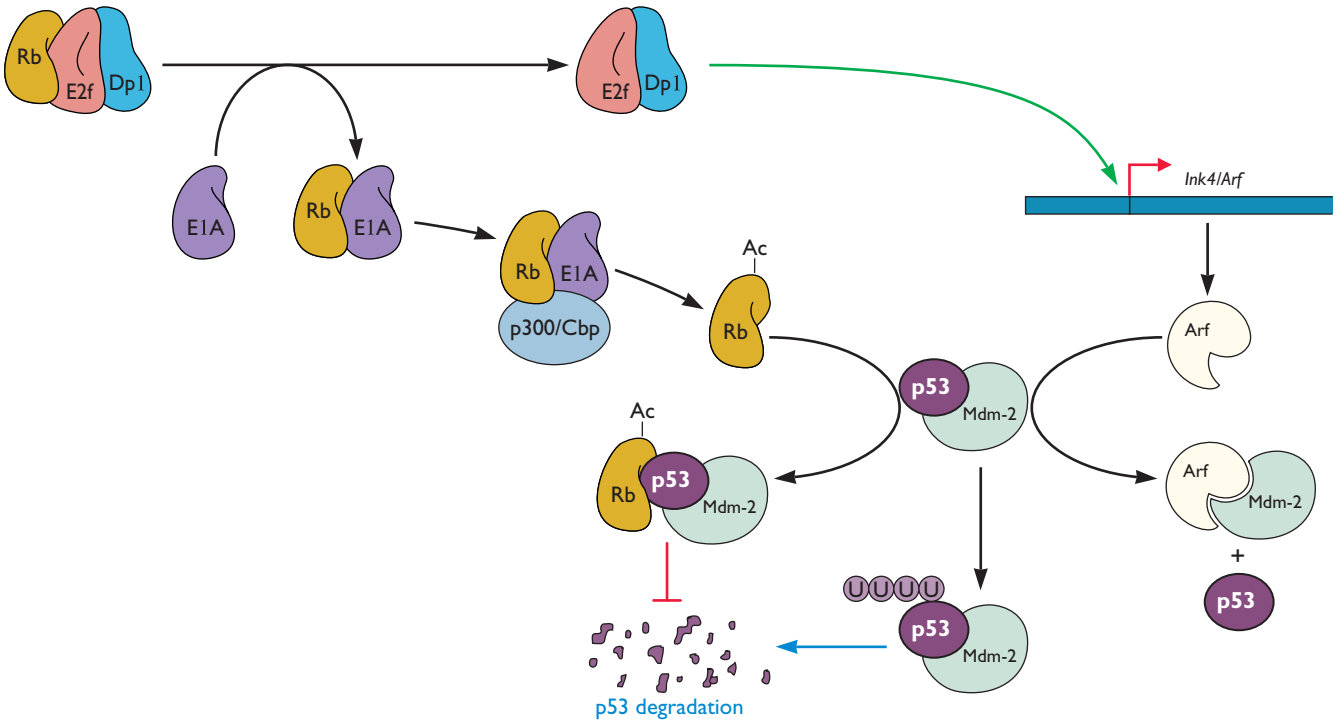
viruses. As we have seen, the transforming proteins of these viruses induce release of E2f family members from association with Rb to promote cell cycle progression. Stabilization of p53 appears to be an inevitable consequence: E2f activates transcription from the promoter of the *Ink4/Arf* gene, which encodes a negative regulator of Mdm-2 (Fig. 7.24).

In contrast to the common mechanism of inactivation of Rb by the different viral proteins discussed above, adenoviral, papillomaviral, and polyomaviral proteins block p53 function in different ways (Fig. 7.25). The human papillomavirus type 16 or 18 E6 proteins bind to both p53 and a cellular ubiquitin protein ligase called the E6-associated protein, and thereby target p53 for proteasome-mediated destruction. Consequently, p53 is cleared from the infected or transformed cell. In conjunction with the viral E4 Orf6 protein, the adenoviral E1B 55-kDa protein also induces increased turnover of p53, but by directing it to a different ubiquitin ligase. In contrast, simian virus 40 LT actually stabilizes the p53 protein upon binding to it, but sequesters this cellular regulator in inactive complexes. The adenoviral E1B 55-kDa protein also binds specifically to the N-terminal activation domain of p53 and can convert the cellular protein from an activator to a repressor of transcription. The results of mutational analyses have correlated the changes in concentration or activity in p53 induced by these viral proteins with their transforming activities.

Despite the mechanistic differences summarized in Fig. 7.25, cells infected by adenoviruses, papillomaviruses, or polyomaviruses all lack functional p53, and are therefore refractory to stimuli that would normally trigger p53-mediated cell cycle arrest or apoptosis.

Alteration of p53 activity via the p300/Cbp proteins. The nuclear phosphoprotein p300 was first identified by virtue of its binding to adenoviral E1A proteins. This protein, and the closely related transcriptional coactivator Creb-binding protein (Cbp), are required for stimulation of transcription by various sequence-specific DNA-binding proteins. It is thought that these coactivators

Figure 7.24 Stabilization of p53 by viral transforming proteins that bind to Rb. As described previously, binding of the adenoviral E1A (or polyomavirus LT or human papillomavirus E7) proteins to Rb allows transcription of E2f-responsive genes. This large set of genes includes the *ink4/arf* gene, and Arf therefore accumulates. Binding of Arf to Mdm2 sequesters this ubiqitin ligase, and hence leads to accumulation of the p53 protein. The E1A proteins also stabilize p53 via p300/Cbp-mediated acetylation of Rb. Acetylated Rb forms a ternary complex with p53 and Mdm2 and blocks p53 degradation. The N-terminal transcriptional activation domain of p53 remains blocked by Mdm-2, but in this form p53 can repress transcription and promote apoptosis.



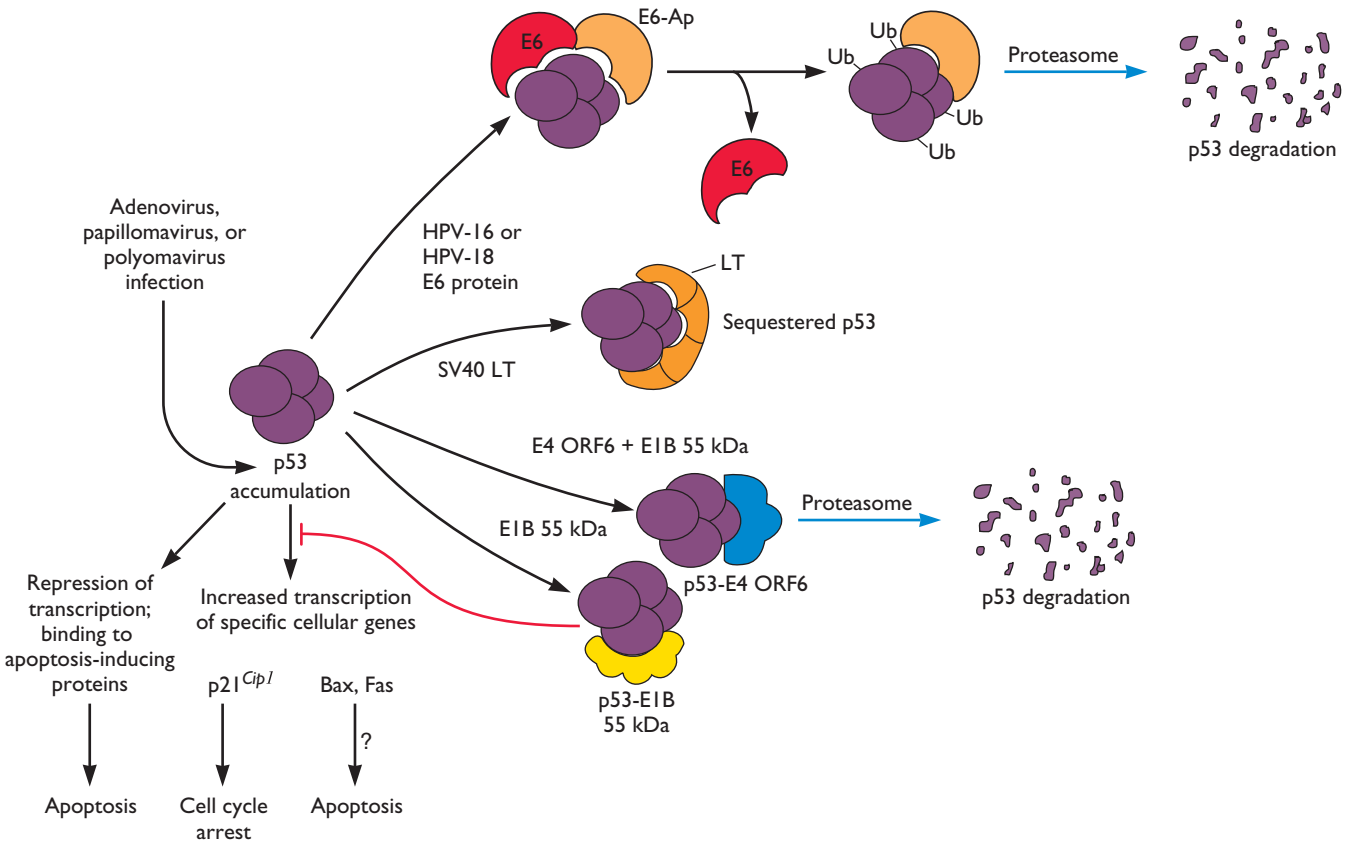


Figure 7.25 Inactivation of the p53 protein by adenoviral, papillomaviral, and polyomaviral proteins. The synthesis of transforming proteins in infected or transformed cells leads to accumulation of p53 (Fig. 7.24). Each of these viral genomes encodes proteins that interfere with the normal function of this critical cellular regulator. The E6 proteins of human papillomavirus types 16 and 18 bind to p53 via the cellular E6-associated protein (E6-Ap). The latter protein is a ubiquitin protein ligase that polyubiquitinylates p53 in the presence of the viral E6 protein, targeting p53 for degradation by the proteasome. Binding of simian virus 40 LT to p53, an interaction that is facilitated by sT, sequesters the cellular protein in inactive complexes. The adenoviral E1B 55-kDa and E4 Orf6 proteins bind to p53 at the N-terminal activation domain and a C-terminal region of p53, respectively. In experimental systems, the former interaction converts p53 from an activator to a repressor of transcription. In transformed rodent cells, it also induces relocalization of p53 from the nucleus to a perinuclear, cytoplasmic body. Binding of the E4 Orf6 and the E1B proteins increases the rate of degradation of p53 via recruitment of an ubiquitin ligase complex, that contains elongins B and C and cullin 5.

improve access of components of the transcriptional machinery to DNA in chromatin templates by acetylating nucleosomal histones. Because the E1A proteins bind to the regions of p300/Cbp that contact other transcriptional regulators, they disrupt complexes containing these cellular proteins. Binding of E1A proteins, and of simian virus 40 LT, to p300 correlates with induction of cell cycle progression and transformation, indicating that inhibition of the histone-modifying role of p300/Cbp proteins is important for the transforming activities of these viral proteins.

As mentioned above, the p53 protein blocks cell cycle progression by stimulating transcription of genes that encode cyclin-Cdk-inhibitory proteins, such as $p21^{Cip1}$. Such stimulation of transcription is mediated by p300/Cbp, which binds to the activation domain of p53 via sequences that can also interact with the E1A proteins. Indeed, binding of E1A proteins to the coactivator inhibits p53-dependent transcription of *Cip1*. Consequently, this interaction blocks the induction of G_1 arrest by p53 following DNA damage.

Tumorigenesis Requires Additional Changes in the Properties of Transformed Cells

The mechanisms described in the preceding sections account for the sustained proliferation and survival of cells transformed by viral oncogenes. However, they are not

necessarily sufficient for the induction of tumors or other types of cancer: tumorigenesis also generally depends on the ability of transformed cells to survive in the face of immune defenses. In some cases, induction of the growth of new blood vessels (angiogenesis) is also required (see "Other Viral Homologs of Cellular Genes" above).

Inhibition of Immune Defenses

The crucial contribution of mechanisms that protect transformed cells from immune defenses against tumorigenesis is illustrated by the properties of rodent cells transformed by oncogenic or nononcogenic human adenoviruses (Box 7.12). As discussed in Chapters 3 and 4, mechanisms that render infected cells refactory to immune defenses are important for the ability of many viruses to replicate in immunocompetent animals. How such mechanisms facilitate the survival of transformed cells and oncogenesis is best understood for herpesviruses associated with human cancers: Epstein-Barr virus and human herpesvirus 8.

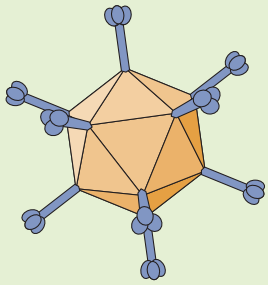
Epstein-Barr virus is associated with Burkitt's lymphoma (a B-cell lymphoma) and nasopharyngeal carcinoma. Although LMP-1 is the only viral gene product that can transform cells in culture, other viral proteins are made in such tumor cells. These products include EBNA-1, which is necessary for replication and maintenance of the episomal viral genome (Volume I, Chapter 9). This protein also contains a sequence that inhibits presentation of EBNA-1 epitopes by major histocompatibility complex (MHC) class I proteins on the cell surface. Consequently, tumor cells cannot be detected so readily by components of the adaptive immune system.

Similarly, several of the human herpesvirus 8 genes that have been implicated in transformation or tumorigenicity encode proteins that inhibit innate or adaptive immune responses. For example, the viral cytokine v-IL-6, which is a B-cell mitogen, also blocks the action of interferon by inhibiting phosphorylation of substrates of the interferon receptor, such as Stat2. In addition, the vFL1P protein, which can enhance the tumorigenicity of murine B cells, inhibits killing by natural killer (NK) cells. The viral genome also encodes proteins that reduce the cell surface expression of MHC class I proteins, block the transport of

BOX 7.12 BACKGROUND

Escape from immune surveillance and the oncogenicity of adenovirus-transformed cells

One of the earliest classifications of human adenovirus serotypes was on the basis of the ability of the viruses to induce tumors in laboratory animals (see the table). Rodent cells transformed with the viral E1A and E1B genes in culture exhibit the tumorigenicity characteristic of the virus. For example, cells transformed by the adenovirus type 12 genes form tumors efficiently when inoculated into syngeneic, immunocompetent animals, whereas cells transformed by adenovirus type 5 DNA induce tumors only in immunoincompetent animals, such as nude mice. This difference was exploited to map the ability of transformed cells to form tumors efficiently in normal animals to a small region of the E1A gene, unique to adenovirus type 12 (and other highly oncogenic adenoviruses). The tumorigenicity of transformed cells was also correlated with repression of transcription of MHC class I genes: the adenovirus type 12 proteins, but not those of adenovirus type 5, inhibit transcription of MHC class I genes by stimulating the binding of a translational repressor and histone deacetylases to the MHC class I enhancers.



In addition, they inhibit the binding of the activator Nf-κb.

The inhibition of MHC class I transcription induced by adenovirus type 12 E1A proteins results in reduced expression of MHC class I proteins on the cell surface, and hence in impaired presentation of antigens to cells of the immune system: recognition of transformed, tumor cells by cytotoxic T lymphocytes depends on MHC class I presentation of antigens. Adenovirus type 12-transformed cells therefore escape immune surveillance and destruction, whereas those transformed by adenovirus type 5 do not.

Yewdell, J. W., J. R. Bennink, K. B. Euger, and R. P. Ricciardi. 1998. CTL recognition of adenovirus-transformed cells infected with influenza virus: lysis by anti-influenza CTL parallels Ad12-induced suppression of class I MHC molecules. *Virology* **162:**236–238.

Zhao, B., S. Huo, and R. P. Ricciardi. 2003. Chromatin repression by COUP-TFII and HDAC dominates activation by NF-κB in regulating major histocompatibility complex class I transcription in adenovirus tumorigenic cells. *Virology* **306:**68–76.

Classification of human adenoviruses on the basis of oncogenicity

Subgroup	Representative serotypes	Oncogenicity in animals
A	12, 18, 31	High: induce tumors rapidly and efficiently
B	3, 7, 21	Low: induce tumors in only a fraction of infected animals, with a long latent period
C	1, 2, 5	None

B-cell receptors to the plasma membrane, or inhibit the complement cascade. Whether these proteins contribute to tumorigenesis remains to be established.

Other Mechanisms of Transformation and Oncogenesis by Human Tumor Viruses

The mechanisms by which some viruses associated with human cancers transform cells and contribute to tumor development cannot be subsumed within the general paradigms discussed in the preceding sections. Our current understanding or the development of these neoplastic disceases, which has taken many years, is described in this section.

Nontransducing, Complex Oncogenic Retroviruses: Tumorigenesis with Very Long Latency

The prototype for the nontransducing oncogenic retroviruses with complex genomes is human T-lymphotropic virus type 1, which is associated with adult T-cell lymphocytic leukemia (ATLL). This disease was first described in Japan in 1977, and has since been found in other parts of the world, including the Caribbean and areas of South America and Africa. The virus, which was isolated in 1980, is now classified as a deltaretrovirus, a group that includes other retroviruses with complex genomes, such as bovine leukemia virus and simian T-cell leukemia virus.

Human T-lymphotropic virus is transmitted via the same routes as human immunodeficiency virus; during sexual intercourse, by intravenous drug use and blood transfusions, and from mother to child. Infection is usually asymptomatic, but can progress to ATLL in about 5% of infected individuals over a period of 30 to 50 years (see Chapter 5). There is no effective treatment for the disease, which is usually fatal within a year of diagnosis. The mechanism(s) by which the virus induces malignancies is still uncertain, but some of the features of ATLL are consistent with a role for a viral regulatory protein. A provirus is found at the same site in all leukemic cells from a given case of ATLL, indicating clonal origin, but there are no preferred chromosomal locations for these integrations. Activation or inactivation of a specific cellular gene is not, therefore, a likely mechanism of transformation. As the genome of human T-lymphotropic virus type 1 does not contain **any** cell-derived nucleic acid, some viral sequences must be responsible for this activity. Surprisingly, however, proviral genes are not expressed in ATLL cells. But viral proteins are made if these cells are placed in culture. The last two features suggest an unusual mechanism of oncogenesis, in which expression of viral genes is necessary for the initiation of transformation but not for its maintenance.

In addition to the conserved genes of all retroviruses, the human T-lymphotropic virus type 1 genome contains a region, denoted X, which encodes a number of regulatory and accessory proteins (Fig. 7.26). One of the best-studied among these is the transcriptional activator Tax. A role for the *tax* gene in oncogenesis is suggested by the observation that transgenic mice carrying *tax* under the control of the viral LTR synthesize this viral protein in muscle tissue and develop multiple soft tissue sarcomas. A current model of how this virus might contribute to oncogenesis proposes that Tax alters the expression of cellular genes encoding proteins that regulate T-cell physiology. Infection by human T-lymphotropic virus type 1 does induce proliferation of T cells and increased synthesis of a number of cellular proteins, among them cytokines (e.g., IL-2 and its receptor) and the product of the *fos* proto-oncogene. Furthermore, the Tax protein inhibits apoptosis and activates Nf-κb, which is required for transcription of a number of genes that encode cellular regulators. Other proteins encoded in the X region may also play a role in oncogenesis, both by down-regulating the synthesis of viral proteins (Tax and Rex) late in infection (thereby avoiding immune recognition), and by affecting the expression of certain cellular proteins on the cell surface. For example, one of the accessory proteins has been found to decrease cell surface expression of MHC class 1 molecules.

A new gene, which is encoded in the **antisense** [(−)] strand of proviral DNA, was recently identified in the human T-lymphotropic virus type 1 genome. Transcription of this *hbz* gene is initiated in the 3′ LTR (Fig. 7.26), and the spliced mRNA encodes a basic leucine zipper protein. This protein forms a heterodimer with the cellular transcription factor Creb, rendering Creb incapable of binding to the Tax-responsive element in the 5′ LTR. Consequently, Tax-mediated transcription is inhibited, an event that may contribute to immune evasion. In addition to this protein function, the *hbz* RNA itself has been found to promote T-cell proliferation by a mechanism that is currently unknown but which could be mediated by RNA interference. The 3′ LTR is conserved in all ATLL cells, but the 5′ LTR is often deleted, or silenced by methylation. It has been proposed, therefore, that the *hbz* RNA may play a vital role in oncogenesis associated with this virus, even at late stages, when Tax is no longer synthesized. Other investigators have hypothesized that at late stages of the disease, no viral products might be required. Rather, genetic changes that accumulate in the DNA of the infected cells may drive oncogenesis. Because the virus-induced oncogenic events occur a long time before ATLL appears, it will be difficult to distinguish among these possibilities. Another challenge to the study of human T-lymphotropic virus has been the inefficiency of infection

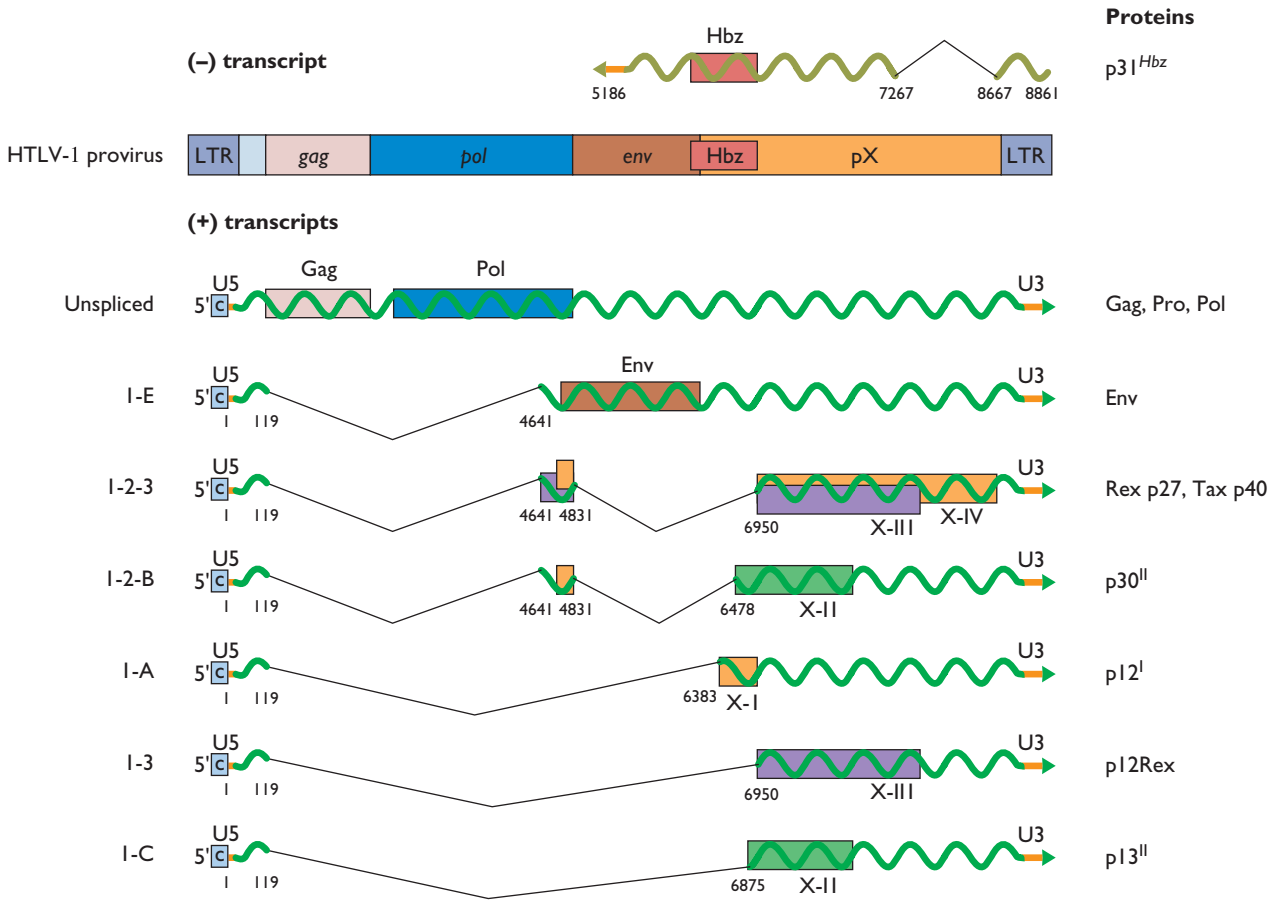


Figure 7.26 Human T-lymphotropic virus type I provirus organization and transcription. Proteins produced from mRNAs transcribed from the DNA (+) strand are shown below, and the spliced transcript produced from the (–) strand is above the provirus map. Boxes in the transcripts represent the sequences encoding the listed proteins. Adapted from D. M. D'Agostino et al., *Cell Death Differ*. **12:**905–915, 2005, with permission.

of T cells with cell-free virions. The recent discovery that dendritic cells can facilitate such infection provides new insight into the parameters that may affect viral infectivity *in vivo*, and should pave the way for more detailed studies with cultured cells (Box 7.13).

Oncogenesis by Hepatitis Viruses

Hepatitis B Virus

Hepatitis B virus is a member of the family *Hepadnaviridae*. The major site of reproduction for all hepadnaviruses is the liver. Infections by these retroid viruses can be acute (3 to 12 months) or lifelong. In humans, the frequency of persistent infection ranges from 0.1 to 25% of the population in different parts of the world. Most persistent infections are acquired neonatally or during the first year of life. Such long-term carriers are at high risk for developing hepatocellular carcinoma (Box 7.1), and as many as 1 million people die of this disease each year. However, study of hepatitis B virus oncogenesis in humans is difficult, because liver cancer arises only after a very long period (decades) of chronic infection. Woodchucks infected with woodchuck hepatitis B virus are used as an animal model for hepadnaviral oncogenesis. Normally, when infected as adults these animals experience only acute infections. However, if they are treated with an immunosuppressing drug (e.g., cyclosporin) before inoculation, or infected in the first few days of life, the infections become persistent and almost all infected animals develop liver cancer by 2 to 4 years of age.

Sustained low-level liver damage is characteristic of persistent infection by hepatitis B viruses. Almost all such damage can be attributed to attack by the host's immune system on infected hepatocytes. The rate of hepatocyte proliferation must increase in such cases to compensate for cell loss.

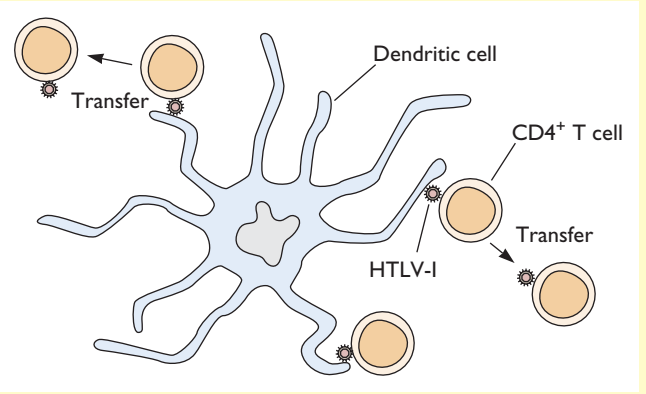
BOX 7.13 EXPERIMENTS

A "hand-off" system for infection with human T-lymphotropic virus

Human T-lymphotropic virus type 1 (HTLV-1) causes ATLL, which is a malignancy of $CD4^+$ T cells. Nevertheless, incubation of cell-free virus particles with isolated $CD4^+$ T cells does not result in productive infection. Consequently, studies of viral transmission and transformation have generally been performed by cocultivating target cells with producing $CD4^+$ T-cell lines that have been killed by irradiation or chemical treatment. Unfortunately, the presence of proviral DNA and viral proteins from the infected T-cell lines in these cocultures has made studies of early events of virus infection in target cells quite difficult.

Dendritic cells play a critical role in the initiation of antiviral immune reponses (Chapters 3 and 4). In some viral infections, such as with human immunodeficiency virus type 1, this normal function of dendritic cells is subverted to facilitate viral transmission (Fig. 6.10). Recently, these cells have been used to establish a tissue culture system for infection of $CD4^+$ T cells with HTLV-1: In these experiments, dendritic cells isolated from human peripheral blood are incubated with virus particles and subsequently cocultured with $CD4^+$ T cells. The dendritic cells are separated from the T cells shortly after their exposure to virus (less than 6 h), to allow for transmission of the virus before the dendritic cells can be infected. Such transmission results in efficient infection and transformation of the $CD4^+$ T cells. The use of this "hand-off" system of infection should greatly facilitate future investigation of the mechanisms of infection and transformation by HTLV-1.

Jones, K. S., C. Petrow-Sadowski, Y. Huang, D. C. Bertolette, and F. W. Ruscetti. 2008. Cell-free HTLV-1 infects dendritic cells leading to transmission and transformation of $CD4^+$ T cells. *Nat. Med.* **14:**429–436.



It is generally accepted that such an increased rate of proliferation over long periods is a major contributor to the development of liver cancer. In addition, the inflammation and phagocytosis that are integral to the immune response can result in high local concentrations of superoxides and free radicals. It is therefore possible that DNA damage and the resulting mutagenesis also contribute to hepadnavirus-induced hepatocellular carcinoma. Consequently, there is considerable incentive for developing antiviral therapies to treat persistent hepatitis B virus infection.

The almost universal presence of integrated fragments of hepadnaviral DNA suggests that this feature plays a role in the development of tumors. Indeed, in woodchucks, activation of a member of the *myc* oncogene family, associated with the nearby integration of woodchuck hepatitis virus DNA, is observed in 90% of hepatocellular carcinomas. In contrast, insertional activation of a common oncogene has not been observed in hepatitis B virus-induced hepatocellular carcinomas in humans.

Other studies suggest that viral proteins encoded by integrated viral DNA sequences may contribute to carcinogenesis in humans. One candidate is the X protein, which is synthesized from integrated viral DNA sequences in many hepatocellular carcinomas. In cell culture systems, and by inference in the infected liver, the hepatitis B virus X protein stimulates transcription from many cellular genes (including proto-oncogenes), both by altering the DNA binding of cellular transcriptional regulators and by activation of signaling via Nf-κb and other pathways. Moreover, one line of transgenic mice developed hepatocellular carcinomas when high concentrations of the X protein were made in liver cells. In another line of transgenic mice, the X protein increased susceptibility to chemical carcinogens. Other evidence suggests that the X protein may affect apoptosis through binding to the cellular p53 protein. These findings, and the alterations in expression of cellular proto-oncogenes and tumor suppressor genes detected in hepatitis B virus-infected liver by using microarray methods, suggest that the viral X protein may contribute to a multistep transformation process. The discovery that truncated envelope proteins of the virus can function as activators of transcription of cellular genes in a transient-expression system has led to the idea that yet another viral gene may play a role in oncogenesis.

The long time required for development of human liver cancer implies that several low-probability reactions must

Table 7.8 The diversity of viral transformation mechanisms

Virus	Viral genetic information in transformed cells			Transformation mechanisms		
	Physical state	Lytic functions	Transforming functions	Cell cycle progression	Apoptosis	Immune modulation or stimulation
Adenovirus						
Human subgroup C and A serotypes	Integrated into host genome by random recombination	Lost during integration	E1A and E1B gene products; some E4 proteins	Deregulated; inactivation of pRB family proteins by E1A proteins	Blocked by inactivation of p53 (E1B 55-kDa and E4 Orf6 proteins) and by the E1B 19-kDa protein, a viral Bcl-2 homolog	Inhibition of transcription of MHC class I genes by subgroup A E1A proteins
Flavivirus						
Hepatitis C virus	Not integrated	Noncytolytic, persistent infection of cells that are rapidly dividing	?	?	?	Immune systems kill infected cells; survivors are selected over years of replication; tumor cells escape elimination
Hepadnavirus						
Hepatitis B virus	Episomal; viral sequences are integrated in some hepatocellular carcinomas	None	X protein; large or truncated middle B surface proteins	Defects in DNA repair; altered transcription of proto-oncogenes (e.g., *c-fos*, *c-myc*) and transcription factors (e.g., Nf-κb); direct interaction of X with proteins in growth-signaling pathways	Both pro- and antiapoptotic effects are seen, depending on conditions; some may be mediated by X protein binding to p53	Continuous tissue damage due to immune cell attack drives abnormal liver proliferation
Herpesvirus						
Epstein-Barr virus	Episomal; replicates in concert with host cell genome	Repressed	LMP-1; EBNA-2 increases production of LMP-1	Deregulated by constitutive signaling from LMP-1	Blocked; LMP-1 induces synthesis of Bcl-2 family proteins and inhibits signaling from death domain proteins	Cytotoxic T cells recognizing EBNA-1 are not produced, because a sequence in this viral protein inhibits the proteasome; LMP-1 signaling induces increased production of MHC class 1 and peptide transporters; EBNA-2 blocks signal transduction cascade induced by interferon

Papillomavirus						
Human papillomavirus types 16 and 18	Integrated into host genome by random recombination	Lost during integration	E6 and E7 proteins	Deregulated by inactivation of Rb family proteins by E7 protein	Blocked by E6 protein-induced degradation of p53	None known
Polyomavirus						
Simian virus 40	Integrated into host genome by random recombination	Lost during integration	LT; sT in some circumstances	Deregulated by inactivation of Rb family proteins by LT and by stimulation of Map kinase cause cascade signaling by sT	Blocked by inactivation of p53 by LT	None known
Retroviruses						
Transducing, e.g., Rous sarcoma virus	Integrated	None	Virally transduced, cell-derived oncogenes	Loss of tissue, developmental, and cell cycle regulation by inappropriate expression of oncogenes	None	
Nontransducing, e.g., Rous-associated viruses 1 and 2	Integrated	None	Insertional mutagenesis—alteration of cellular proto-oncogenes (or their expression) in vicinity of proviral integration site	Loss of tissue, developmental, and cell cycle regulation by inappropriate expression of wild-type or truncated proto-oncogenes	None	Viral superantigen (murine mammary tumor virus, Sag protein) stimulates T cells and induces proliferation of B cells
Long latency, e.g., human T-lymphotropic virus type 1	Integrated	None	Viral transactivor proteins (Tax); Hbx protein and RNA	Stimulates T-cell proliferation by induction of Nf-κb and IL-2	?	Abnormal T-cell growth proliferation is a critical early event, but other currently unknown changes are required

take place. The relative importance of viral proteins and the indirect effects of immune damage described above, remains to be determined.

Hepatitis C Virus

As noted previously, hepatitis C virus is a member of the family *Flaviviridae* and possesses a (+) strand RNA genome. Its discovery in 1989 established the etiology of what had been known previously as non-A, non-B hepatitis, a disease contracted by a small fraction of transfusion recipients, who developed liver cancer years later. Routine screening of the blood supply has since reduced this mode of infection, but the virus is still transmitted by intravenous drug abusers and through the use of contaminated needles. Fortunately, the virus is not easily transmitted and seems to require direct blood-to-blood contact to be passed from one individual to another. Nevertheless, an estimated 170 million people are infected worldwide with one of six major genotypic variants (clades) that are specific for different geographical locations. Approximately 75 to 85% of infected individuals develop a persistent infection; among these, 1 to 5% will develop hepatocellular carcinoma. Although a small percentage, this still amounts to 1.3 million to 7.2 million cases of liver cancer worldwide.

Like hepatitis B virus, hepatitis C virus is hepatotropic. Chronic infection of hepatocytes leads to their destruction by the immune system and formation of fibrotic scars that obstruct the passage of blood (a life-threatening condition known as cirrhosis). As not all patients with cirrhosis also develop cancer, host-specific factors must play a role. Some evidence has suggested that certain viral proteins (capsid, envelope, and NS5A) can block the normal response of hepatocytes to apoptotic signals, and may therefore contribute to oncogenesis. Such ideas are difficult to test because there is no good animal model for hepatitis C virus-mediated hepatocellular carcinoma, and until recently it was not even possible to propagate the virus in tissue culture. However, as with hepatitis B virus, the indirect effects of immune-mediated inflammation and oxidative damage are thought to be the major contributors to cirrhosis and cancer. Whether viral proteins have a modulating role is yet to be determined.

Perspectives

As noted at the beginning of this chapter, viruses do not cause cancer as an essential part of their infectious cycles. Nevertheless, the discovery that viruses can do so, initially made a century ago, was the harbinger of the spectacular progress in understanding the molecular basis of oncogenesis and transformation made within the past 3 decades. Because tumor cells grow and divide when normal cells do not, elucidation of the mechanisms of transformation inevitably has been accompanied by the tracing of the intricate circuits that regulate cell proliferation in response to both external and internal signals. The remarkable discovery that the transforming gene of the acutely transforming retrovirus Rous sarcoma virus was a transduced cellular gene paved the way for identification of many cellular proto-oncogenes, and the elucidation of the signal transduction pathways in which the proteins encoded by them function. Indeed, in several cases, we can now describe in atomic detail the mechanisms by which mutations introduced into these genes during or following their capture into retroviral genomes lead to constitutive activation of signaling. These viral genes and their cellular counterparts that have acquired specific mutations in tumors are **dominant** oncogenes. In contrast, studies of a hereditary juvenile cancer in humans, retinoblastoma, had indicated that neoplastic disease can also develop following the loss of function of specific genes, which were therefore named **tumor suppressor** genes. Our current appreciation of the critical roles played by the products of such tumor suppressor genes in the control of cell cycle progression stems directly from studies of transforming proteins of adenoviruses, papillomaviruses, and polyomaviruses.

The initial cataloging of viral transforming genes and the properties of the proteins they encode suggested a bewildering variety of mechanisms of viral transformation. With the perspective provided by our present understanding of the circuits that control cell proliferation, we can now see that the great majority of these mechanisms fall into one of two general classes: viral transformation can be the result of either constitutive activation of cytoplasmic signal transduction cascades or disruption of nuclear pathways that negatively regulate cell cycle progression. In both cases, viral proteins or transcriptional control signals override the finely tuned mechanisms that normally ensure that cells duplicate their DNA and divide only when external and internal conditions are propitious.

Such an integrated view of the mechanisms by which viruses belonging to very different families can transform cells is intellectually satisfying. Nevertheless, not all aspects of the complex interactions that can occur between viruses and their host cells are yet understood (Table 7.8). We do not fully appreciate why, in some cases, a single viral oncogene is sufficient for transformation of cells in culture whereas, in others, several viral functions are required. Nor do we understand why viral proteins that apparently alter the same cellular pathway can differ in their transforming potentials. For example, the constitutively active tyrosine kinase encoded in the *v-src* gene of Rous sarcoma virus is sufficient for both transformation of primary cells in culture and induction of sarcomas in animals. In contrast, activation of the c-Src tyrosine kinase by polyomaviral mT

allows transformation of established cell lines, but the viral LT protein is also required for transformation of primary cells. As these examples illustrate, some of the more subtle aspects of the mechanisms of viral transformation have yet to be explained. However, the greatest challenge is posed by the mechanisms of tumorigenesis.

Transformation of cells in culture is not necessarily accompanied by acquisition of the ability to form tumors in animals. This dissociation is evident in the etiology of some human cancers associated with viral infections. For example, infection by Epstein-Barr virus, which immortalizes human B cells in culture by mechanisms that we can describe in some detail, is but one of several factors implicated in the development of Burkitt's lymphoma. Similarly, the increased rate of proliferation of liver cells following host immune responses to persistent hepatitis B virus infection makes a major contribution to the development of hepatocellular carcinoma. A deeper appreciation of the parameters that determine a host's response to transformed cells will clearly be necessary if we are to understand the complex process of tumorigenesis. Oncogenic viruses and cells transformed by them will undoubtedly prove to be as valuable in this formidable endeavor as they have been in elucidation of the molecular basis of control of cell proliferation, and the derangement of these processes during transformation.

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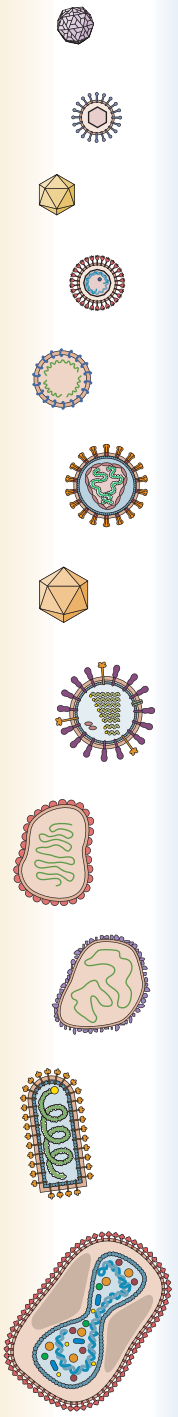

8

Vaccines

Nothing shocks me. I'm a scientist.
INDIANA JONES

Introduction

We survive the continuous barrage of viral infections because our natural defense systems recognize and defend against these ever-changing pathogens. Conversely, any successful virus infection must have modulated or bypassed such defenses. The acquired immunodeficiency syndrome (AIDS) pandemic, the appearance of severe acute respiratory syndrome (SARS), as well as periodic influenza virus epidemics (and fears of pandemics) serve as witnesses to the frailties of natural defenses. Human ingenuity has provided several adjunct actions to augment natural defenses, which have had dramatic effects on our survival and longevity. Two of the most effective are the use of vaccines to stop viral infections or ameliorate disease, and the use of drugs to treat viral diseases. There are several examples of interventions that, when properly applied, are remarkably effective.

The proviso "when properly applied" in the previous sentence warns of the complexity of host-virus interactions and the many difficulties encountered when attempting to control disease. We have learned that any agent or method that blocks viral replication or reduces viral pathogenesis selects for viral mutants that are capable of bypassing the blocking agent. We also know that any intervention affecting a host enzyme or process carries inherent risks, because the normal function of that enzyme or process may be compromised. Indeed, resistance, toxicity, and immunopathology are major problems in any virus control program. In this and the next chapter, we discuss vaccines and antiviral drugs separately, but the principles and problems of blocking viral replication and pathogenesis are similar.

The Historical Origins of Vaccination

Smallpox: a Historical Perspective

Smallpox has been called the most destructive disease in history; infection by smallpox virus killed, crippled, or disfigured over 1 in 20 of all humankind. In the 20th century alone, more than 300 million people succumbed to this

disease. Nevertheless, it was the first to be eliminated from the natural environment by directed human intervention. It is fascinating to follow the convoluted path of discovery that made it possible to orchestrate the demise of such a deadly agent.

Several thousand years ago, the Chinese knew and wrote about diseases that occur only once in a lifetime. The practical aspects of this observation were recognized by Chinese and Indian physicians of the 11th century who injected pus from smallpox lesions into healthy individuals in hope of inducing mild disease that would provide lifelong protection (a process later called **variolation**). Despite such early insight, little progress in controlling the disease was made for centuries. The dangerous practice of variolation was introduced into Europe at the beginning of the 18th century. Unfortunately, many people contracted smallpox from variolation, and the practice not only never became popular, but actually was prevented by law in many countries.

The horror of smallpox was vanishing from our collective consciousness until the specter of bioterrorism appeared in the late 20th century. Consequently, there is renewed interest in the virus and the vaccine that was so effective. The eradication story begins with Edward Jenner (1749–1823), a country doctor and naturalist, who was well known at the time for a seminal paper titled "Observations on the Natural History of the Cuckoo." At first glance, Jenner seems an unlikely candidate to conceive of, and establish, the means by which natural infection by smallpox was eventually eradicated. However, he was a careful and thoughtful observer of his patients. Jenner's

BOX 8.1 BACKGROUND

The current U.S. smallpox vaccine

The smallpox vaccine in the U.S. stockpile to protect civilian and military personnel against deliberate dissemination of smallpox virus is live vaccinia virus that was grown on the skin of calves.

This vaccine stockpile is more than 30 years old, although its potency is asserted to be high. This vaccine, called Dryvax, which is the only currently licensed smallpox vaccine, was manufactured by Wyeth. It is a lyophilized preparation of the strain "New York City calf lymph," which was derived from a seed stock of the New York City Board of Health strain that was passed 22 to 28 times on young calves. The lyophilized calf lymph is reconstituted with 50% glycerol, 0.25% phenol, and 0.005% brilliant green. Distribution of this vaccine was discontinued by Wyeth in 1983.

The vaccinia virus vaccine has been used in millions of vaccinations. The evidence of a productive immune response (called a "take") is a common local reaction of pustular lesions that occur 6 to 10 days after vaccination. The vaccine causes rare but serious adverse reactions, including dermatological reactions, central nervous system disorders, and general disseminated disease. During the program in the United States in which every child was vaccinated, approximately seven to nine deaths per year were attributed to vaccination, with the highest risk occurring in infants. Inadvertent administration of the vaccine to immunodeficient individuals or to people with certain skin diseases resulted in a significantly larger number of adverse reactions.

In 2002, the U.S. government announced plans to immunize military personnel and frontline civilian health care workers. Subsequently, the vaccine will be made available to the general public on a voluntary basis.

In today's world, with an AIDS pandemic and the common use of immunosuppressive drugs for transplant patients, the risk of inadvertent infection by the live vaccine virus and subsequent adverse reactions is considerably higher than it was in the 1950s. New vaccines that are safe and efficacious must be prepared to replace the aging stockpile.

Harrison, S., B. Alberts, E. Ehrenfeld, L. Enquist, H. Fineberg, S. McKnight, B. Moss, M. O'Donnell, H. Ploegh, S. Schmid, K. Walter, and J. Theriot. 2004. Discovery of antivirals against smallpox. *Proc. Natl. Acad. Sci. USA* **101:**11178–11192.

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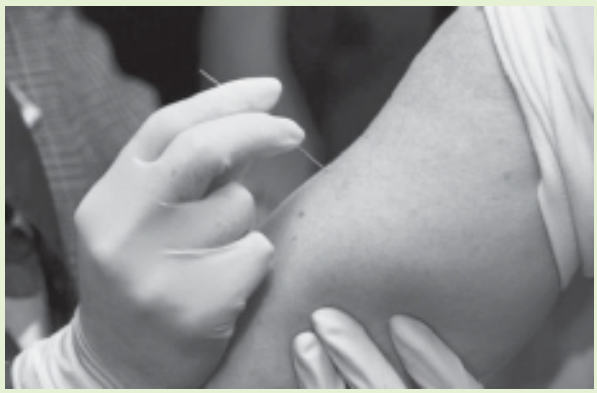
Rosenthal, S., M. Merchlinsky, C. Kleppinger, and K. Goldenthal. 2001. Developing new smallpox vaccines. *Emerg. Infect. Dis.* **7:**920–926.

The image is from *Public Health Bulletin* vol. 52, no. 3, spring 2003, California County of Orange Health Care Agency. (http://www.ochealthinfo.com/newsletters/phbulletin/2003/2003-spring.htm) with permission.



insight was that milkmaids appeared to be spared the facial disfigurations of smallpox, because they had cowpox infections on their hands. He put this idea to the test on 14 May 1796, when he injected pus from a cowpox lesion on the finger of milkmaid Sarah Nelmes under the skin of James Phipps, a healthy young boy. Phipps developed a fever and a lesion typical of cowpox at the site of infection. Two weeks later, Jenner then deliberately infected Phipps with smallpox, and the boy survived this potentially lethal challenge. Needless to say, such an experiment would not be possible today.

Despite this amazing result, the Royal Society rejected Jenner's paper describing these experiments, and so he ultimately resorted to publishing his work privately. While it is Jenner's name that has been remembered, his friend William Woodville, a prominent physician, was responsible for the first large-scale clinical trial that confirmed Jenner's observations. Thanks to Woodville's careful clinical research, the idea of harnessing the body's natural defenses as a public health measure was placed on firm ground.

As is the case for many early discoveries, the scientific world was not prepared to exploit Jenner's new findings. It took 100 more years before the next practical vaccine for a viral disease appeared. Louis Pasteur prepared a rabies vaccine from dried, infected rabbit spinal cord and introduced the term **vaccination** (from *vacca*, Latin for "cow") in honor of Jenner's pioneering work. Even with Pasteur's success, other antiviral vaccines were slow to follow, largely because viruses were difficult to identify, propagate, and study. Indeed, the next vaccines (against yellow fever and influenza viruses) did not appear until the mid-1930s.

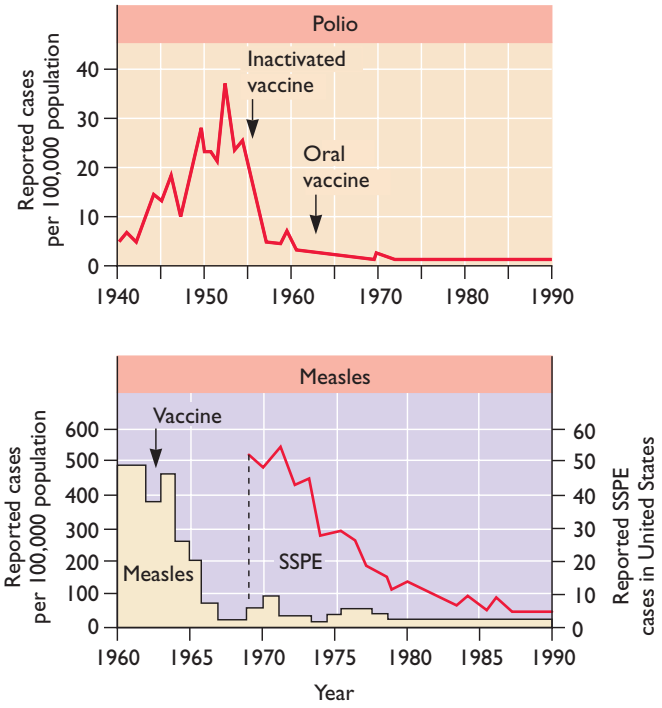


Figure 8.1 Profiles of successful vaccination campaigns. The consequences of poliovirus **(top)** and measles virus **(bottom)** infection have been reduced or eliminated in the United States after massive vaccination programs. SSPE is a rare but fatal brain disease that can occur 10 to 15 years after primary measles virus infection. Blocking primary measles virus infection by vaccination also eliminates SSPE. Adapted from C. A. Janeway, Jr., P. Travers, M. Walport, and M. Shlomchik, *Immunobiology: the Immune System in Health and Disease* (Current Biology Limited, Garland Publishing Inc., New York, NY, 2001), with permission.

Large-Scale Vaccination Programs Can Be Dramatically Effective

Ideally, vaccination mobilizes the host immune system **to prevent** virus infections. As more and more individuals become immune, the transmission cycle of host-host spread in a population is disrupted. As illustrated by the eradication of smallpox, a vaccine can provide a remarkably effective antiviral defense (Box 8.1). However, for reasons discussed below, it will be difficult to eradicate other common viral diseases, including those caused by poliovirus, measles virus, mumps virus, and rubella virus. Even though the World Health Organization (WHO) has made eradication of poliomyelitis and measles the highest priority, progress has been slow for a variety of reasons (Fig. 8.1 and Box 8.2). As an example of the magnitude of the effort, the World Health Organization once immunized 127 million Indian children against poliovirus in more than 650,000 villages in a single day.

Measles, one of the world's five major killer of children, and subacute sclerosing panencephalitis (SSPE), a rare but lethal brain infection also caused by measles virus, are rapidly disappearing in the United States because of effective vaccines (Fig. 8.1). However, unlike the polio vaccine, which can be given orally, the current measles vaccine requires two injections. This requirement alone imposes a number of logistical and practical problems that will complicate worldwide elimination of this virus. In addition, other factors conspire to make measles eradication problematic. The infection is highly contagious, and it is difficult to detect infected individuals. The reproduction number (R_0; see Chapter 10) of measles virus provides a measure of its contagious nature. R_0 is the number of secondary infections produced by an infected person in a fully susceptible population. The R_0 for smallpox virus is estimated to be between 5 and 7, but R_0 for measles virus is between 12 and 18. Amazingly, one person infected with measles infects two to three times as many susceptible people as does someone infected with smallpox.

BOX 8.2

DISCUSSION

The poliomyelitis eradication effort: should vaccine eradication be next?

The worldwide effort to eradicate poliomyelitis, launched by the World Health Assembly in 1988, has stalled. The goal for eradication was set to be the year 2000, but it was not to be. Many setbacks necessitated shifting the target date forward to the present target of 2010.

Enthusiasm was high during the first 12 years of the campaign, when the number of cases of the disease fell from an estimated 350,000 to 2,971. Now, the initial optimism is gone, replaced by doubt over whether eradication is realistic in light of the biological and political realities that have emerged in the course of the campaign. Furthermore, cessation of vaccination, which was to follow eradication, now seems ill advised. Why have the hopes engendered by this once popular program plummeted?

The strategy to eradicate polio makes use of large-scale immunization campaigns with live attenuated poliovirus vaccine. These vaccine strains were known to revert to neurovirulence and cause vaccine-associated poliomyelitis. However, it was thought initially that vaccine-derived poliovirus strains do not circulate efficiently in the population, and that once wild-type poliovirus was eliminated, cessation of vaccination would eradicate the cases of vaccine-associated disease. Unfortunately, the 2000 outbreak of poliomyelitis in Hispaniola shattered this incorrect assumption. In this outbreak, a total of 21 confirmed cases were reported, all but 1 of which occurred in unvaccinated or incompletely vaccinated children. However, subsequent analyses showed that the viruses responsible for the outbreak were derived from Sabin poliovirus type 1 administered in 1998 and 1999. The neurovirulence and transmissibility of these viruses were indistinguishable from those of wild-type poliovirus type 1.

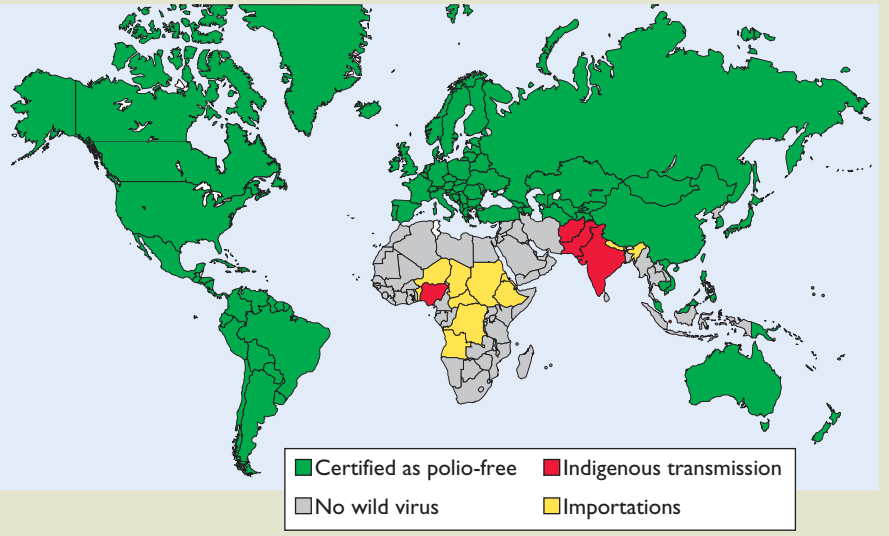
Evidence of circulating vaccine-derived poliovirus was subsequently identified elsewhere. In Egypt, type 2 vaccine-derived poliovirus circulated from 1983 to 1993 and was associated with 32 reported cases. Vaccine-derived strains are also thought to have caused outbreaks of poliomyelitis in Africa and Asia in the 1980s. Vaccine-derived polioviruses caused more than 100 cases of poliomyelitis in Nigeria in 2005 and 2006. Unchecked, and with continued circulation, vaccine-derived strains could eventually cause outbreaks as large as those caused by wild-type strains. The previously underestimated threat of vaccine-derived polioviruses now makes the plan to cease vaccination unacceptable.

During the eradication campaign, countries are certified as free from wild-type polioviruses when the virus cannot be isolated for a 3-year period. As of 2007, poliovirus is endemic in only four countries: Afghanistan, India, Nigeria, and Pakistan. Failure to eliminate transmission of wild polioviruses in these countries is probably a consequence of insufficient vaccine coverage due to politics and war. For example, it is difficult to deliver polio vaccine to the border of Pakistan and Afghanistan, where skirmishes occur regularly. In the north of India, children continue to contract polio despite multiple immunizations. In this case, poor sanitation, crowding, poverty, and infection with other microbes likely contribute to vaccine failure. In 2003, immunization was halted in Nigeria due to fears that the vaccine was contaminated. This led to a resurgence of polio in Nigeria, which then spread across central Africa where immunization also had been reduced, and eventually spread to Yemen, Indonesia, and northern India. Although immunization in Nigeria resumed a year later, many children in that country still do not receive vaccine. The recent outbreak of vaccine-associated polio in Nigeria is a consequence of low immunization rates, which allow circulation and evolution of vaccine-derived polioviruses.

Certification of a region as polio-free means only that wild polioviruses cannot be detected, not that they are absent. Unfortunately, the surveillance network is not infallible. For example, orphan polioviruses, with genotypes unrelated to known strains have been identified, suggesting a failure of some point in the surveillance chain.

These considerations lend strength to the conclusion that polio eradication, followed by cessation of immunization, is not a realistic goal and that the program should be modified to ensure the protection of as many individuals as possible from

Globally reported incidence of poliomyelitis in 2008. The Americas, Western Pacific, and European regions have been declared poliomyelitis free by the WHO. The number of cases has declined from an estimated 350,000 in 1988 to ca. 1,300 in 2008. At the same time, the number of countries in which poliovirus is endemic has decreased from >125 to 4.



poliomyelitis. The vaccine of choice for this purpose would be one that does not revert to neurovirulence. Furthermore, the use of a killed vaccine should bring the circulation of vaccine-derived polioviruses in check. This strategy, combined with surveillance to provide information on the extent of circulation of poliovirus strains, will provide protection of the population for the foreseeable future.

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Minor, P. D. 2004. Polio eradication, cessation of vaccination and re-emergence of disease. *Nat. Rev. Microbiol*. **2:**473–482.

Eradicating a Viral Disease: Is It Possible?

The concept and possibility of eradicating a viral disease deserves careful contemplation. Viruses have survived countless bouts of selection during evolution, so the hubris of declaring a viral disease eradicated is obvious. Nevertheless, in 1978, the Director General of the WHO announced that smallpox was eradicated. Indeed, since that time no natural cases of smallpox have been reported (although accidental laboratory infections have occurred). What makes global elimination conceivable? A number of features are important, as summarized in Table 8.1, but two are absolutely essential: the viral infectious cycle must take place in a single host, and infection (or vaccination) must induce lifelong immunity. By definition, a vaccine that renders the host population immune to subsequent infection by a virus **that can grow only in that host** effectively eliminates the virus. In contrast, a virus with alternative host species in which to propagate cannot be eliminated by vaccination of a single host population: other means of blocking viral spread are required.

Table 8.1 Features of smallpox that enabled its eradication

Virology and disease aspects
No secondary hosts; this is a human-only virus
Long incubation period
Infectious only after incubation period
Low communicability
No persistent infection
Subclinical infections are not a source of infection
Easily diagnosed
Immunology
Infection confers long-term immunity
One stable serotype
Effective vaccine is available
Vaccine is stable and cheap
Social and political aspects
Severe disease with high morbidity and mortality
Considerable savings to developed countries where infection is not endemic
Eradication from developed countries demonstrated its feasibility
Few cultural or social barriers to case tracing and control

National Programs for Eradication of Agriculturally Important Viral Diseases Differ Substantially from Global Programs

National programs typically are established for economically important livestock diseases. The goals are to keep a country free of a particular viral disease even though the disease may still be present in other countries. For example, the United States and Canada have been declared to be free of foot-and-mouth disease, but infections still occur in parts of Europe and South America. Indeed, a devastating epidemic occurred in the United Kingdom in 2001 and a laboratory outbreak occurred in 2007. Such national programs can be successful only when supplemented with broad governmental enforcement and border security, as animals in the virus-free country are constantly exposed to sources from outside the country. Obviously, other principles of control must be implemented. For example, surveillance and containment strategies must be mobilized quickly and aggressively to identify and stop the spread from niche outbreaks. A common practice is to slaughter every host animal in farms at increasing distances surrounding an outbreak site (the so-called "ring-slaughter" program [Box 8.3]). Because acute infections spread rapidly from the outbreak site by many routes, and do so before identifiable symptoms are visible, the ring-slaughter containment often is breached unknowingly. To deal with this fact, preemptive slaughter of **all** animals on "at-risk" farms may be required. Obviously, the faster an outbreak is identified, the more likely the success of containment actions. Unfortunately, on-farm diagnostic tools that provide guaranteed identification of pathogens before symptoms are visible are simply not available. A false-positive identification of an outbreak will have serious consequences.

The strategies devised originally for national veterinary virus control take on new significance when the possibility

that human and animal viruses may be used for nefarious purposes is considered. We cannot be sure that deadly, frightening, or economically devastating viruses are not hidden away for use as weapons, or to inflict terror on an unsuspecting and unprotected public (Box 8.4).

Vaccine Basics

Immunization Can Be Active or Passive

Ideally, **active immunization** with modified virions or purified viral proteins induces an immunologically mediated resistance to infection or disease. In contrast, **passive immunization** introduces the **products** of the immune response (e.g., antibodies or stimulated immune cells) obtained from an appropriate donor(s) directly into the patient. Passive immunization is a preemptive response, usually given when a virus epidemic is suspected. In 1997, consumption of contaminated fruit led to a widespread outbreak of hepatitis A infections in the United States. Pooled human antibodies (also called immune globulin) were administered in an attempt to block the spread of infection and reduce disease. Immune globulin contains the collective experience of many individual infections and provides instant protection against some. The standard procedure for smallpox vaccination with live vaccinia virus requires that so-called "vaccine immune globulin" be available should disseminated vaccinia occur. When stimulated immune cells (e.g., T cells) are used, the process often is called **adoptive transfer**. Passive immunization is expected to produce short-term effects, depending on the biological half-lives of the antibodies or immune cells. Mothers passively immunize their babies through colostrum (antibody-rich first milk), or by transfer of maternal antibody to the fetus via the placenta, providing a protective umbrella against a number of pathogens (Fig. 8.2). This protective effect can be detrimental if active immunization of infants is attempted too early, as maternal antibody may block the vaccine from stimulating immunity in the infant.

Active Vaccines Stimulate Immune Memory

Vaccines work primarily because the immune system can recall the identity of a specific virus years after the initial encounter, a phenomenon called **immune memory** (Box 8.5). While the molecular aspects of the establishment and maintenance of memory continue to be active topics of research and debate, the resounding practical success of immunization

BOX 8.3 BACKGROUND

Stopping epidemics in agricultural animals by culling and slaughter

Vaccination of agriculturally important animals such as cattle and swine may not be cost-effective or may run afoul of government rules that block the shipping and sale of animals with antibodies to certain viruses. The 2001 foot-and-mouth disease epidemic in the United Kingdom provides a dramatic example of how viral disease is controlled when vaccination is not possible. The solution that stopped the epidemic was mass slaughter of **all animals** surrounding the affected areas and chemical decontamination of farms. It is estimated that 6,131,440 animals were destroyed in less than a year before the spread of foot-and-mouth disease virus was contained.

Animal slaughter is often the only alternative available to officials dealing with potential epidemic spread. For example, in recent years, millions of chickens in Hong Kong were killed to stop an influenza virus epidemic with potential to spread to humans. In 2002, millions of chickens in California were slaughtered to stem the spread of Newcastle disease virus in major poultry factories.

Keeling, M. J., M. E. J. Woolhouse, R. M. May, G. Davies, and B. T. Grenfell. 2003. Modeling vaccination strategies against foot-and-mouth disease. *Nature* **421:**136–142.

Kitching, R., A. Hutber, and M. Thrusfield. 2005. Factors relevant to predictive modelling of the disease. *Vet. J.* **69:**197–209.

Woolhouse, M., and A. Donaldson. 2001. Managing foot-and-mouth. The science of controlling disease outbreaks. *Nature* **410:**515–516.

BOX 8.4 DISCUSSION

Should laboratory stocks of smallpox virus be destroyed?

In 2007, WHO officials agreed to postpone for 4 years a decision on when to destroy the world's remaining stores of smallpox virus. In 2010 the WHO will conduct a review of all completed and proposed research to enable members of the 2011 assembly to reach a consensus on when to destroy existing variola virus stocks.

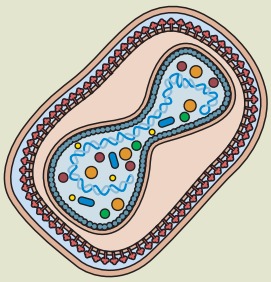
Russia and the United States are the only countries known to hold stocks of the smallpox virus. Since the disease was eradicated in the late 1970s, the WHO has, on several occasions, delayed destroying the virus to permit research on smallpox vaccines and treatments, particularly in light of concerns about bioterrorist attacks.

The important issues are the following:

- Should we destroy biodiversity and gene pools that are not well understood?
- Is smallpox virus simply a bad collection of genes that should be destroyed?
- Are stocks of smallpox virus necessary for development of vaccines and antivirals?
- How do we ensure that all stocks have been destroyed?

Fenner, F. 1996. History of smallpox, p. 25–37. *In* H. Koprowski and M. B. A. Oldstone (ed.), *Microbe Hunters, Then and Now*. Medi-Ed Press, Bloomington, IL.

Henderson, D. A., and F. Fenner. 2001. Recent events and observations pertaining to smallpox virus destruction in 2002. *Clin. Infect. Dis.* **33:** 1057–1059.



in stimulating long-lived immune memory stands as one of humankind's greatest medical achievements.

Immune memory is maintained by dedicated T and B lymphocytes that remain after an infection has waned. These cells provide a heightened ability to respond quickly to a subsequent infection (Fig. 8.3). Antiviral vaccines establish immunity and memory without the pathogenic events typical of the initial encounter with a virulent virus. Ideally, an effective vaccine is one that induces and maintains significant concentrations of specific antibodies (products of B cells) in serum and at points of viral entry. At the same time, T cells responsible for specific cellular immunity must be maintained in a precursor state, ready to make their lethal products (e.g., granzymes and perforins) when challenged by subsequent infection.

Two Central Concepts about Vaccines: Protection from Infection or Protection from Disease

It is critical to understand two fundamental ideas about vaccination and the potential outcomes. It is possible to immunize an individual so that a subsequent infection is stopped before it starts. In this case, the immune response from vaccination is strong enough such that sufficient humoral and cell-based immune effectors are produced and maintained for long periods. Second, it also is possible to immunize an individual so that infection is not blocked immediately, but the increased immune protection provided by vaccination delays or ameliorates disease. In this case, the immune response in the vaccinated individual is said to be "primed." As a result, the subsequent cooperation of vaccine-induced immune effectors and infection-induced molecules may clear the invading virus, but **only** after infection begins. It also is possible that the pathogen may not be eliminated because the vaccine response or the natural response (or both) to the infection is not adequate. In this case, vaccination might only delay the appearance of disease. A further possibility is that immunized individuals may be infected, might mount an active immune response, and will have little or no disease, but the immune response does not eliminate the agent. These infections are said to be persistent or latent (see Chapter 5), and can occur naturally in the absence of vaccination.

Vaccines Must Be Safe, Efficacious, and Practical

The major prerequisites for an effective vaccine are presented in Table 8.2. The overriding requirement for any vaccine is safety: the vaccine cannot cause undue harm. For example, it is imperative that infectious particles and viral nucleic acids be undetectable in vaccines containing inactivated virions or viral proteins. If a live vaccine is used, virulent revertants must be exceedingly rare or undetectable. Contamination of vaccines with adventitious agents, such as other microbes introduced during production, must be avoided. These safety ideals are easy to state, but absolute safety is impossible to guarantee. Human error during vaccine production and testing, as well as limits of process biochemistry and cell biology, are simple facts of life. Rare

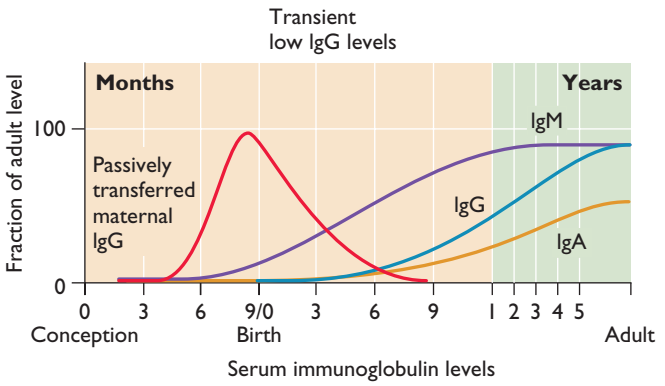


Figure 8.2 Passive transfer of antibody from mother to infant. The fraction of the adult concentration of various antibody classes is plotted as a function of time, from conception to adulthood. Newborn babies have high levels of circulating IgG antibodies derived from the mother during gestation (passively transferred maternal IgG), enabling the baby to benefit from the broad immune experience of the mother. This passive protection falls to low levels at about 6 months of age as the baby's own immune response takes over. Total antibody concentrations are low from about 6 months to 1 year after birth, which may lead to susceptibility to disease. Premature infants are particularly at risk for infections because the level of maternal IgG is lower and their immune system is less well developed. The time course of production of various isoforms of antibody (IgG, IgM, and IgA) synthesized by the baby is indicated. Adapted from C. A. Janeway, Jr., P. Travers, M. Walport, and M. Shlomchik, *Immunobiology: the Immune System in Health and Disease* (Current Biology Limited, Garland Publishing Inc., New York, NY, 2001), with permission.

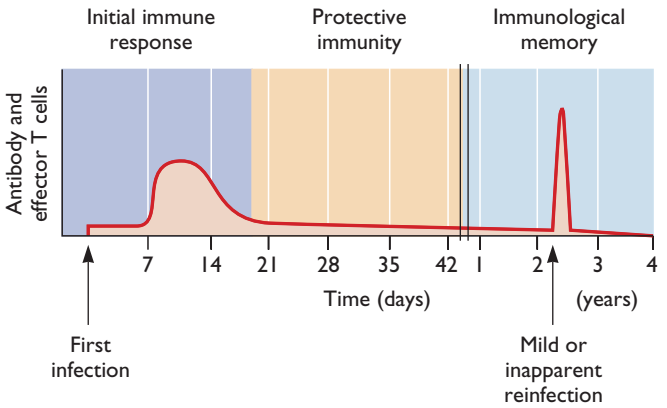


Figure 8.3 Antibody and effector T cells are the basis of protective immunity. The relative concentration of antibody and T cells is shown as a function of time after first (primary) infection. Antibody levels and numbers of activated T cells decline after the primary viral infection is cleared (purple). Reinfections at later times (years later), even if mild or inapparent, are marked by rapid and robust immune response because of "memory." Adapted from C. A. Janeway, Jr., P. Travers, M. Walport, and M. Shlomchik, *Immunobiology: the Immune System in Health and Disease* (Current Biology Limited, Garland Publishing Inc., New York, NY, 2001), with permission.

side effects or immunopathology often can be identified only after millions of people have been vaccinated (Box 8.6). Furthermore, live vaccine agents have the potential to spread to nonvaccinated individuals in a population. For example, the smallpox vaccine is not given intentionally to immunosuppressed individuals, because they cannot contain the infection and may die. These individuals obviously are at risk in a major vaccination program with the current smallpox vaccine (Box 8.1). Untoward side effects or other safety issues have been the death knell of many otherwise effective vaccines. Safety is paramount because vaccines are given to millions of people, some of whom may never be exposed to the virus targeted by the vaccine.

The next requirement is that the vaccine must induce protective immunity **in a significant fraction of the population.** Not every individual in the population need be

BOX 8.5 EXPERIMENTS

A natural "experiment" demonstrating immune memory

A striking example of immune memory is provided by a natural "experiment" in the 18th and 19th centuries on the Faroe Islands in the northern Atlantic Ocean. In 1781 a devastating measles outbreak drastically reduced the islands' population. For the next 65 years, the islands remained measles free and the surviving population flourished. In 1846 measles struck again, infecting over 75% of the population with similar devastating results. An astute Danish physician noted that none of the aged people who survived the 1781 epidemic were infected. However, their age-matched peers who had not been infected earlier were ravaged by measles.

This natural experiment illustrates two important points: immune memory lasts a long time, and memory is maintained during this time without reexposure to the virus.

Ahmed, R., and D. Gray. 1996. Immunological memory and protective immunity: understanding their relation. *Science* **272:**54–60.

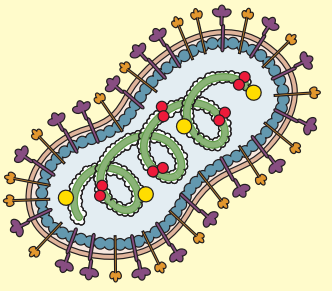


Table 8.2 Requirements of an effective vaccine

Safety	The vaccine must not cause disease
Side effects must be minimal	
Induction of protective immune response	Vaccinated individuals must be protected from illness due to pathogen
	Proper innate, cellular, and humoral responses must be evoked by vaccine
Practical issues	Cost per dose must not be prohibitive
	The vaccine should be biologically stable (no genetic reversion to virulence; able to survive use and storage in different surroundings)
	The vaccine should be easy to administer (oral delivery preferred to needles)
	The public must see more benefit than risk

immunized to stop viral spread, but a sufficient number must become immune to impede virus transmission. Virus spread stops when the probability of infection drops below a critical threshold. This effect has been called **herd immunity**. The actual threshold is agent and population specific, but it generally corresponds to 80 to 95% of the population acquiring vaccine-induced immunity. The herd immunity threshold is calculated as $1 - 1/R_0$. For smallpox this number is 80 to 85%, while for measles it is 93 to 95%. No vaccine is 100% effective, and as a result, the level of immunity is not equal to the number of people immunized. In fact, we know that when 80% of a population is immunized with measles vaccine, about 76% of the population actually is immune, clearly well below the 93 to 95% required for herd immunity. Obviously, achieving such high levels of immunity by vaccination is a daunting task. Moreover, if the virus remains in other populations or in alternative hosts, reinfection is always possible. Closed populations (e.g., military training camps) can be immunized easily, but extended populations spread over large areas of a country present serious logistical and social problems. In addition, public complacency, or reluctance to be immunized, is dangerous to any vaccine program (Box 8.6). Unfortunately, epidemics can occur if the immunity of a population falls below a critical level because an available vaccine is not used.

The protection provided by a vaccine must also be long-term, lasting many years. Some vaccines cannot provide lifelong immunity after a single administration. In such cases, inoculations given after the initial vaccination (booster shots) to stimulate waning immunity are effective, but may be impractical to administer in large populations. Protective immunity also requires that the proper immune response be mounted. Primary infection by some viruses, such as poliovirus, can be blocked only when a robust **antibody response** is evoked by vaccination. On the other hand, a potent **cellular immune response** is required for protection against herpesviral disease. To maximize effectiveness, a vaccine must be tailored to fit its viral target.

Outbred populations always have varied responses to vaccination. Some individuals exhibit a robust response, while others may not respond as well (a "poor take"). While many factors are responsible for this variability, the age and health of the recipient are major contributors. For example, the influenza virus vaccine available each year is far more effective in young adults than in the elderly. Weak immune responses to vaccination pose several problems. Obviously, protection against subsequent infection may be inadequate, but another concern is that upon such subsequent infection, replication will occur in the presence of weak immune effectors. Mutants that can escape the host's immune response can then be selected, and may spread

BOX 8.6 DISCUSSION

The public's view of risk-taking is a changing landscape

Whooping cough used to be a major lethal disease of children until the introduction of the DPT vaccine (diphtheria, pertussis, and tetanus), which virtually eliminated the disease. Immunization resulted in some common, mild side effects: about 20% of children experienced local pain and a feeling of being tired. In addition, about 1 immunized child in 1,000 had more severe side effects. Given that whooping cough was well known to be a child killer, these side effects were acceptable.

However, the vaccine is exceptionally effective and whooping cough was nearly eliminated. Now some parents assess the risk of immunization side effects to be unacceptable, and whooping cough is being seen again in clinics. The risk posed by the vaccine has not changed, but in the face of reduced threat of natural disease, the perceived risk of vaccination was elevated.

Johnson, B. 2001. Understanding, assessing, and communicating topics related to risk in biomedical research facilities. *ABSA Anthology of Biosafety IV—Issues in Public Health,* Chapter 10. http://www.absa.org/0100johnson.html.

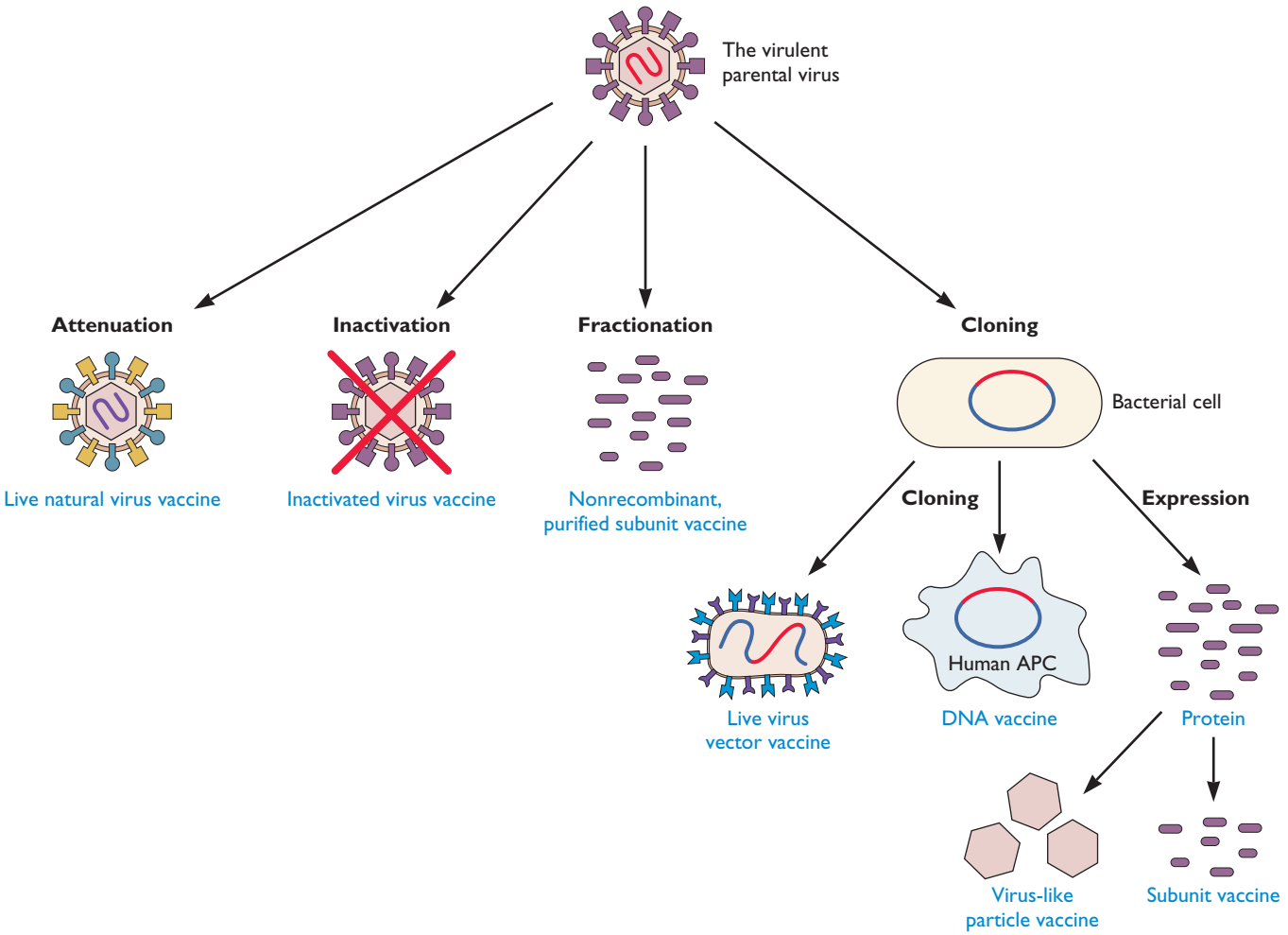


Figure 8.4 How to make vaccines.

in the immunized population. Indeed, vaccine "escape" mutants are well documented.

Important practical requirements for an effective vaccine include stability, ease of administration, and low cost. If a vaccine can be stored at room temperature, rather than refrigerated or frozen, it can be used where cold storage facilities are limited. When a vaccine can be administered orally rather than by injection, more people will accept the vaccination. The WHO estimates that a vaccine must cost less than $1 per dose if much of the world is to afford it. However, the research and development costs for a modern vaccine are in the range of hundreds of millions of dollars. Another, often prohibitive expense is covering the liability of the vaccine producer. Liability expenses can be astronomical in a litigious society and have forced many companies to forgo vaccine development completely. Unfortunately, there is an inherent conflict between providing a good return on investment to vaccine developers and supplying vaccines to people and government agencies with a limited ability to bear the cost.

The Fundamental Challenge

Given the remarkable success of smallpox, measles, and polio vaccines, it might seem reasonable to prepare vaccines against all viral diseases. Unfortunately, designing and producing an effective vaccine are exceedingly difficult. Despite considerable progress in research, we cannot predict with confidence the efficacy or side effects of different vaccine preparations. We lack sufficiently detailed knowledge of the important mechanisms of immune protection against most viral infections, so the basic design of a vaccine is not always obvious. Even questions such as "Is a neutralizing antibody response important?" or "Is a cytotoxic T-lymphocyte (CTL) response essential?" cannot be answered with certainty even for our most common viral infections. Will a vaccine induce sufficient immunity to

block infection completely, or will it delay disease onset? In fact, only when a vaccine is effective, or more often, when it fails, can we learn what actually does or does not constitute a protective response. The regulatory network of gene products required to reproduce an effective antiviral response is understood only in outline. To complicate the situation, even when an experienced vaccine manufacturer sets out to develop, test, and register a new vaccine, the process can take years. For example, it took 22 years to develop and license a relatively straightforward hepatitis A vaccine. The fundamental challenge is to find ways to capitalize on the discoveries in molecular virology and medicine to speed up the process of vaccine development. Despite many problems, those involved in vaccine research and development remain optimistic because of our remarkable history of success and our increasingly detailed knowledge of the molecular nature of viruses and viral disease.

The Science and Art of Making Vaccines

At present, we can outline four basic approaches to produce vaccines (Fig. 8.4). Each approach starts with a pathogenic virus of interest. A vaccine developer may produce large quantities of the virulent virus of interest and chemically inactivate it (**killed vaccine**), may attenuate the pathogenicity through laboratory manipulation (**live, attenuated vaccine**), may produce individual proteins free of the viral nucleic acid (**subunit vaccines**), or may clone all or portions of the viral genome to give rise to recombinant DNA vaccines of several types (**recombinant vaccines**). The most common, commercially successful vaccines simply comprise attenuated or inactivated virions. Their preparation is based on principles that would be understood by Pasteur (Table 8.2 and Boxes 8.1, 8.7, and 8.8).

Basic Approaches

Inactivated or "Killed" Virus Vaccines

The inactivated poliovirus, influenza virus, hepatitis A virus, and rabies virus vaccines are examples of effective inactivated vaccines administered to humans (Table 8.3). In addition, these vaccines are widely used in veterinary medicine. To prepare such a vaccine, virions of the virulent virus are isolated and inactivated by chemical or physical procedures. These treatments eliminate the infectivity of the virus, but do not compromise the antigenicity (i.e., the ability to induce the desired immune response). Common techniques include treatment with formalin or β-propriolactone, or extraction of enveloped virus particles with nonionic detergents.

Theoretically, inactivated vaccines are very safe, but accidents can and do happen. In the 1950s, a manufacturer of Salk polio vaccine did not inactivate it completely. As a result, more than 100 children developed disease after vaccination. Incomplete inactivation and contamination of vaccine stocks with potentially infectious viral nucleic acids have been singled out as major problems with this type of vaccine.

Vaccination is currently the most important measure for reducing influenza virus-induced morbidity and mortality. In the United States alone, influenza virus infections may cause as many as 50,000 deaths every year and cost at least $12 billion in health care. Every year, millions of citizens seeking to avoid infection receive their "flu shot," which contains several strains of influenza virus that have been predicted to reach the United States that year. The magnitude of this undertaking is noteworthy. For example, between 75 million and 100 million doses of inactivated vaccine must be manufactured annually. Typically, these vaccines are formalin-inactivated whole virions or

BOX 8.7 BACKGROUND

The response to infection and vaccination

After a natural infection, viral proteins that can be recognized by the immune system are made in the infected cell. The production of progeny virions and their subsequent spread to other cells amplifies the response. However, vaccination may not reproduce all aspects of this response. Several important variables include the following.

- Infection by live attenuated viruses may provoke an immune response that is qualitatively different from that stimulated by infection with virulent virus.
- In the case of killed vaccines or subunit vaccines, no new viral proteins are made after injection. The immune system therefore **must** recognize only the input material.
- Inactivated virus particles and virion subunits often stimulate an antibody response, but they rarely stimulate an effective CTL response.
- Inactivated virus particles or virion subunits may not persist in the body long enough to establish an effective immune memory response.

Ada, G. L. 1994. Vaccines and the immune response, p. 1503–1507. *In* R. G. Webster and A. Granoff (ed.), *Encyclopedia of Virology*. Academic Press, San Diego, CA.

BOX 8.8 BACKGROUND

Our best vaccines are based on old technology

It seems ironic in this age of modern biology that the mutations in many of our common vaccine strains were not introduced by site-directed mutagenesis of genes known to be required for viral virulence, but rather were isolated by selection of mutants that could replicate in various cell types. The vaccines were produced with little bias as to how to reduce virulence.

Despite the old technology, the vaccines are relatively safe and remarkably effective. Consequently, their analysis has led to the identification of important attenuating mutations, as well as parameters affecting the protective immune response. The current vaccines not only provide protection but also are the foundation for future vaccines.

Painting of Louis Pasteur examining the dried spinal cord of an infected rabbit used to prepare an attenuated strain of rabies virus. Image courtesy of the Pasteur Institute (Photothèque/Relations Presse et Communication externe, Institut Pasteur, Paris, France).

detergent- or chemically disrupted virions (often called a "split" virus preparation). The viruses, which are mass-produced in embryonated chicken eggs, can be natural isolates or reassortant viruses constructed to express the appropriate hemagglutinin (HA) or neuraminidase (NA) gene from the virulent virus.

Currently, a typical influenza vaccine dose is standardized to comprise 15 μg of each viral HA protein, but it contains other viral structural proteins as well. The efficacy of these vaccines varies considerably. They are reportedly about 60 to 90% effective in healthy children and adults younger than 65 years exposed to virus strains in the vaccine. The influenza virus vaccines are all highly immunogenic in young adults, but less so in the elderly, immunosuppressed individuals, and people with chronic illnesses. Protection against illness correlates with the concentration of serum antibodies that react with viral HA and NA proteins produced after vaccination. Immunization may also stimulate limited mucosal antibody synthesis and CTL activities, but these responses vary widely.

The envelope proteins of influenza viruses change by antigenic drift and shift as the virus replicates in various animal hosts around the world (see Chapter 5). Consequently, protection one year **does not guarantee protection the next.** To deal with this ever-changing agent, vaccine manufacturers must reformulate the vaccine **every year** so that it contains antigens from the predicted next generation of viruses. A committee of the Food and Drug Administration chooses the particular strains, in conjunction with WHO-designated laboratories that monitor influenza infections. Timing is critical, as the final decision for the virus composition in the vaccine must be made within the first few months of each year to allow sufficient time for production of the vaccine. Any delay or error in the process, from prediction to manufacture, has far-reaching consequences, given the millions of people who are vaccinated and expect safe protection. Even if the vaccine contains the appropriate viral antigens, and is made promptly and safely, inactivated influenza virus vaccines have the potential to cause side effects in some individuals who are allergic to the eggs in which the vaccine strains are grown. As an example of other problems, the H5N1 avian virus that first infected humans in Hong Kong in the 1990s was extraordinarily cytopathic to chicken embryos, making it difficult to propagate for vaccine purposes. Reassortants had to be constructed by placing the new H5N1 segments in less cytopathic viruses. The risk, of course, is that such reassortants will not provide the proper immune protection against the original strain.

Live Attenuated Virus Vaccines

Successful live attenuated vaccines are effective for at least three reasons: viral replication occurs and stimulates an immune response, progeny virions often are contained at the site of replication and do not spread to other sites, and the infection induces mild or inapparent disease (Fig. 8.5). Less virulent (attenuated) viruses can be selected by growth in cells other than those of the normal host, or by propagation at nonphysiological temperatures (Fig. 8.6). Mutants able to propagate better under these selective conditions arise during viral replication. When such mutants are isolated, purified, and subsequently tested for pathogenicity in appropriate models, some may be less pathogenic than their parent. Temperature-sensitive and cold-adapted mutants are often less pathogenic than the parental virus, because of reduced capacity for replication and spread in the warm-blooded host. Cold-adapted influenza viruses with mutations in almost every gene are licensed vaccines. In the case of viruses with segmented genomes (e.g., arenaviruses, orthomyxoviruses,

Table 8.3 Viral vaccines licensed in the United States

Disease or virus	Type of vaccine	Indications for use	Schedule
Adenovirus	Live attenuated, oral	Military recruits	One dose
Hepatitis A	Inactivated whole virus	Travellers, other high-risk groups	0, 1, and 6 mo
Hepatitis B	Yeast-produced recombinant surface protein	Universal in children, exposure to blood, sexual promiscuity	0, 1, 6, and 12 mo
Influenza	Inactivated viral subunits	Elderly and other high-risk groups	One dose seasonally
Influenza	Live attenuated	Children 2–8 yr old, not previously vaccinated with influenza vaccine	Two doses at least 1 mo apart
		Children 2–8 yr old, previously vaccinated with influenza vaccine	One dose
		Children, adolescents, and adults 9–49 yr old	One dose
Japanese encephalitis	Inactivated whole virus	Travelers to or inhabitants of high-risk areas in Asia	0, 7, and 30 days
Measles	Live attenuated	Universal vaccination of infants	12 mo of age; 2nd dose, 6 to 12 yr of age
Mumps	Live attenuated	Universal vaccination of infants	Same as measles, given as MMR
Papilloma (human)	Yeast- or SF9-produced virus-like particles	Females 9–26 yr old	Three doses
Rotavirus	Live reassortant	Healthy infants	2, 3, and 6 mo or 2 and 4 mo of age depending on vaccine
Rubella	Live attenuated	Universal vaccination of infants	Same as measles, given as MMR
Polio (inactivated)	Inactivated whole viruses of types 1, 2, and 3	Changing: commonly used for immunosuppressed where live vaccine cannot be used	2, 4, and 12–18 mo of age, then 4 to 6 yr of age
Polio (live)	Live, attenuated, oral mixture of types 1, 2, and 3	Universal vaccination; no longer used in United States	2, 4, and 6–18 mo of age
Rabies	Inactivated whole virus	Exposure to rabies, actual or prospective	0, 3, 7, 14, and 28 days postexposure
Smallpox	Live vaccinia virus	Certain laboratory workers	One dose
Varicella	Live attenuated	Universal vaccination of infants	12 to 18 mo of age
Varicella-zoster	Live attenuated	Adults 60 yr old and older	One dose
Yellow fever	Live attenuated	Travel to areas where infection is common	One dose every 10 yr

bunyaviruses, and reoviruses), attenuated, reassortant viruses may be obtained after mixed infections with pathogenic and nonpathogenic viruses.

Live oral poliovirus vaccine comprises three attenuated strains selected for their reduced neurovirulence. Type 1 and 3 vaccine strains were isolated by the passage of virulent viruses in different cells and tissues until mutants with reduced neurovirulence in laboratory animals were obtained (see Fig. 8.7 for a description of how type 3 was derived). The type 2 component was derived from a naturally occurring attenuated isolate. The mutations responsible for the attenuation phenotypes of all three serotypes are shown in Fig. 8.7. Curiously, each of the three serotypes contains a different mutation in the 5′ noncoding region that may affect translation. Alterations in capsid proteins are thought to influence viral assembly.

The live measles virus vaccine currently in use was derived from a virulent virus isolated in 1954 by John Enders, called the Edmonston strain. Attenuated viruses were isolated following serial passages through various types of cells (Fig. 8.8). Even though this approach was empirical, the virions that were isolated replicated poorly at body temperature and caused markedly less disease in primates. The vaccine strain harbors a number of mutations, including several that affect the viral attachment protein.

The live varicella-zoster vaccine is currently the only licensed human herpesvirus vaccine. It has proved to be safe and effective in children and adults, providing significant protection against infection by this prevalent human herpesvirus. Recently, the vaccine has been licensed for use in previously infected adults (over 60 years of age) to protect against recurrent disease (herpes zoster, or shingles).

Live attenuated viruses are administered by injection (e.g., measles-mumps-rubella [MMR] and varicella-zoster vaccines) or by mouth (e.g., poliovirus, rotavirus, and adenovirus vaccines). The highly effective Sabin poliovirus

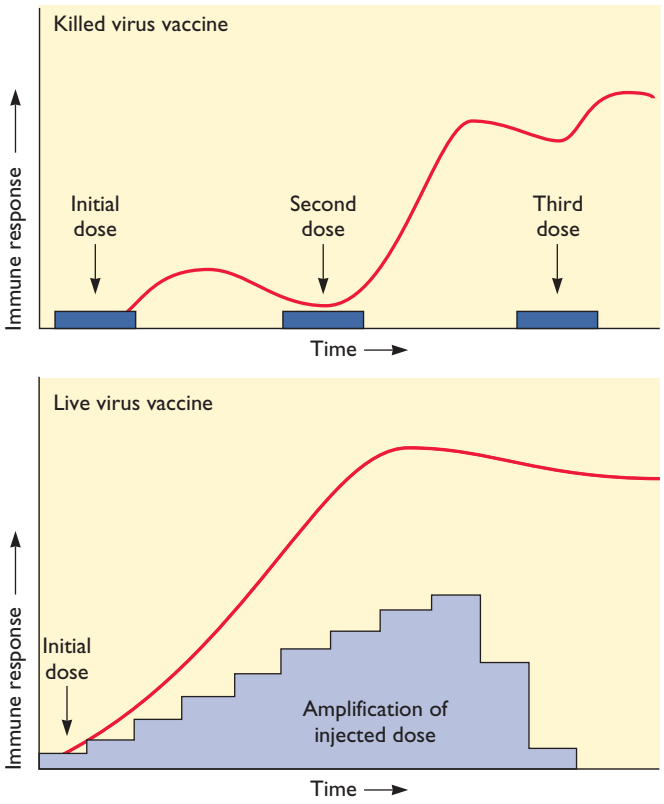


Figure 8.5 Comparison of the predicted immune responses to live and inactivated viruses used in vaccine protocols. (Top) Immune responses plotted against time after injection of a killed virus vaccine (red curve). Three doses of inactivated virions were administered as indicated. **(Bottom)** Results after injection of a live attenuated virus vaccine. A single dose was administered at the start of the experiment. The filled histogram (lavender-colored area) under the curve displays the titer of infectious attenuated virus. Redrawn from C. A. Mims et al., *Mims' Pathogenesis of Infectious Disease*, 4th ed. (Academic Press, Inc., Orlando, FL, 1995), with permission.

vaccine is administered as drops to be swallowed, and enteric adenovirus vaccines are administered as virus-impregnated tablets. One virtue of the oral delivery method for enteric viruses is that it mimics the natural route of infection and, therefore, has the potential to induce the natural immune response. Another is that it bypasses the traditional need for hypodermic needles required for intramuscular or intradermal delivery. Live attenuated respiratory viruses (e.g., the live influenza virus vaccine) can also be delivered by nasal spray, simulating the natural route of infection and immune response.

Live attenuated virus vaccines have inherent problems. For example, despite reduced spread of attenuated viruses in the vaccinee, some viral shedding occurs and these virions are available to infect unvaccinated individuals (Box 8.2). Given the high rate of mutation associated with RNA virus replication, a reversion to virulence should not surprise us. Such reversion is one of the main obstacles to developing effective live attenuated vaccines and is formally equivalent to the emergence of drug-resistant mutants (see "Resistance to Antiviral Drugs" in Chapter 9). While virulent revertants are a serious problem, considerable insight into viral pathogenesis and the immune response can be obtained by determining the changes responsible for increased virulence.

Because our knowledge about virulence is so limited, it is difficult to predict how a live attenuated virus will behave in individuals and in the population. The attenuating mutations may lead to unexpected diversions from the natural infection and expected host response. For example, the attenuated virus may be eliminated from the vaccinated individual before it can induce a protective response, it may infect new niches in the host with unpredictable effects, or it may initiate atypical infections (e.g., slow or chronic infections) that can trigger immunopathological responses of unknown etiology, such as **Guillain-Barré syndrome.** This syndrome may follow viral illness or vaccination and is characterized by rapidly progressing, symmetric weakness of the extremities. It is the most frequent cause of acute generalized paralysis. Vaccine side effects, whether real or not, often have a detrimental effect on public acceptance of national vaccine programs (Box 8.9).

Reversion is not the only problem for live attenuated vaccines, as illustrated by the following informative example. The varicella-zoster live vaccine generally is quite stable and not prone to genetic drift or reversion. Indeed, reversion of the attenuating mutations has been exceedingly difficult to document. However, in studies where viral genomes were isolated and sequenced from individuals who developed vaccine-associated rash, scientists found an unexpected, nonrandom selection of mutations in the isolated viruses. These mutations were not reversions of attenuating mutations, but, rather, had preexisted at a very low level in stocks of the vaccine virus. They were selected in the vaccinated individuals who experienced rashes. The implication was that viral, rather than host, factors were responsible for the rare vaccine side effect of severe rash. This unexpected discovery points to the importance of defining the genetic purity of live-vaccine stocks.

Ensuring purity and sterility of the product is a problem inherent in the production of biological reagents on a large scale. If the cultured cells used to propagate attenuated viruses are infected with unknown viruses, the vaccine may well contain these adventitious agents. For example, in the 1950s, early lots of poliovirus vaccine were grown in monkey cells that were unknowingly infected with the polyomavirus simian virus 40. It is estimated that 10 million to 30 million persons received one or more doses of live simian virus 40

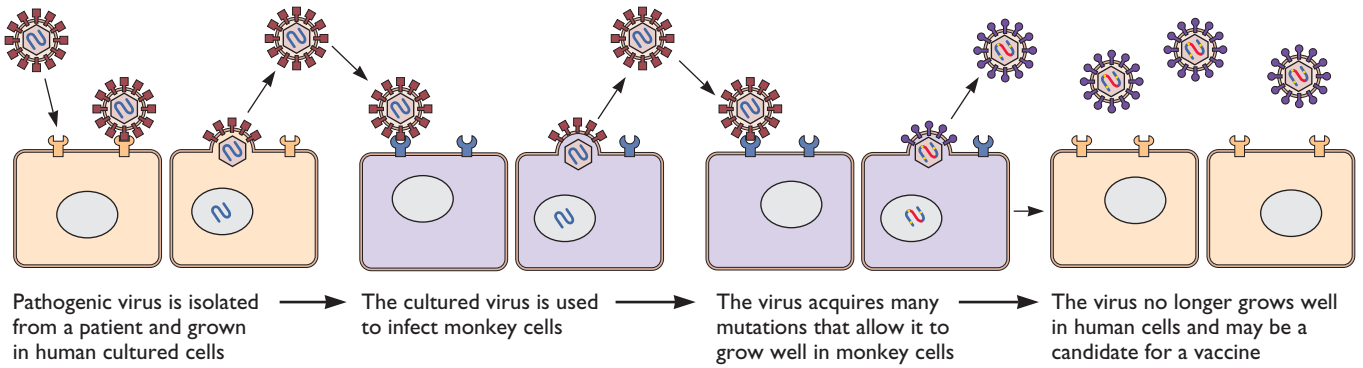


Figure 8.6 Viruses specific for humans may become attenuated by passage in nonhuman cell lines. The four panels show the process of producing an attenuated human virus by repeated transfers in cultured cells. The first panel depicts isolation of the virus from human cells (yellow). The second panel shows passage of the new virus in monkey cells (lavender). During the first few passages in nonhuman cells, virus yields may be low. Viruses that grow better can be selected by repeated passage, as shown in the third panel. These viruses usually have several mutations, facilitating growth in nonhuman cells. The last panel shows one outcome in which the monkey cell-adapted virus now no longer grows well in human cells. This virus may also be attenuated (have reduced ability to cause disease) after human infection. Such a virus may be a candidate for a live vaccine if it will induce immunity but not disease. Adapted from C. A. Janeway, Jr., P. Travers, M. Walport, and M. Shlomchik, *Immunobiology: the Immune System in Health and Disease* (Current Biology Limited, Garland Publishing Inc., New York, NY, 2001), with permission.

in contaminated vaccines. Many even developed antibody to simian virus 40 virion proteins. Some concern exists that rare tumors may be linked to this inadvertent infection, but proving cause and effect has been difficult (see Chapter 7).

Alternatives to the classical empirical approach to attenuation based on modern virology and recombinant DNA technology can now be applied. Viral genomes can be cloned, sequenced, and synthesized. Their genetic information can be analyzed using animals and cultured cells. Viral genes required for pathogenesis in model systems can be identified in systematic fashion. In many cases, attenuation can be deliberately achieved by genetic manipulation. For example, deletion mutations with exceedingly low probabilities of reversion can be created (Fig. 8.9). In another approach that relies on genome segment reassortment of influenza viruses and reoviruses, genes contributing to virulence are replaced with those from related but nonpathogenic viruses. No matter which technology is applied to achieve attenuation, the genetic engineer and the classical virologist must satisfy the same fundamental requirements: isolation or construction of an infectious agent with low pathogenic potential that is, nevertheless, capable of inducing a long-lived, protective immune response.

Subunit Vaccines

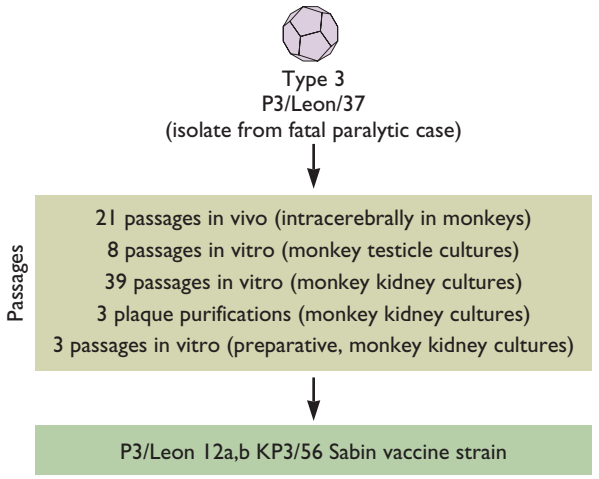
A vaccine may consist only of a subset of viral proteins, as demonstrated by successful hepatitis B and the "split" influenza vaccines. Vaccines formulated with purified components of viruses, rather than the intact virion, are called subunit vaccines. We can deduce what viral proteins to include in a vaccine by determining which are recognized by the antibodies and CTLs found in individuals recovering from disease. Although the most obvious proteins would be those present on the virion surfaces, in fact, any viral protein could be a target.

Synthetic peptides of about 20 amino acids or more in length can induce specific antibody responses when chemically coupled to certain protein carriers that can be taken up, degraded, and presented by major histocompatibility complex (MHC) class II proteins. In principle, synthetic peptides should be the basis for an extremely safe, well-defined vaccine. To date, however, peptide vaccines have had little success, mainly because synthetic peptides are expensive to make in sufficient quantity, and the antibody response they elicit is often weak and short-lived. A weak immune response can be far worse than no response at all, because viral escape mutants may be selected. Given the simplicity of the antipeptide response (usually a single epitope is represented by the peptide), selection of escape mutants is highly probable.

Recombinant DNA Approaches to Subunit Vaccines

Recombinant DNA methods enable cloning of appropriate viral genes into nonpathogenic viruses, bacteria, yeasts, insect cells, or plant cells to produce the immunogenic protein(s). As only a portion of the viral genome is required for such production, there can be no contamination of the resulting vaccine with the original virus, solving a major safety problem inherent in inactivated whole-virus vaccines. Viral proteins can be made inexpensively in large quantities by engineered organisms

A Derivation of Sabin type 3 attenuated poliovirus



B Determinants of attenuation in the Sabin vaccine strains

Virus	Mutation (location/nucleotide position)
P1/Sabin	5'-UTR nt 480 VP1 aa 1106 VP1 aa 1134 VP3 aa 3225 VP4 aa 4065
P2/Sabin	5'-UTR nt 481 VP1 aa 1143
P3/Sabin	5'-UTR nt 472 VP3 aa 3091

C Reversion of P3/Sabin

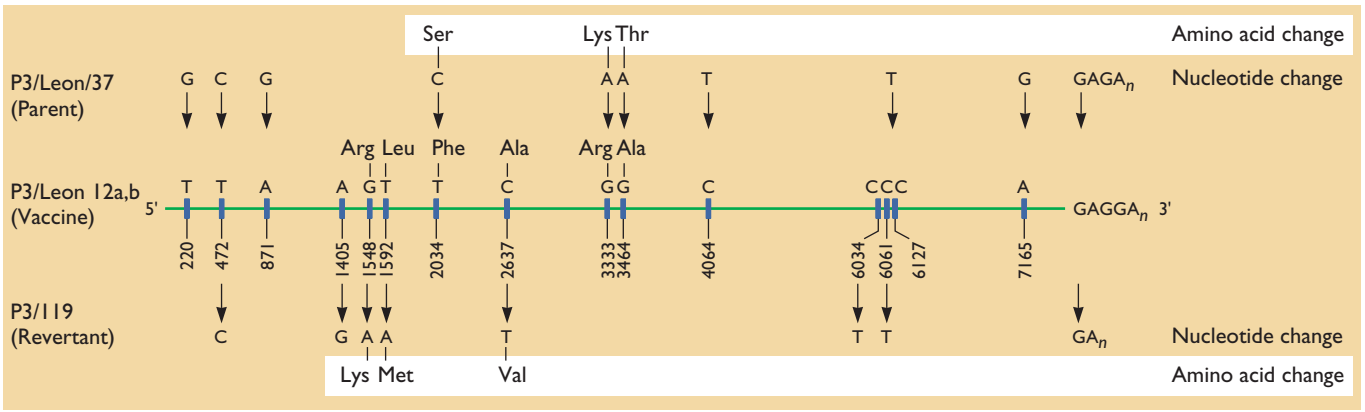


Figure 8.7 Live attenuated Sabin oral poliovirus vaccine. (A) All three viral serotypes may cause poliomyelitis. Therefore, the Sabin vaccine is administered as a mixture of three different strains that are representatives of poliovirus serotypes 1, 2, and 3. Shown is the derivation of the type 3 vaccine strain, called P3/Sabin (the letter P means poliovirus). The parent of P3/Sabin is P3/Leon, a virus isolated from the spinal cord of an 11-year-old boy named Leon, who died of paralytic poliomyelitis in 1937 in Los Angeles. P3/Leon virus was passaged serially as indicated. At various intervals, viruses were cloned by limiting dilution, and the virulence of the virus was determined in monkeys. An attenuated strain was selected to be the final P3/Sabin strain included in the vaccine. **(B)** Determinants of attenuation in all three strains of the Sabin vaccine. The mutations responsible for the reduced neurovirulence of each serotype of the live poliovirus vaccine are indicated (5′-UTR is the 5′ untranslated region, VP1 to VP4 are the viral structural proteins; nt, nucleotide). **(C)** Reversion of the P3/Sabin vaccine strain. Differences in the nucleotide sequences of the virulent P3/Leon strain and the attenuated P3/Sabin vaccine strain are shown above the cartoon of the parental viral RNA (green) and of a virulent virus (P3/119) isolated from a case of vaccine-associated poliomyelitis (below).

under conditions that simplify purification and quality control. For example, problems with egg allergies after vaccination can be eliminated completely when influenza virus proteins are synthesized in *Escherichia coli* or yeasts.

Unfortunately, many candidate subunit vaccines fail because they do not induce an immune response sufficient to protect against infection. Protection against infection (often called "challenge") is the "gold standard" of any vaccine. To achieve this standard, many variables must be assessed: the nature, dose, and virulence of the challenging virus, as well as the route of immunization and the age and health of the host, come into play. The immune repertoire evoked by a live-virus infection may be only partially represented in a response to a subunit vaccine. In particular,

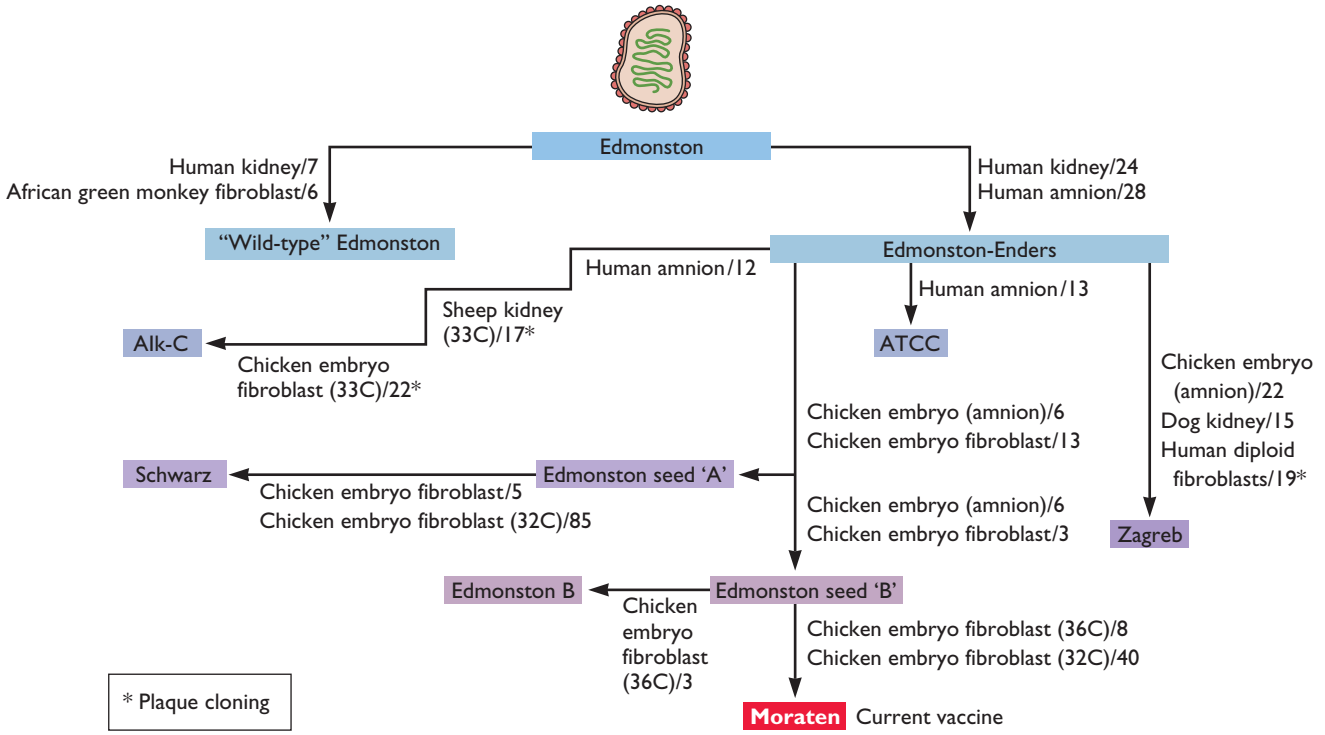


Figure 8.8 Live attenuated measles virus vaccine. Passage histories of live attenuated measles virus vaccines derived from John Enders' original isolate of Edmonston virus (top, blue). The current vaccine is called the Moraten strain (bottom, red). The cells used in passaging the virus to select attenuated mutants are indicated. The temperature during growth is given in parentheses, and the number of passages of virus in the particular cell or cell line follows the slash. An asterisk indicates that a single plaque was picked for further propagation. Adapted from D. D. Richman, R. J. Whitley, and F. G. Hayden (ed.), *Clinical Virology,* 2nd ed. (ASM Press, Washington, DC, 2002), with permission.

purified protein antigens rarely simulate the appearance of mucosal antibodies, particularly immunoglobulin A (IgA).

Virus-Like Particles

Capsid proteins of nonenveloped and some enveloped virions may self-assemble into virus-like particles. Virus-like particles have virtually identical capsid structure to virions, but unlike authentic virions, the capsids are empty; they contain no nucleic acid. These empty capsids retain most of the conformational epitopes not found on purified or unstructured proteins. Consequently, unlike simple subunit vaccines of pure proteins, virus-like particle vaccines often induce authentic neutralizing antibodies and other protective responses after injection. Importantly, because the particles are completely noninfectious, inactivation with formalin or other agents typically used to inactivate live-virus vaccines are not required. This fact presents at least two more advantages: immunogenicity is not affected (formalin and other alkylating chemicals often alter the immunogenicity of the inactivated virions), and concerns about efficiency of inactivation are avoided. Virus-like particle vaccines have proven to be particularly attractive for viruses that replicate poorly in cell culture.

The highly successful hepatitis B subunit vaccine comprises virus-like particles produced in yeast. This vaccine contains a single viral structural protein (the surface antigen) that assembles spontaneously into virus-like particles, whether made in yeast, *E. coli,* or mammalian cell lines. Formation of particles is critical, as purified monomeric capsid protein does not induce a protective immune response. In mice, as little as 0.025 μg of virus-like particles can elicit antibody production without the presence of an adjuvant. Typically, in the human vaccine, 10 to 20 μg of virus-like particles per dose is administered in three doses over a 6-month period, and more than 95% of recipients develop antibody against the surface antigen.

The virus-like particle vaccine effective against human papillomavirus infections is attracting considerable attention. More than 80% of sexually active women will be infected with several serotypes of human papillomavirus during their lifetime. As a result, many of them will develop genital warts and also cervical carcinoma. There are many serotypes of

BOX 8.9 DISCUSSION

National vaccine programs depend on public acceptance of their value

November 2001: Doctors warn of possible measles, mumps, or rubella epidemic

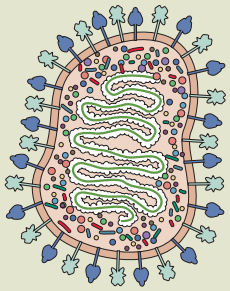
Doctors in Devon [United Kingdom] are warning of a possible outbreak of measles, mumps or rubella as the number of children immunised with MMR vaccine drops.

Update: July 2006

Thirty British doctors call for responsible media coverage amid doubts about the MMR vaccine in the United Kingdom. The number of vaccinated children in the United Kingdom declined from 93% in 1995 to 83% in 2005. Doctors cite a dramatic rise of measles in 2005.

From the BBC News Service, November 2001 and July 2006

The measles-mumps-rubella (MMR) vaccine has proved to be effective in reducing the incidence of these highly contagious and serious diseases. The economic benefit in the United States from use of the MMR vaccine has been estimated to exceed $5 billion per year. However, in the past few years, the press has reported anecdotal studies that link the MMR vaccine to autism. Infants can receive many immunizations early in life, and the symptoms of autism often first appear at the time of these immunizations. The MMR vaccine was singled out as a potential link to autism in some studies. Of prime concern



was that thimerosal, a mercury compound with potential neurological effects, was used as a preservative in vaccines. As a result of the publicity and parental concern, public confidence has been shaken in most developed countries. In some, people are refusing to have their children vaccinated. For example, the immunization rate has fallen significantly in the United Kingdom and, consequently, the incidence of measles infection is on the rise.

In the United States, the Centers for Disease Control and Prevention and the National Institutes of Health recognized the need for an independent group to examine the hypothesized MMR-autism link and address other vaccine safety issues. The committee concluded that "The evidence favors rejection of a causal relationship at the population level between MMR vaccine and autistic spectrum disorders (ASD)," and that "a consistent body of epidemiological evidence shows no association at a population level between MMR and ASD." Other leading medical groups, the American Academy of Pediatrics, the WHO, and British health authorities have come to similar conclusions for largely the same reasons. In 1999, the Food and Drug Administration ordered the elimination of thimerosal from children's vaccines, despite evidence that the mercury exposure was too low to be responsible for neurological defects.

Unfortunately, public confidence in government proclamations is not always high. Moreover, members of the lay public are not well trained to analyze data collected from complex studies that seek to find correlations, or to assess cause and effect. It makes no difference if the public perceives that cancer is caused by high-tension lines or that autism is caused by vaccination: proving (or disproving) cause and effect to a disbelieving public is an exceedingly difficult task.

Information on the Immunization Safety Review Committee can be found at http://www.iom.edu/ImSafety. The full report can be purchased or read online at http://www.nap.edu/catalog/ 10101.html. Copies of *Immunization Safety Review: Measles-Mumps-Rubella Vaccine and Autism* are available for sale from the National Academy Press; call (800) 624-6242 or (202) 334-3313 (in the Washington, DC, metropolitan area), or visit the National Academy Press home page at http://www.nap.edu.

the virus, but serotypes 6, 11, 16, and 18 cause 70% of cervical cancers and 90% of genital warts. It had been known for some time that the human papillomavirus L1 capsid protein forms virus-like particles when synthesized in a variety of heterologous systems. Upon testing, these empty capsids proved to be exceptional inducers of a protective immune response. As a result, a quadrivalent, virus-like particle vaccine effective against the four major serotypes of the virus was formulated. In 2006, the Food and Drug Administration approved this formulation as the first vaccine to be developed to prevent cervical cancer induced by a virus.

DNA Vaccines

In 1992, scientists developed a variation of the subunit vaccine approach that is showing exceptional promise. The **DNA vaccine** consisted simply of a DNA plasmid encoding a viral gene that can be expressed inside cells of the animal to be immunized. In the simplest case, the plasmid encodes only the immunogenic viral protein under the control of a strong eukaryotic promoter. The plasmid DNA, produced in bacteria, can be prepared free of contaminating protein and has no capacity to replicate in the vaccinated host (Fig. 8.10). Remarkably, unlike the requirements for standard protein subunit vaccines, no adjuvants or special formulations are necessary to stimulate an immune response. The vaccine can be delivered by injection of an aqueous solution containing a few micrograms of the plasmid DNA into muscle or skin tissue. Another effective delivery method uses a "gene gun" that literally shoots DNA-coated microspheres through the skin to introduce the plasmid into dermal tissue. Because

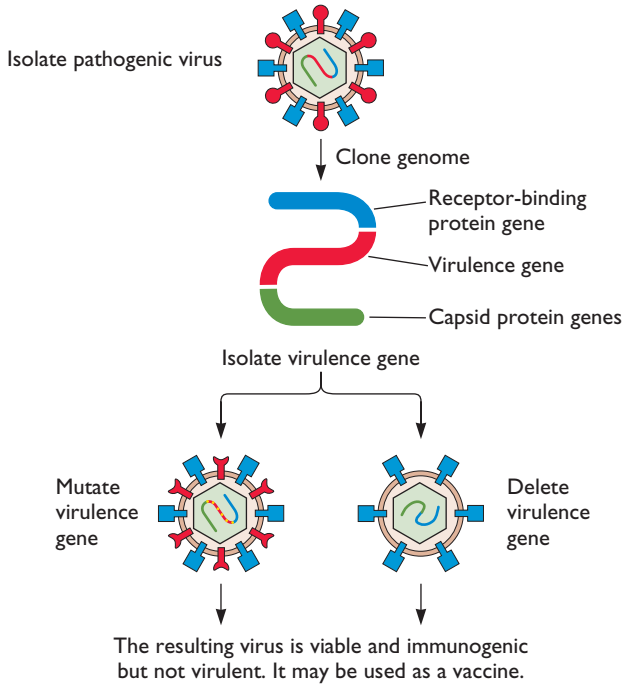


Figure 8.9 Construction of attenuated viruses by using recombinant DNA technology. Once the genome of a pathogenic virus is cloned in a suitable system, deletions, insertions, and point mutations can be introduced by standard recombinant DNA techniques. If the cloned genome is infectious or if mutations in plasmids can be transferred to infectious virus, it is possible to mutate viral genes systematically to find those required for producing disease. The virulence gene can then be isolated and mutated, and attenuated viruses can be constructed. Such viruses can be tested for their properties as effective vaccines. The mutations in such attenuated viruses may be point mutations (e.g., temperature-sensitive mutations) or deletions. Multiple point mutations or deletions are preferred to reduce or eliminate the probability of reversion to virulence. Adapted from C. A. Janeway, Jr., P. Travers, M. Walport, and M. Shlomchik, *Immunobiology: the Immune System in Health and Disease* (Current Biology Limited, Garland Publishing Inc., New York, NY, 2001), with permission.

the viral protein is made *de novo* inside a cell, a fraction of it is presented on the surfaces of producing cells by MHC class I molecules, and the cells are recognized by T cells. Both the humoral response and CTLs can be stimulated by DNA vaccination, and, most important, the vaccine protects against challenge in some animal models. The striking property of DNA vaccination is that a relatively low dose of immunizing protein seems sufficient to induce long-lasting immune responses: the quantity of encoded protein produced is perhaps in the nanogram range or less, as determined by sensitive reporter proteins such as luciferase.

The type of immune response is dictated by the method of inoculation. A Th1 response predominates after injection of an aqueous DNA solution into muscle. In this method, DNA rapidly disappears from the site of injection and is found in the spleen, where the immune response occurs. Animals vaccinated in this way usually synthesize high concentrations of gamma interferon (IFN-γ), interleukin-2 (IL-2), and IL-12, but only low levels of IL-4 or IL-10. In contrast, after DNA immunization by gene gun, the DNA either enters cells directly or is taken up by skin cells and keratinocytes to produce the viral protein. Langerhans dendritic cells in the skin then acquire the protein and move to the draining lymph node. The response induced often is more typical of a Th2 response, with synthesis of IL-4 and antibodies, not IFN-γ and CTLs.

DNA vaccines are effective without addition of adjuvants. The reason is that plasmid DNA itself has intrinsic adjuvant activity in mammalian cells, presumably due to its recognition by Toll-like receptors such as Tlr9. When DNA with unmethylated CpG sequences binds Tlr9 on dendritic cells and monocytes, a burst of IL-12 and IFN-γ ensues and activates Th1 cells. These findings explain why techniques that bypass the dendritic cell Tlr9 protein, such as gene guns that place DNA directly into target cells, often activate the Th2 antibody response rather than the Th1 CTL response.

From a basic research point of view, much remains to be investigated. A technique called **gene shuffling**, which can be applied to produce diverse coding sequences, may have utility in DNA vaccine technology (Box 8.10). Another variation on the single-gene DNA vaccine is a **genomic vaccine**: a library of all the genes of a particular pathogen is prepared in multiple DNA vaccine vectors. The entire plasmid mixture is injected into an animal. Such a vaccine has the potential to present every gene product of the pathogen to the immune system. Because each plasmid encodes a unique mRNA, it should be straightforward to determine the gene(s) necessary and sufficient to induce a protective immune response.

We cannot be sure if DNA vaccine technology has a commercial future. Safety of DNA vaccines is a prime concern. Some possible dangers include integration of plasmid DNA leading to insertional mutagenesis, induction of autoimmune responses such as anti-DNA antibodies, and induction of immune tolerance.

Live Attenuated Viral Vectors and Foreign Gene Expression

Genomes of nonpathogenic viruses can be constructed to produce selected viral proteins that can immunize a host against the pathogenic virus (see Volume I, Chapter 3). This approach merges subunit and live attenuated virus vaccine technologies. Genes from a pathogenic virus are cloned in a nonpathogenic viral vector and used to infect the animals to be immunized. In theory, the vector provides the benefits of a viral infection with respect to stimulating an immune response to the expressed proteins, but with none

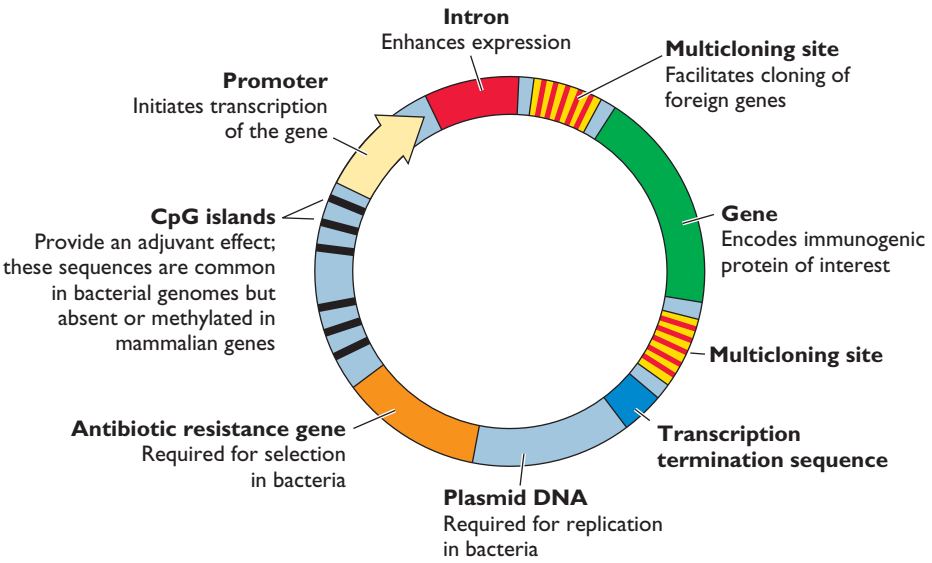


Figure 8.10 Functional components of a DNA vaccine expression vector. The system is based on a plasmid, typically from *E. coli*, carrying its own origin of replication and an antibiotic resistance gene as a selectable marker. A critical component is a strong eukaryotic promoter to initiate transcription of the cloned gene. Expression may be enhanced by including an intron sequence (to facilitate transport of messenger RNA [mRNA] from the nucleus). A multicloning site offers restriction enzyme sites to facilitate cloning of the antigen-encoding gene downstream of the strong promoter and intron. A short untranslated termination sequence provides a polyadenylation signal as well as stability to the antigen-encoding mRNA. CpG-rich sequences can be added and are often found naturally in the bacterial plasmid DNA. Such sequences function as an adjuvant to stimulate the immune response. Adapted from M. Oyaski and H. Ertl, *Sci. Med.* **7**:30–39, 2000, with permission.

of the pathogenesis associated with a virulent virus. However, any replicating viral vector has the potential to produce pathogenic side effects, particularly if injected directly into organs or the bloodstream (see Box 4.7). The immune response to such hybrid viruses is not always predictable, particularly if children, the elderly, and immunocompromised individuals are to be treated.

Poxviruses, such as vaccinia virus, often are used as vaccine vectors. Highly attenuated vaccinia virus mutants have been isolated and may be the basis for the next generation of vaccinia virus-based vaccines. A wide variety of systems are available for the construction of vaccinia virus recombinants that cannot replicate in mammalian cells, but allow the efficient synthesis of cloned gene products that retain their immunogenicity. Attenuated vaccinia virus vectors can accommodate more than 25 kb of new genetic information. Vaccinia virus recombinants can also be used to dissect the immune response to a given protein from a pathogenic virus. This application is illustrated in Fig. 8.11. Other poxviruses, including raccoonpox, canarypox, and fowlpox viruses, offer additional possibilities because they are able to infect, but not replicate in, humans.

The successful use of an oral rabies vaccine for wild animals in Europe and the United States demonstrates that recombinant vaccinia virus vaccines have considerable potential. Recombinant vaccinia virus genomes encoding the major envelope protein of rabies virus yield virions that are formulated in edible pellets to be spread in the wild. The pellets are designed to attract the particular animal to be immunized (e.g., foxes or raccoons). Ideally, the animal eats the pellet, is infected by the recombinant virus, and responds with a protective immune response to subsequent rabies virus infection. While effective, this vaccine approach must be applied with care. Given the rare but serious side effects possible when vaccinia virus infects humans, inadvertent human infection by these wildlife vaccines must be avoided.

The rhabdovirus vesicular stomatitis virus is a particularly promising vaccine vector (see Volume 1, Chapter 3). This virus causes an acute infection of short duration producing vesicular lesions in tongue, teats, and hooves of cattle, pigs, and horses. It also can infect humans, causing mild symptoms. Molecular virologists have studied this virus for many years and have developed techniques to construct attenuated mutants and recombinants that direct the synthesis of almost any protein. Infection of mice and rhesus monkeys induces strong cellular and humoral responses to any foreign protein that has been made after viral infection. The virus is attractive for vaccine technology, because

BOX 8.10 EXPERIMENTS

DNA shuffling: directed molecular evolution

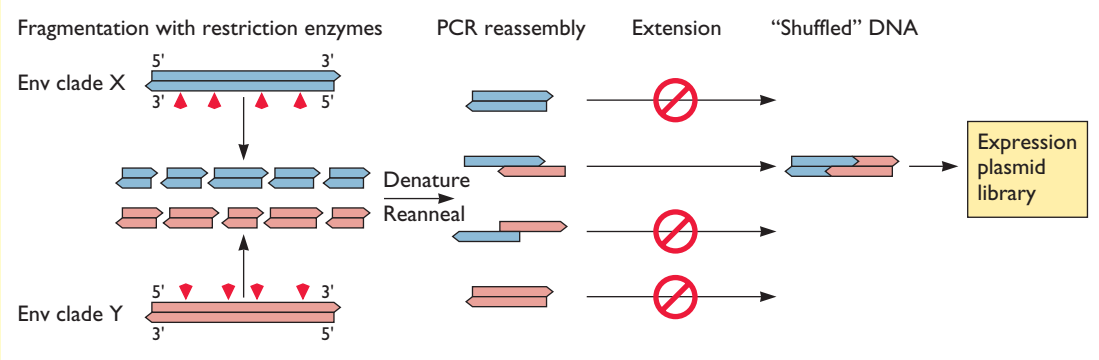
DNA shuffling enables scientists to assemble and analyze new combinations of DNA fragments not found in nature. The utility of this approach for vaccine development is illustrated by the following example. Many different clades of HIV exist, and neutralizing antibodies often recognize the Env protein from one clade, but not others. Some scientists suggest that an important component of a vaccine would be a protein that induces an antibody response capable of recognizing the Env proteins from all known viral clades.

DNA shuffling may provide technology to achieve this goal. DNA encoding Env proteins from various clades would be mixed, digested with restriction enzymes, denatured, reannealed, and amplified by the polymerase chain reaction (PCR). Some of the single-stranded DNA fragments from the *env* gene of one clade will anneal to similar (but not identical) DNA sequences derived from another *env* gene. The 3′ ends of these hybrids are extended in the PCR, and single-stranded regions are copied by DNA polymerase. A mixture of chimeric double-stranded DNA fragments is produced, representing not only all combinations of *env* genes, but also many not found naturally. Expression libraries created by insertion of the new shuffled DNA segments into suitable plasmids would then be screened for production of new proteins that bind antibodies specific for Env proteins, or that induce antibodies capable of neutralizing a broad spectrum of virus isolates.

Optimists assert that DNA shuffling can be used to make effective, safe, multivalent DNA vaccines, and also can be used to boost the potency of known vaccines.

Stemmer, W. P. C. 1994. Rapid evolution of a protein *in vitro* by DNA shuffling. *Nature* **370:**389–391.

Whalen, R. G., R. Kaiwar, N. W. Soong, and J. Punnonen. 2001. DNA shuffling and vaccines. *Curr. Opin. Mol. Ther.* **3:**31–36.



high titers can be obtained easily and it can be delivered by a mucosal route without the need for injection. Initial experiments are promising: when rhesus monkeys were vaccinated with recombinant vesicular stomatitis viruses expressing the human immunodeficiency virus type 1 (HIV) *env* and *gag* genes, and then challenged with a strain of human virus that causes AIDS in monkeys, all animals remained healthy for over a year, with low or undetectable viral loads. In contrast, all nonvaccinated, challenged monkeys progressed to AIDS in less than 5 months.

Two general problems arise with live-virus vector vaccines. First, the host usually is immunized against the viral vector, as well as the vaccine antigen. As a result, subsequent uses of the particular vector may result in a weak response, no response, or an immunopathological response. Second, introducing live viral vectors, even though they are attenuated, into the population at large may have long-term effects. For example, immunocompromised individuals may be infected, with adverse consequences.

Vaccine Technology

Most Killed and Subunit Vaccines Rely on Adjuvants To Stimulate an Immune Response

Charles Janeway revealed what he called "immunologists' dirty little secret": inactivated virions or purified proteins often do not induce the same immune response as live attenuated preparations, unless mixed with a substance that stimulates early processes in immune recognition, particularly the inflammatory response. Such immunostimulatory substances are called **adjuvants**. Development of these substances has been largely empirical, although as our understanding of the various regulators of immune responses increases, more specific and powerful molecules are being discovered. The fundamental understanding of adjuvant action is that adjuvants stimulate early intrinsic and innate defense signals, and therefore can shape subsequent adaptive responses. Adjuvants work in at least three different ways: by presentation of antigen as particles,

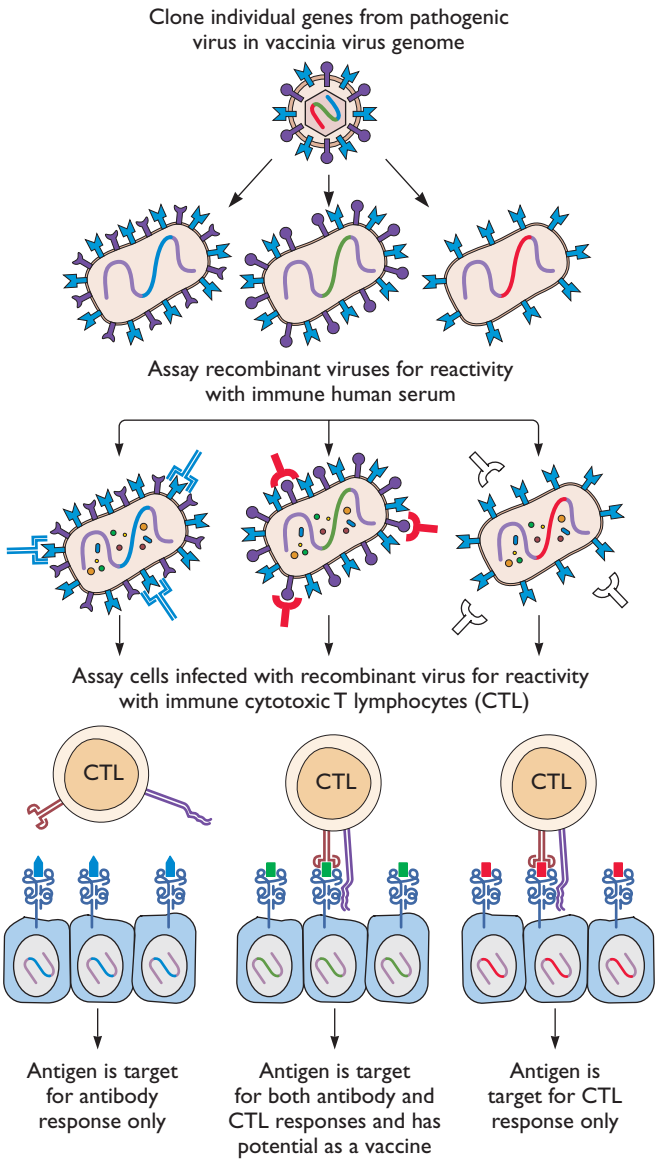


Figure 8.11 Use of recombinant vaccinia viruses to identify and analyze T- and B-cell epitopes from other viral pathogens. As illustrated, it is possible to determine if a particular viral protein contains a B-cell epitope (binds antibodies), a T-cell epitope (recognized by CTLs), or both. Subsequent site-directed mutational analysis of the viral genes enables precise localization of these epitopes on the viral protein. Adapted from C. A. Janeway, Jr., P. Travers, M. Walport, and M. Shlomchik, *Immunobiology: the Immune System in Health and Disease* (Current Biology Limited, Garland Publishing Inc., New York, NY, 2001), with permission.

by localization of antigen to the site of inoculation, and by direct stimulation of the intrinsic and innate immune responses. The immune system can be stimulated directly when adjuvants mimic or induce cellular damage or alter homeostasis (sometimes called "danger" signals), or when they engage intrinsic cellular defense receptors.

Adjuvants vary in composition, from complex mixtures of killed mycobacteria and mineral oil (the classic complete Freund's adjuvant) to lipid vesicles or mixtures of aluminum salts (Table 8.4). Some adjuvants, like alum (microparticulate aluminum hydroxide gel), are widely used for human vaccines such as hepatitis A and B vaccines. Others, such as complete Freund's adjuvant, are used only in research. Freund's adjuvant is extremely potent, but is not licensed for use in humans because it causes tissue damage and other undesirable effects. Two of the active components in Freund's adjuvant have been identified as muramyl dipeptide and lipid A, both potent activators of the inflammatory response. We now understand that the strong adjuvant effects of this complex adjuvant are due, at least in part, to the mycobacterial DNA present in the emulsion that activates the Tlr9 pathogen recognition protein. Less toxic derivatives, along with saponins and so-called block copolymers (linear polymers of clustered hydrophobic and hydrophilic monomers), are promising adjuvants.

As noted above, virus-like particles do not require adjuvants to stimulate strong immune responses. It may be possible to produce other viral protein complexes with adjuvant activity. For example, liposomes and lipid micelles can be prepared with viral proteins exposed on the surface (immune-stimulating complexes [ISCOMs]). These preparations are being evaluated in human trials (Table 8.4).

Some new adjuvants under study enable exogenous proteins to enter the major MHC class I pathway normally reserved for proteins synthesized *de novo*. Preparations with saponins or muramyl tripeptide-phosphatidylethanolamine induce macrophages and dendritic cells to synthesize IL-12, which stimulates Th1 immune responses. Others include molecules that stimulate the pattern recognition receptors that provide the initial responses to infection (Chapter 3). Administration of a natural or synthetic ligand for a particular Tlr, triggers a rapid and targeted immune response to a coinjected foreign protein in laboratory animals.

Vaccine researchers speak of "tailoring" a vaccine by using different combinations of adjuvant and vaccine to induce a protective immune response. The principle is promising, but much work remains to be done to demonstrate the safety and efficacy of this approach.

Delivery

Improvement of administration or delivery is an important goal of vaccine developers. At present, vaccines can be administered by a variety of methods, including the traditional hypodermic needle injection and the "air gun" injection of liquid vaccines under high pressure through the skin. Other formulation methods under consideration include new emulsions, artificial particles, and direct injection of fine powders through the skin. Oral delivery of vaccines can be effective in stimulating appearance of

Table 8.4 Vaccine delivery systems and adjuvants[a]

Adjuvant	Formulation and properties
Aluminum salts	Aluminum hydroxide or phosphate (alum). Form precipitates with soluble antigen, making the complexes more immunogenic; form antigen "depot" at site of injection; complement activation.
Emulsions	Freund's complete adjuvant: antigen suspended in water/mineral oil emulsion with killed *M. tuberculosis* bacteria or muramyl di- or tripeptide to stimulate strong T-cell responses. Freund's incomplete adjuvant: antigen suspended in water-in-mineral oil emulsion.
Microspheres	Antigen encapsulated in polymers of lactic and glycolic acids. They are biodegradable and cause slow release of antigen.
ISCOMs	Immune stimulating complexes composed of glycosides, a purified saponin from the plant *Quillaja saponaria*, cholesterol, phospholipids, and antigens. Form spheres of 30–40 nm in diameter that incorporate antigen.
Nucleic acid vaccines	Genes encoding antigens expressed from strong promoters are introduced directly to muscle or skin by using physical methods or liposomes, leading to intracellular protein production and presentation of antigen to the immune system.
Engineered viruses	Genes encoding foreign antigens are introduced into a viral genome (the vector) such that the new protein made following infection. Common viral vectors are vaccinia virus, adenovirus, and baculovirus. Many other viruses can also be modified to express foreign genes.

[a] See B. Guy, *Nat. Rev. Microbiol.* **5**:505–517, 2007, for more information.

IgA antibodies at mucosal surfaces of the intestine, and in inducing a more systemic response. However, oral delivery is not always possible, because the protective surfaces and enzymes of the oral cavity and alimentary tract block or destroy many vaccines. Specially constructed edible plants that make viral proteins represent an attractive, new approach. Transgenic plants can be engineered, or plant viruses with genomes encoding immunogenic proteins can be used to infect food plants. Early experiments are promising: when such a plant is eaten, antibodies to the viral structural protein can be demonstrated in the animal's serum. Another oral vaccine formulation technology is based on principles discovered by research on enteric viruses that survive passage through the stomach. Biopolymers that mimic the protective action of capsids can be formulated with vaccine components to resist the low pH and digestive enzymes of the stomach, but dissolve in the more hospitable regions of the upper intestinal tract. Such coating technology has been used for oral delivery of adenovirus vaccines and the vaccinia virus-based wildlife rabies vaccine discussed previously.

Immunotherapy

Patients already infected with viruses that cause persistent infections, or that reactivate from latency in the face of an immune response, present special problems. **Immunotherapy** provides the already-infected host with antiviral cytokines, other immunoregulatory agents, antibodies, or lymphocytes **over and above those provided by the normal immune response** in order to reduce viral pathogenesis. Immunotherapy can be administered by introduction of the purified compounds, or of a gene encoding the immunotherapeutic molecule (a gene therapy approach). In at least the case of the varicella-zoster vaccine, a large boost of the same live, attenuated vaccine given to children will stimulate the immune response of latently infected adults such that recurrent disease (e.g., zoster or shingles) is markedly reduced. A live attenuated virus or a DNA vaccine can be modified to synthesize cytokines that stimulate the appropriate immune response. If a live attenuated vaccine is used, care must be taken as it is possible that the altered immune response will have unexpected effects, such as increased virulence, persistence, and pathogenesis.

It is possible to isolate lymphocytes from patients, infect these cells with a defective virus vector (e.g., a retrovirus) encoding an immunoregulatory molecule, and then introduce the transduced cells back into the patient (*ex vivo* approach). If these cells survive and synthesize the protein, the patient's immune response may be bolstered. If stem cells are transduced, a long-term effect may be achieved, as these cells replicate and propagate the transgene.

We know that immunotherapy using cytokines can be effective. Historically, it was pioneered by using interferons to treat a variety of diseases, including those caused by persistent viruses. For example, IFN-α is approved in the United States for treatment of chronic hepatitis caused by hepatitis B and C viruses. Its effect on chronic hepatitis B virus infection is remarkable: as many as 50% of treated patients have no detectable infection. This result illustrates the potential of immunotherapy. However, similar treatment of hepatitis C virus infection has been much less successful for reasons that are not clear. Limitations of IFN therapy (and probably cytokine therapy in general) are that the biological activity of IFN is not sustained for a prolonged time, side effects are significant, patients older than 50 tend to be less responsive, and treatment is expensive.

Immunomodulating agents, including IFN, cytokines that stimulate the Th1 response (e.g., IL-2), and certain chemokines, are being studied individually and in combinations for their ability to reduce virus load and complications of persistent infections caused by papillomavirus and human immunodeficiency virus. Cytokines that stimulate natural killer cells (e.g., IL-12 and IFN-γ) may have promise as well. An alternative to using cytokines or immunoregulatory molecules is the introduction of more antibodies or activated lymphocytes into an infected individual. Care must be taken when introducing immune-modulating genes into viral genomes. For example, cloning of the IL-4 gene into mousepox virus, with the hope that this recombinant would produce a strong antibody response, led to a hypervirulent virus. A fundamental problem with these approaches is that immune stimulation can lead to pathology or provide new avenues for virus spread.

The Quest for an AIDS Vaccine

In 1984, several years after HIV was identified, officials in the U.S. government predicted that an AIDS vaccine would be available within 3 years. Although research has continued for nearly 25 years with unprecedented intensity, we still do not know how to make a vaccine that will protect against AIDS. Such ill-founded optimism has a precedent in the history of vaccination. Poliovirus was isolated in 1908, and 3 years later Simon Flexner of the Rockefeller Institute announced that a vaccine would be prepared in 6 months. Almost 50 years of research on basic poliovirus biology was necessary to provide the knowledge of pathogenesis and immunity that allowed the development of effective poliomyelitis vaccines (Table 8.5).

Formidable Challenges

Despite an investment of more than 1.5 billion dollars toward the development of an HIV vaccine since 1996, it is safe to say that the prospect of a licensed vaccine appearing even by the next decade is remote. The reasons for this lack of progress lie deep in HIV biology and the interaction of the virus with the host immune system. Although a vigorous immune response is induced after infection, this response is not effective in clearing it. Not only that, but viral proteins actually derail subsequent immune responses to the extent that the host often dies due to other microbial infections. As a consequence, HIV pathogenesis is almost completely one sided, in that patients never spontaneously recover. At this time, we do not know what constitutes protective immunity and have few, if any, clues to guide development of a prophylactic or therapeutic vaccine. Vaccine developers are resigned to testing their hypotheses in surrogate systems or even in the clinic before any progress can be made. A number of approaches, including inactivated virions, subunit vaccines based on single viral proteins, and passive immunization, have already been tested with no obvious success. In particular, subunit vaccines, although capable of inducing strong antibody responses, are markedly inefficient in eliciting a CTL response. Therapeutic vaccines administered to infected individuals may have value, if administered in combination with a drug regimen that protects the immune system. Live attenuated HIV vaccines, modeled after the successful live poliovirus vaccine, present difficult scientific and ethical problems: the risks associated with injecting thousands of healthy uninfected volunteers with a living (albeit attenuated) virus are currently considered unacceptable. More promising, however, are vaccines based on recombinant adenovirus, poxvirus, vesicular stomatitis virus, or alphavirus genomes that encode various HIV proteins. Such recombinant vaccines have proved to be potent activators of CTLs as well as of humoral responses. A promising area of research is combination of a DNA vaccine to provide strong cellular and humoral responses (DNA priming) and a live attenuated virus vaccine (vector boosting). Nevertheless, truth be told, in the last 15 years, more than 30 vaccine candidates have been tested and all have failed. As a sobering example of the paucity of basic knowledge, a recent book on HIV vaccines stated that "… there is a general, but unproven notion, that both humoral and cellular immune responses will ultimately be required for a successful AIDS vaccine."

Table 8.5 When can we expect an HIV vaccine?

Viral vaccine	Yr when etiologic agent was discovered	Yr when vaccine was developed in the United States	No. of yr elapsed
Polio	1908	1955	47
Measles	1953	1983	30
Hepatitis B	1965	1981	16
Rotavirus	1970	1998	28
Hepatitis A	1973	1995	22
HIV	1983	None yet	>25

The Central Issues

Lentivirus replication requires integration of a DNA copy of the viral genome into the host genome. Such a close relationship with the host often results in a true latent infection where cells with integrated proviral DNA produce no proteins. These reservoirs of infection remain invisible to immune recognition. Consequently, the infection is maintained in the face of a vigorous immune response. Some scientists consider this the crux of the challenge: how do we induce an immune response **superior** to the normal response that apparently is not good enough to clear the infection? How do we eliminate immune escape mutants?

At a minimum, we can make a short list of our expectations for an HIV vaccine (Table 8.6). It should be safe, of course, but it also must be effective in preventing infection in most of the vaccinated people. The protection should last for many years and be effective against as many of the diverse HIV strains as possible. The vaccine should not be so complicated that it cannot be produced on a large scale for a reasonable price. It should be stable with a significantly long shelf life so that it can be distributed, stored, and delivered when needed. Finally, it should be easy to administer. If anything has been learned over the past 25 years of managing HIV infections in the clinics around the world, it is that a predictive relationship exists between the viral load (as measured by the number of viral RNA copies in the circulation) and disease progression: patients with low viral loads progress more slowly to disease. The hope is that a vaccine that will mimic the response resulting in low viral load can be developed. What this immune response is remains a matter of conjecture. The window of opportunity to block a primary infection, integration, and dissemination within a host is very small.

There are complicated social, ethical, and political issues that must be addressed, when vaccines are to be tested in humans (Table 8.7). For example, can vaccine trials be conducted so that appropriate placebo controls can be evaluated? Vaccine developers face costly litigation if unanticipated reactions occur. On a larger scale, significant political issues arise when decisions are made by Western leaders about vaccines to be used in developing countries. Resolution of these problems will not be easy.

Table 8.6 Challenges of developing an HIV vaccine[a]

1. Provirus is integrated in host genome
2. Infected cells transmit the infection (cell-cell spread)
3. Must deal with high mutation rate/immune selection
4. Virus infects areas of reduced immune surveillance (brain, testes)
5. Infection compromises immune function
6. Must induce the proper balance of Th1 and Th2 responses

[a]For more details, see Y. Bhattacharjee, *Science* **318:**28–29, 2007.

Despite the formidable challenges, scientists in academia and industry must work with public health scientists and government policy makers to muster the political and scientific might to solve the problems. The HIV pandemic is already extracting an inestimable cost and, sadly, shows no signs of diminishing.

Perspectives

The goal of modern vaccine research is to formulate vaccines that can be tailored to particular infections, and that will produce a safe and protective defense. We have had noteworthy success with new vaccine formulations such as the virus-like particle preparations effective in protecting against hepatitis B and papillomavirus infections. However, our lack of progress with others reminds us that viral infection and pathogenesis are complex and poorly understood processes. Because of the complexity of host-virus interactions, the problems of controlling pathogenesis are daunting. What we don't know about both processes is humbling.

Even when available, vaccines can have unexpected side effects, people may refuse to accept them, and societies may not use or be able to pay for them. In addition, we have learned that it is difficult to intervene in any complex

Table 8.7 Three phases of vaccine trials[a]

Phase	Time	Action
I	12–18 mo	Safety. A small group of seronegative individuals is given the vaccine and observed for adverse affects and tested for their immune responses.
II	1–2 yr	Safety, dose optimization, immunogenicity. Several hundred seronegative individuals are given the vaccine. Initial and long-term immune responses at various doses are measured.
III	Several years	Safety and efficacy. Several thousand individuals are enrolled. One group receives the vaccine, and the other receives a placebo control. The extent of virus transmission and the effect of the vaccine on virus replication are determined.

[a]Adapted from J. A. Levy, *HIV and the Pathogenesis of AIDS*, 3rd ed. (ASM Press, Washington, DC, 2007).

host-parasite interaction without unanticipated effects. Indeed, we often find out how little we know when we test vaccines in the real world: formulations that worked in the lab fail in the field. Furthermore, it is inevitable that viral mutants able to escape immune defenses will arise.

An important principle is that the built-in survival mechanisms of virus and host provide both opportunities and problems in our quest to control viral disease. However, it is telling that essentially all successful vaccines on the market today have been developed empirically. We anticipate that the situation will change as we learn more about the molecular mechanisms of antiviral immune defense and the epidemiology of infections.

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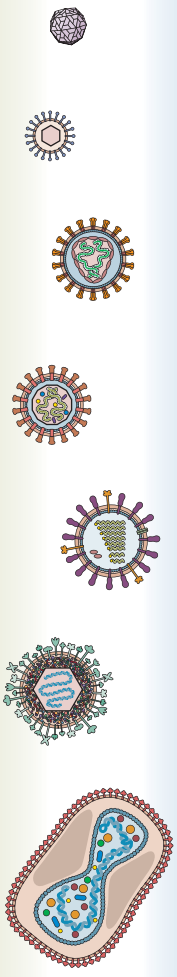


9

Antiviral Drugs

You can't go back and you can't stand still,
If the thunder don't get you, then the lightning will.

JERRY GARCIA
The Wheel (words by Robert Hunter)

Introduction

Some viral infections can be controlled effectively by public health measures and vaccines. However, for many others, these measures have no effect, are not available, or cannot be applied. Antiviral drugs are intended to fill a portion of this void. However, despite almost 50 years of research, our armamentarium of such drugs remains surprisingly small. The current arsenal comprises fewer than 50 drugs, and most of these are directed against human immunodeficiency virus (HIV) and herpesviruses (Table 9.1). It is important to understand why we are in this precarious situation.

Paradox? So Much Knowledge, So Few Antivirals

Because we know so much about some viruses, this dearth of antiviral drugs is unexpected. There are many reasons for the lack of therapies, but a primary reason is that antiviral drugs **must** be safe. This simple goal often is unattainable, as viruses are parasites of cellular mechanisms, and compounds interfering with viral growth often have adverse effects on the host. Another reason is that antiviral compounds must be extremely potent, virtually 100% efficient in blocking viral growth. Even modest replication in the presence of an inhibitor provides the opportunity for resistant mutants to prosper. Achieving the potency to block viral replication **completely** is remarkably difficult. Additionally, many medically important viruses cannot be propagated conveniently in the laboratory (e.g., hepatitis B virus and papillomaviruses), and for some human viruses, there are no available small-animal models (e.g., measles virus and hepatitis C virus). The testing and development of safe, potent, and efficacious compounds that block infection by these viruses pose a significant challenge. Finally, as noted in Chapter 5, many acute infections are of short duration, and by the time the individual feels ill, viral replication is completed and infected cells are being cleared. Antiviral drugs must be given early in the infection, or prophylactically to populations at risk. It takes time, not only to identify the specific viral pathogen but also to obtain and dispense the drug (Box 9.1). The lack of rapid

Table 9.1 The antiviral repertoire[a]

Targets	Viruses[b]	Examples of compounds approved
Virion uncoating	Influenza A	Amantadine, rimantadine
DNA polymerase	Herpesviruses (HSV-1, HSV-2, VZV, CMV, EBV, HHV-6, HHV-7, HHV-8)	Nucleosides: acyclovir, valacyclovir, ganciclovir, valganciclovir, penciclovir famciclovir, brivudin,[c] foscarnet
	Herpesvirus (CMV)	Acyclic nucleoside phosphonates: cidofovir, tenofovir
	HIV	
Reverse transcriptase	HIV	Nucleosides: zidovudine, didanosine, zalcitabine, stavudine, lamivudine,[d] abacavir
		Nonnucleosides: nevirapine, delavirdine, efavirenz
Viral protease	HIV	Saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir
Viral neuraminidase	Influenza A and B virus	Zanamivir, oseltamivir
Inosine monophosphate dehydrogenase	HCV, RSV	Ribavirin[e]

[a]Data from E. De Clercq, *Nat. Rev. Drug Discov.* **1:**13–25, 2002.

[b]Abbreviations: CMV, cytomegalovirus; EBV, Epstein-Barr virus; BCAR, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide; HBV, hepatitis B virus; HCV, hepatitis C virus; HHV, human herpesvirus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; IMP, inosine 5′-monophosphate; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; RSV, respiratory syncytial virus; VZV, varicella-zoster virus.

[c]Birivudin is approved in some countries, for example, Germany.

[d]Lamivudine is also approved for the treatment of HBV.

[e]Ribavirin is used in combination with interferon-α for HCV.

BOX 9.1 DISCUSSION

We can put a person on the moon, but we cannot cure the common cold

While the title phrase of this box is timeworn and trite, it contains elements of truth that underlie some basic problems of finding treatments to common, acute infections. The common cold is a syndrome caused by many different viruses, including rhinoviruses (about 50% of all common colds), adenoviruses, coronaviruses, and others. Several fundamental problems confound the quest for a cure for the common cold.

- The common cold is not a life-threatening disease, but a mild, inconvenient illness. Antiviral drugs typically are not sold over the counter. Will people see their physicians to obtain a prescription every time they experience a drippy nose, congestion, or cough?
- By the time the symptoms of a common cold are evident, viral replication has reached its peak. Therefore, by the time an antiviral is given, it may be too late for significant effect.
- Drugs and vaccines may have to be given to millions of healthy people to be effective. Short-term and long-term safety of any treatment must be ensured.

In 2001, a new antiviral compound called pleconaril was tested in humans. This compound is effective against rhinoviruses, but not coronaviruses or adenoviruses. It binds to the hydrophobic pocket under the canyon in the receptor-binding sites of many rhinoviruses (Volume I, Chapter 5). Pleconaril is thought to act by stabilizing the particle so that uncoating and genome release cannot occur. In human trials, symptoms were reduced and the time course of disease was shortened by a day or two. Consequently, the compound has yet to be approved for widespread use.

An effective treatment of the common cold simply may be one that targets mechanisms of symptom production, not the viral proteins that promote infection and replication. As the symptoms are the result of immunopathology, targeting immune cells or soluble mediators may be a solution. We have incomplete knowledge about the mediators of immune defense against cold viruses, but it may be that there will be overlap among the host responses to different viruses giving rise to the common symptoms.

Hayden, F. G., T. Coats, K. Kim, H. Hassman, M. M. Blatter, B. Zhang, and S. Liu. 2002. Oral pleconaril treatment of picornavirus-associated viral respiratory illness in adults: efficacy and tolerability in phase II clinical trials. *Antiviral Ther.* **7:**53–65.

diagnostic reagents alone has hampered the development and marketing of antiviral drugs to treat many acute viral diseases, despite the existence of effective therapies.

Historical Perspective

The first large-scale effort to find antiviral compounds began in the early 1950s and focused on inhibitors of smallpox virus replication. At that time, virology was in its infancy, and smallpox was a worldwide scourge. In the 1960s and 1970s, drug companies expanded efforts because of increased knowledge and understanding of the viral etiology of common diseases, as well as their remarkable progress in the discovery of antibiotics to treat bacterial infections. They launched massive screening programs to find chemicals with antiviral activities. Despite much effort, there was relatively little success. One notable exception was amantadine (Symmetrel), approved in the late 1960s for treatment of influenza A virus infections. These antiviral discovery programs were called **blind screening**, because random chemicals and natural-product mixtures were tested for their ability to block replication of a variety of viruses in cell culture systems. "Hits" were then purified, and fractions were tested in various cell and animal models for safety and efficacy. Promising molecules, called "leads," were modified systematically by medicinal chemists to reduce toxicity, increase solubility and bioavailability, or improve biological half-life. As a consequence, hundreds if not thousands of molecules were made and screened before a specific antiviral compound was tested in humans. Moreover, the mechanism by which these compounds inhibited the virus was often unknown. For example, the mechanism of action of amantadine was not deduced until the early 1990s, almost 30 years after its discovery!

Discovering Antiviral Compounds

With the advent of modern molecular virology and recombinant DNA technology, the random, blind-screening procedures described above have been all but discarded. Instead, viral genes essential for growth can be cloned and expressed in genetically tractable organisms, and their products can be purified and analyzed in molecular and atomic detail. The life cycles of many viruses are known, revealing numerous targets for intervention (Table 9.2). Inhibitors of these processes can be found, even for viruses that cannot be propagated in cultured cells.

The New Lexicon of Antiviral Discovery

Mechanism- and cell-based assays, high-throughput screens, small interfering RNA screens, *in silico* screens, rational drug design, and combinatorial chemistry are the approaches of modern antiviral discovery (Fig. 9.1). Despite all the modern technology, this process does not find drugs (compounds approved and licensed for use in humans), but, rather, detects hits and highlights strategies for developing them as leads. The hard work begins when a lead compound is found. Will the compound get to the right place in the body at the appropriate concentration (**bioavailability**)? Will it

Table 9.2 Some viral targets for antiviral drug discovery[a]

Function	Lead compound or example	Virus
Attachment	Peptide analogs of attachment protein	HIV
Penetration and uncoating	Dextran sulfate, heparin	HIV, herpes simplex virus
mRNA synthesis	Interferon	Hepatitis A, B, and C viruses; papillomavirus
	Antisense oligonucleotides	Papillomavirus, human cytomegalovirus
Protein synthesis		
Initiation	Interferon	Hepatitis A, B, and C viruses; papillomavirus
IRES elements	Ribozymes; antisense oligonucleotides	Flaviviruses and picornaviruses
DNA replication		
Polymerase	Nucleoside, nonnucleoside analogs	Herpesviruses, HIV, hepatitis B virus
Helicase/primase	Thiozole ureas	Herpes simplex virus
Processing/packaging	Benzimadolazoles	Herpesviruses
Nucleoside scavenging		
Thymidine kinase	Nucleoside analog	Herpes simplex virus, varicella-zoster virus
Ribonucleotide reductase	Inhibitors of protein-protein interaction of large and small subunits	Herpes simplex virus
Assembly		
Protease	Peptidomimetics	HIV
Virion integrity	Nonoxynol-9	
Lipid raft disruption	β-Cyclodextrins	HIV, herpes simplex virus

[a] Data from E. De Clercq, *Nat. Rev. Drug Discov.* **1**:13–25, 2002; S.-L. Tan, A. Pause, Y. Shi, and N. Sonenberg, *Nat. Rev. Drug Disc.* **1**:867–881, 2002.

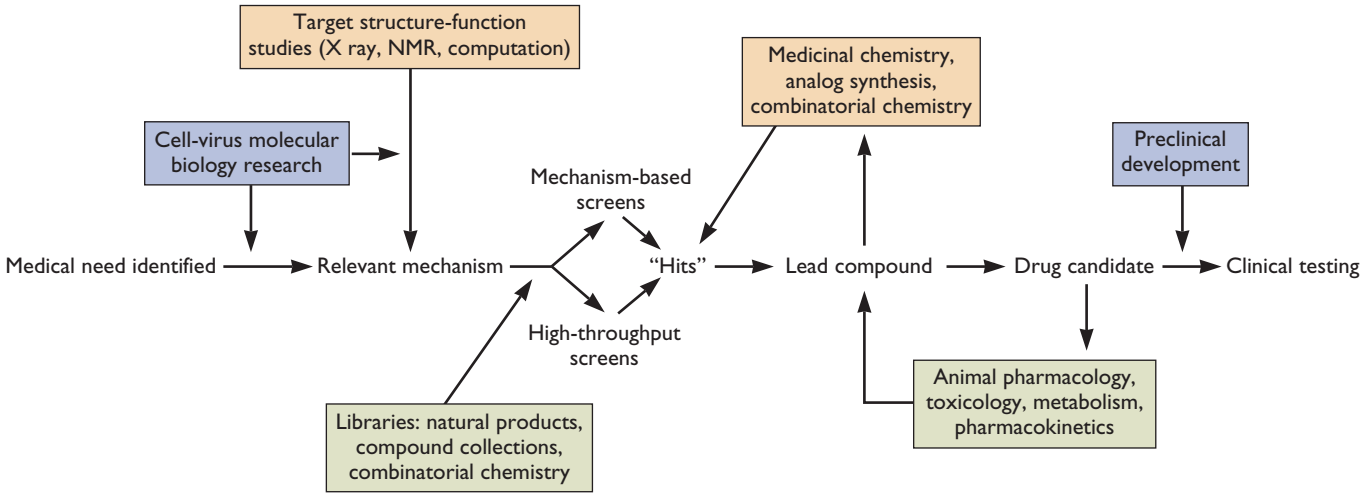


Figure 9.1 Path of drug discovery. The flow of information and action followed by modern drug discovery programs that ultimately yield compounds that can be tested clinically for efficacy is illustrated.

persist in the body long enough to be effective (**pharmacokinetics**)? Will it be toxic? Answers to these questions require considerable time, effort, and money.

Screening for Antiviral Compounds

Genetics and Drug Discovery

Viral targets. When blind screening was the only method to find antiviral compounds, the mechanism by which the compound blocked virus replication was often unknown for years after the compound was discovered. Modern antiviral discovery methods focus on known mechanisms that are essential for viral replication (mechanism-based screens). These essential mechanisms are defined by genetics and by our knowledge of viral genomes. Viral genomes can be manipulated to provide the **proof of principle** for an antiviral compound. For example, if the hypothesis is that inhibition of a particular viral enzyme blocks virus replication and disease, then construction of a mutant virus in which the gene of interest is inactivated or deleted provides a rigorous test. If the mutant is unable to replicate, or cannot cause disease in model systems, the investigator has increased confidence that an inhibitor will have antiviral activity. However, care must be taken to avoid overinterpretation of these studies. An obvious caveat is that an inhibitor bound to the protein under study certainly is not the same situation as complete absence of that protein.

Host targets. In the past, finding host gene products required for viral replication was difficult and often impossible for basic research scientists. As a consequence, no pharmaceutical company directed resources to this problem. This situation has now changed. Because the human genome sequence is now available, as are the genome sequences of the common laboratory animals, the roles of host genes in viral replication and pathogenesis can be assessed using RNA interference technology.

Mechanism-Based Screens

As the name "mechanism-based screen" implies, the specific virus-encoded enzyme or molecular interaction to be inhibited is known (or predicted), and compounds that affect this particular mechanism are sought. Enzymes, transcriptional activators, cell surface receptors, and ion channels are popular targets. Often this screening is done with purified protein in formats that facilitate automated assay of many samples. One example of a mechanism-based screen designed to identify inhibitors of a viral protease is given in Fig. 9.2.

Cell-Based Screens

In cell-based assays, essential elements of the specific mechanism to be inhibited (e.g., a viral enzyme plus a readily assayable substrate) are engineered into an appropriate cell. One example is illustrated in Fig. 9.3 for a bacterially based screen. Similar approaches work well in other cells as well. Such assays can provide information, not only about inhibition of the target reaction, but also about cytotoxicity and specificity. New approaches now include technology to measure more than one event at a time by using several different reporter molecules, or optical methods to track movement of a labeled molecule inside the cell.

An interesting variation of the cell-based assay is the **mini-replicon**. Certain viruses cannot be propagated readily in standard cultured cells (e.g., hepatitis C virus or human papillomavirus) or are dangerous enough to require high biological containment (e.g., smallpox virus and the hemorrhagic fever

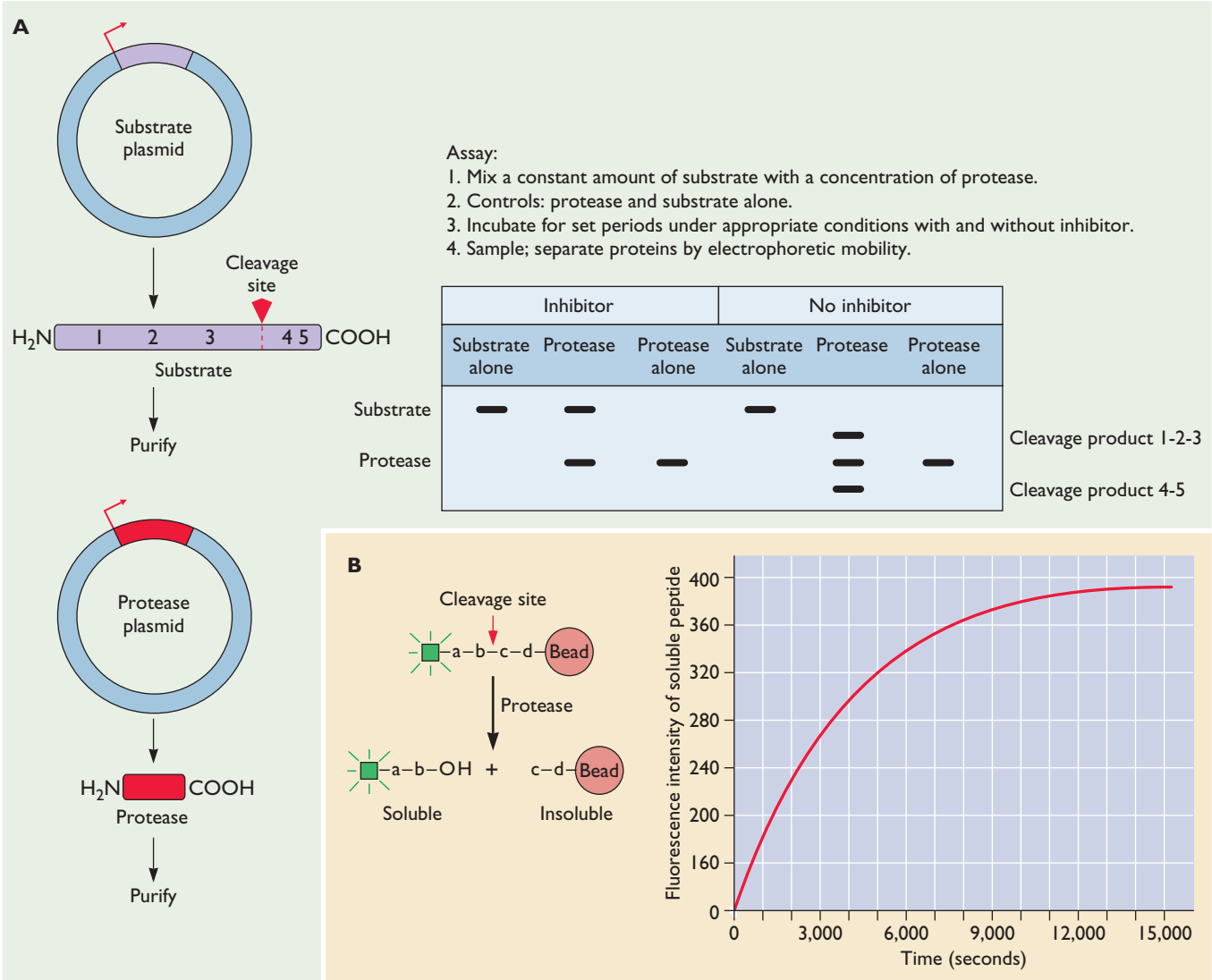


Figure 9.2 Screens for inhibitors of viral proteases. (A) A mechanism-based assay for the activity of a viral protease. The plasmids used to produce the protease substrate and the protease are indicated by circles at the left. The resulting proteins are indicated below. The substrate protein has a defined cleavage site for the protease (red arrowhead). The protease is purified as an active enzyme from *E. coli* extracts. The assay is performed as indicated on the right. A specific inhibitor of the protease activity is added to one set of reactions. A diagram of a typical electropherogram is illustrated. Experiments of this type can be used to determine kinetic parameters of enzyme activity and inhibition. **(B)** Fluorogenic, mechanism-based screen for a viral protease. The substrate is a short peptide encoding the protease cleavage site. A fluorogenic molecule is covalently joined to the N terminus of the peptide, and the entire complex is attached via its C terminus to a polystyrene bead. When the peptide-bead suspension is exposed to active protease, the peptide is cleaved such that the fluorogenic N terminus is released into the soluble fraction, which can be quickly and cleanly separated from the insoluble beads containing the nonfluorogenic product as well as the fluorogenic unreacted substrate. Protease activity is assayed by the appearance of soluble fluorescent peptide as a function of time as shown.

viruses). The minireplicon system comprises a set of plasmids, which, when introduced into cells, sponsor the synthesis of viral replication proteins that promote replication of an engineered viral genome segment marked with a reporter gene (the minireplicon). Replication can be monitored by assaying for reporter gene expression. Inhibitors that block replication can be discovered, analyzed, and developed for therapeutic use. Indeed, the minireplicon system was absolutely essential for discovery and testing of the first lead compounds capable of blocking human hepatitis C virus replication.

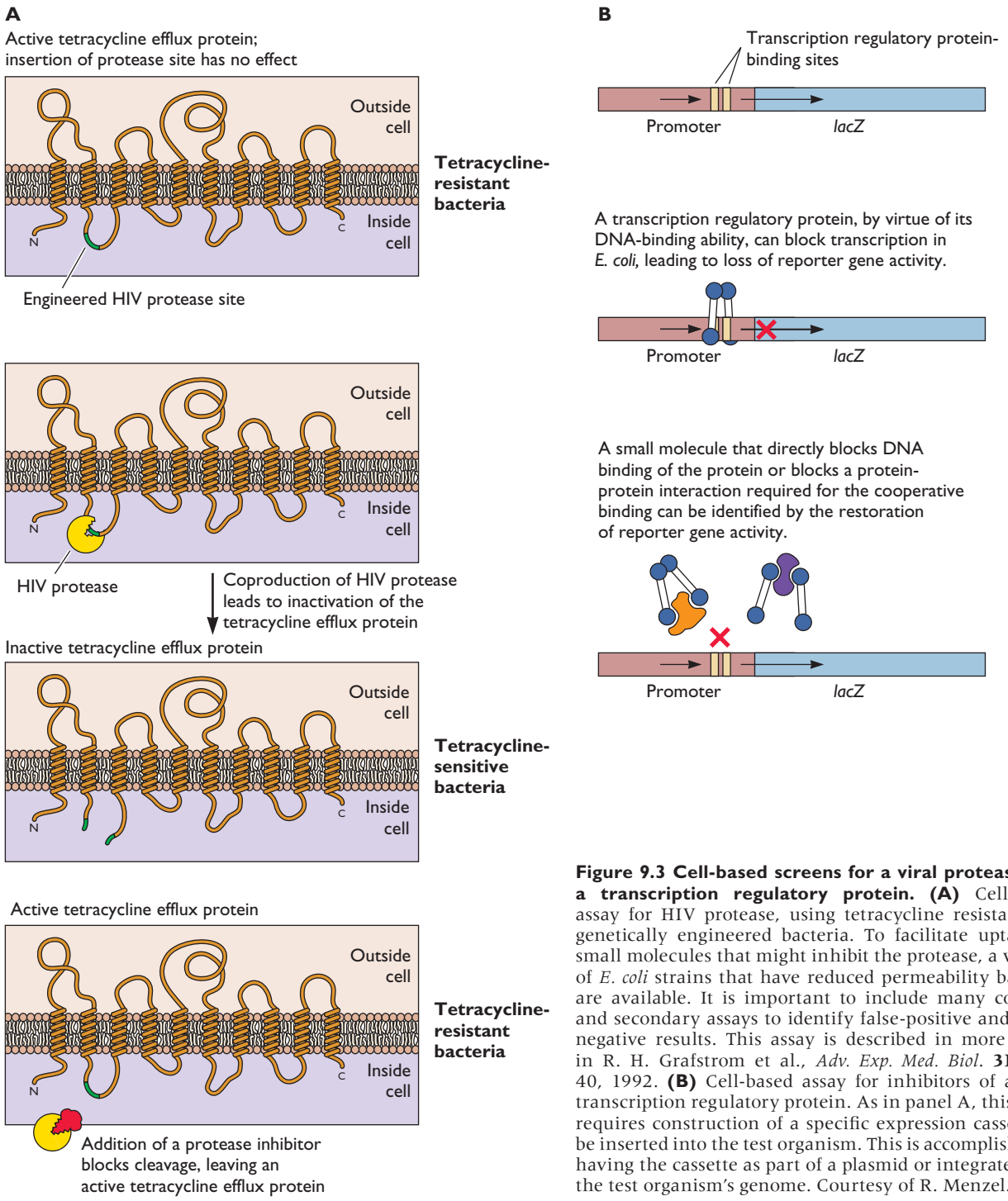


Figure 9.3 Cell-based screens for a viral protease and a transcription regulatory protein. (A) Cell-based assay for HIV protease, using tetracycline resistance of genetically engineered bacteria. To facilitate uptake of small molecules that might inhibit the protease, a variety of *E. coli* strains that have reduced permeability barriers are available. It is important to include many controls and secondary assays to identify false-positive and false-negative results. This assay is described in more detail in R. H. Grafstrom et al., *Adv. Exp. Med. Biol.* **312:**25–40, 1992. **(B)** Cell-based assay for inhibitors of a viral transcription regulatory protein. As in panel A, this assay requires construction of a specific expression cassette to be inserted into the test organism. This is accomplished by having the cassette as part of a plasmid or integrated into the test organism's genome. Courtesy of R. Menzel.

High-Throughput Screens

High-throughput screens are mechanism- or cell-based screens that allow very large numbers of compounds to be tested in an automated fashion. It is not unusual for pharmaceutical companies to examine more than 10,000 compounds per assay per day, a rate inconceivable for early antiviral drug hunters. Compounds to be screened typically are arrayed in multiwell, interchangeable plastic dishes with a few microliters or less of compound solution per well. Robots then apply these compounds to other

plastic dishes containing the cell-free or cell-based screen, and after incubation, the signal created by the reporter gene (or other output) is read and recorded. Data are stored in computers for retrieval and analysis. Not only numerical data but also images of cells or reactions can be captured, stored, and analyzed. These screens are called **high-content screens** because they examine more than one parameter. For example, using antibodies, it is possible to monitor the import of transcriptional regulators from their site of cytoplasmic synthesis to their site of action in the nucleus. By using a robotic microscope and high-speed camera/computer system, thousands of microdishes with cells and particular inhibitors of nuclear import can be screened quickly. Inhibitors can be identified because the antibody complex remains in the cytoplasm and does not enter the nucleus.

Sources of Chemical Compounds Used in Screening

Many pharmaceutical and chemical companies maintain large libraries of chemical compounds. Usually, a sample of every compound synthesized by the company for any project is archived, and its history is stored in a database. Chemical libraries of half a million or more distinct compounds are not unusual for a large company. Other kinds of libraries containing natural products collected from all over the world, including "broths" from microbial fermentations, extracts of plants and marine animals, and perfusions of soils containing diverse mixtures of unknown compounds, can be searched for components that may have antiviral activities. Another type of chemical library is now available from application of **combinatorial chemistry**, a technology that provides drug hunters access to unprecedented numbers of small, synthetic molecules for screening (Fig. 9.4). Before implementation of this technology, a medicinal chemist could reliably synthesize and characterize only about 50 compounds a year. Combinatorial chemistry can provide all possible combinations of a basic set of modular components, often on uniquely tagged microbeads or other chemical supports, such that active compounds in the mixtures can be traced, purified, and identified with relative ease. Making thousands of compounds in days is now routine.

Designer Antivirals and Computer-Based Searching

Structure-Based Drug Design

Structure-based design depends on knowing the atomic structure of the target molecule, usually provided by X-ray crystallography. Computer programs, known and predicted mechanisms of enzyme action, fundamental

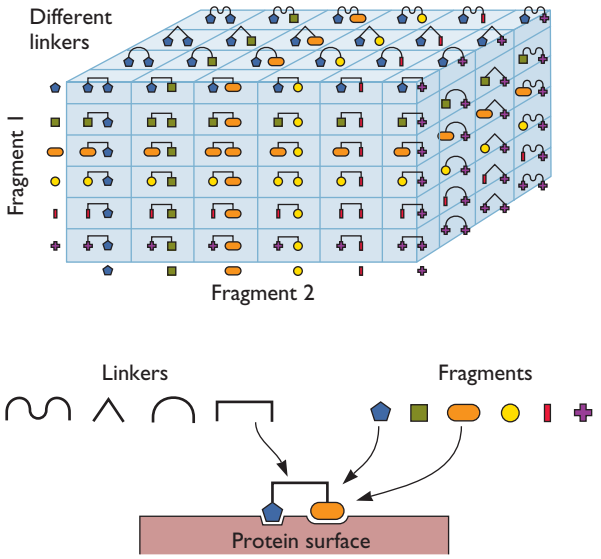


Figure 9.4 Combinatorial chemistry and the building-block approach to chemical libraries. Small organic molecules predicted to bind to different pockets on the surfaces of proteins can be grouped into subsets of distinctive chemical structures (different colored symbols). With automated procedures, these chemical entities can be joined together by various chemical linkers (lines) to produce a large but defined library of small compounds. For example, if assembled pairwise with 10 linkers, a collection of 10,000 small molecules yields a library of 1 billion new combinations. These defined chemical libraries allow a detailed exploration of the binding surfaces of complex proteins. Adapted from P. J. Hajduk et al., *Science* **278:**497–499, 1997, with permission.

chemistry, and personal insight all aid an investigator in the design of ligands that bind at a critical site and inhibit protein function. Currently, the atomic structures of tens of thousands of macromolecules, including important viral proteins, have been determined. HIV protease inhibitors may be the most successful antiviral agents that have been designed by structure-based analyses (Fig. 9.5).

Genome Sequencing Provides New Information for Antiviral Drug Discovery

We now have available the sequences of many viral genomes and those of the corresponding hosts. These sequences provide a wealth of information to define new targets for antiviral drug discovery. High-density arrays of DNA fragments on a DNA chip the size of a microscope slide can be used to assess the expression of thousands of genes in a single experiment (microarray analyses). Such global analysis of complicated processes in host-parasite interactions is unprecedented. This technology enables scientists to identify the changes in messenger RNA (mRNA) concentration that vary in response to viral infection in

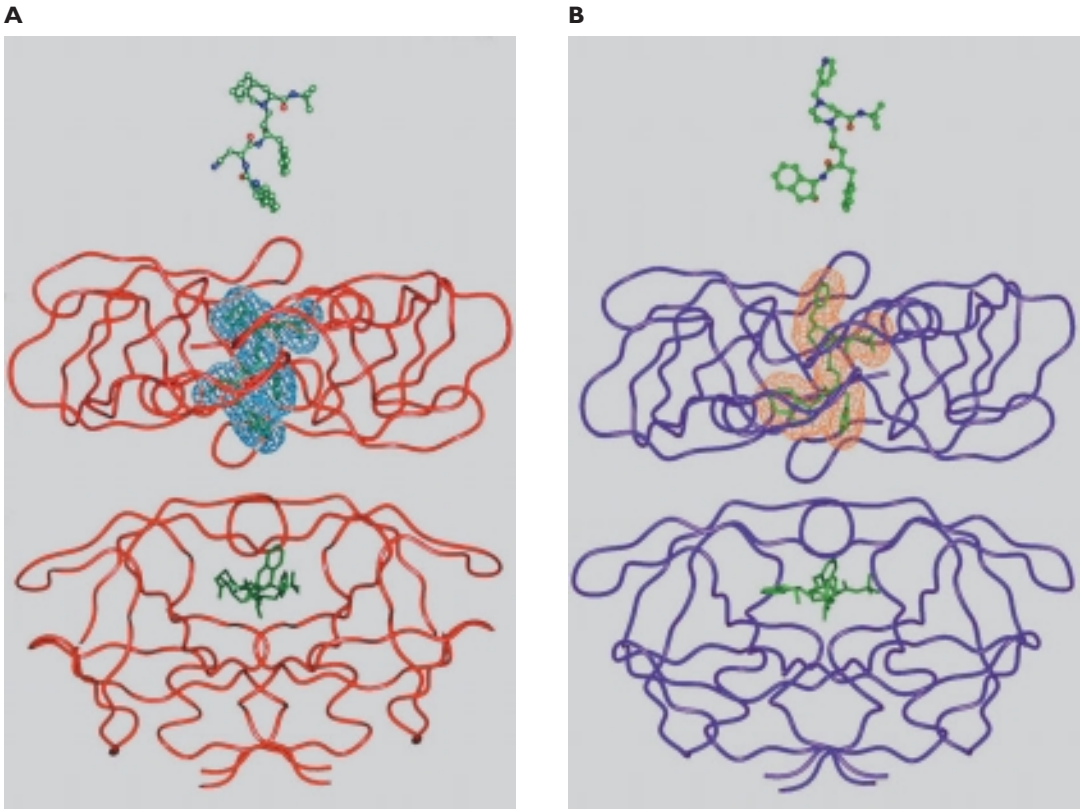


Figure 9.5 Structure of the HIV type 1 protease with the inhibitors indinavir and saquinavir. Structures of the viral protease dimer with bound AIDS drugs saquinavir (Roche) and indinavir (Merck), as well as the structures of these drugs by themselves, are shown in panels A and B, respectively. **(A)** Tracing of the main chain of the protease in two views rotated by 90 degrees (middle and bottom), with the ball-and-stick model of saquinavir (top), saquinavir covered by a net representing the atomic surface (middle), and stick representation of saquinavir alone (bottom). **(B)** Similar images of indinavir. The images were prepared by J. Vondrasek and are reproduced from the protease database (http://srdata.nist.gov/hivdb/) (J. Vondrasek et al., *Nat. Struct. Biol*. **4:**8, 1997), with permission.

various tissues. Given the power of RNA interference technology to reduce or eliminate expression of selected host gene, it is now possible to identify host pathways essential for every step in viral replication, from entry to egress. The race is on to identify which proteins in these various cellular pathways can be targeted for antiviral attack. We can be sure that new human antiviral gene products and new defensive pathways will be identified in the not too distant future. In addition, microarray and proteomic technology is assisting in the identification of toxic or adverse responses to lead antiviral compounds long before they are tested in animals.

In Silico *Drug Discovery*

With all the advances in the "-omics" (genomics, proteomics, metabolomics, and structural genomics), a curious conundrum has emerged. We have an enormous number of potential targets for drug discovery, but a paucity of lead compounds that engage these targets. Similarly, due to advances in chemistry, the number of chemical structures that could be tested as antiviral compounds has increased dramatically. In fact, one estimate suggests that as many as 10^{47} quadrillion chemicals can be made to interact with human protein targets. Some structural biologists think that the atomic structures of the vast majority of possible enzymatic active sites are present in our current structural databases. Nevertheless, no one would seriously think of urging medicinal chemists and biologists to do random screening of these active sites with all the compounds. However, many companies now are using computers to take on this daunting task. ***In silico* drug discovery** is the systematic testing of chemicals and their interaction with known protein structures by using computers. Such

virtual screening is accomplished by iterative docking of each chemical into the active site of a protein target. When a small molecule "fits" into a pocket, the matching of shape and charges is estimated and recorded. A modern one-teraflop computer is capable of screening 200 million chemicals for 5,000 protein targets in a year (about 31.5×10^{18} floating point operations per year). A computer that is 1,000 times faster is expected to be functional in 2010. Further advances are expected, as methods are developed to predict protein structures from gene sequence with some confidence. This paradigm of virtual screening has been called "genome-to-drug-to-lead" and has the potential to reduce the formidable manpower requirements for chemistry and biology. It remains to be seen what will come out of the *in silico* drug discovery pipeline.

The Difference between "R" and "D"

Antiviral Drugs Are Expensive To Discover, Develop, and Bring to the Market

It is not unusual for the cost of bringing an antiviral drug to market to approach or even exceed half a billion dollars (Table 9.3). Even with modern methods, it is common for thousands of leads to yield but one promising candidate for further development (Fig. 9.6). Research and lead identification, the "R" of "R&D," represent only the beginning of the process of producing a drug. The "D" of "R&D" is development, comprising all the steps necessary to take an antiviral lead compound through safety testing, scale-up of synthesis, formulation, pharmacokinetic studies, and clinical trials. With rare exceptions, it takes 5 to 10 years after the initial lead is found to get a drug to the market, even with the latest technology. Decisions made by drug companies often are influenced strongly by these remarkable costs. Every time a lead compound fails to exhibit a desirable feature, the research director must decide whether to rework the failed compound or to stop the project (Box 9.2).

Antiviral Drugs Must Be Safe

As in vaccine development, safety is the overriding concern of any company developing an antiviral drug. Toxicity to test cells and animals is the first indication that a compound may not be safe. More promising leads are discarded because of toxicity than for any other reason. Toxicity can be described in terms of the **cytotoxic index** (for cells) or the **therapeutic index** (for hosts). These indices are defined as the dose that inhibits viral

Table 9.3 Critical points for "drug hunters" seeking commercially viable antiviral compounds

Virological issues
"Proof of principle" must be obtained as soon as possible during discovery process.
What is the molecular mechanism?
Does the drug work in cell and in animal models as predicted?
Compound should block viral spread early to limit cytopathology and host cytokine/inflammatory response
Drug must be potent and block replication completely; must not make an infection "persistent" by slow replication and spread
Resistance to the antiviral drug must be manageable
Mutants resistant to any antiviral compound are guaranteed
Resistance mutations arise when any virus is permitted to replicate; antiviral compounds select for these spontaneous mutants
Viruses with resistance to other drugs must not be resistant to your drug
The virulence of resistant mutants must be understood
Noncompliance by patient may select for drug resistance or may encourage the persistent infection; multiple dosing, stringent dietary requirements, unpleasant taste, or side effects affect compliance
Toxicities should be anticipated particularly for compounds given to control persistent infections
Long-term human toxicology studies (years) are difficult, expensive, and often not possible
Short-term toxicology studies may not predict side effects arising when drugs are taken over an extended time (years)
Commercial issues
Compound should be safe with no side effects
Compound should be inexpensive to manufacture
Compound should be easy to formulate and deliver; a pill to be swallowed is much preferred over injection
Compound must satisfy an unmet medical need; i.e., it must be better than any competitive drug or, better yet, have no competition
Ultimately, a profit should be possible; the market should be large enough to enable a profit to be made

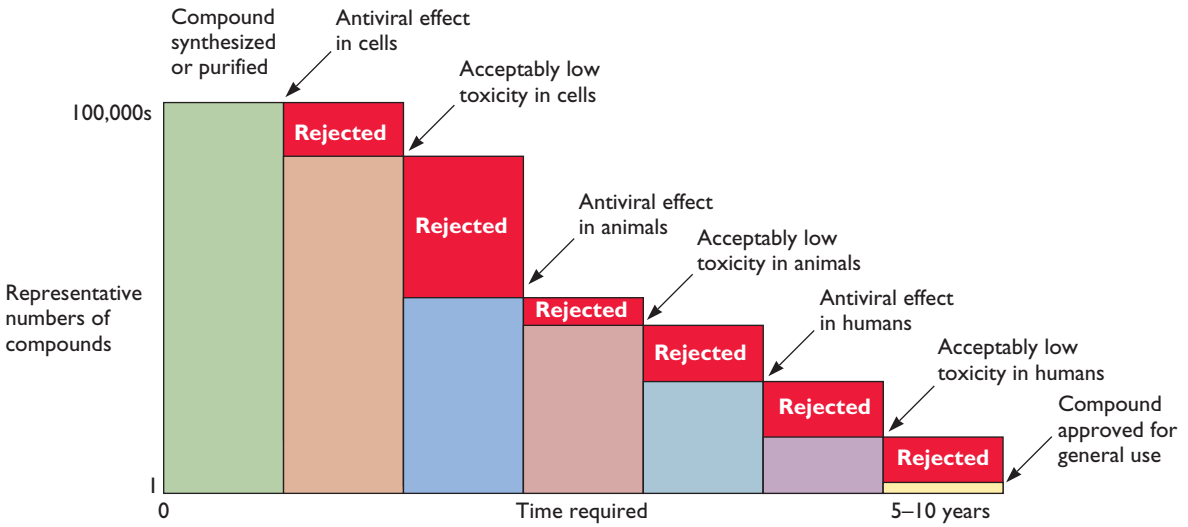


Figure 9.6 Up the down staircase of drug discovery. Many compounds must be tested before a commercially viable antiviral drug will become available. The attrition rate is very high (red "rejected" label), as hundreds of thousands of chemicals are tested in multiple steps taking several years before one compound emerges as a drug. A few of the significant hurdles in the process are illustrated.

replication, divided by the dose that is toxic to cells or host The smaller the index, the better; indices of 1/1,000 or smaller are preferred. Traditional approaches to determining toxicity and efficacy can be difficult and time-consuming for many reasons, including the fact that initial lead compounds often are not very soluble in aqueous solutions, or may have only modest antiviral activities. New technologies, such as DNA microarray analyses and proteomics studies, are valuable in identifying mechanisms and sources of toxicity.

No human trial can be initiated without detailed toxicology studies of diverse animal species. Compounds that may be used in long-term treatment must be evaluated for toxicity, allergic effects, mutagenicity, teratogenicity, and carcinogenicity. Many drugs will be given to children, to pregnant women, and to individuals with reduced immune responses, all situations that present special problems. Safety overrides efficacy in most cases. On the other hand, when there are no other effective treatments, as in the early days of the acquired immunodeficiency syndrome

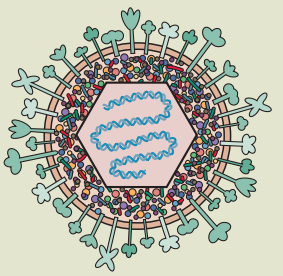
BOX 9.2 DISCUSSION

New drugs, new mechanisms—no interest?

Recently, two pharmaceutical companies independently discovered a new class of drug that inhibits herpes simplex virus replication. These compounds are targeted to the DNA helicase-primase that is essential for viral replication. They represent the first new anti-herpes simplex virus drugs since acyclovir was developed in the 1970s. The helicase-primase inhibitors are more potent than acyclovir and its derivatives in animal models, and have remarkable potential.

However, neither company has plans to develop the inhibitors or to test them in people. The reason is that acyclovir is a safe, effective drug, and the expense of taking a new drug through clinical trials is enormous. Marketing strategy asserts that it is not cost-effective to compete with a proven drug. The reality is that companies must make choices about where to put their resources.

Crumpacker, C. S., and P. A. Schaffer. 2002. New anti-HSV therapeutics target the helicase-primase complex. *Nat. Med.* **8:**327–328.

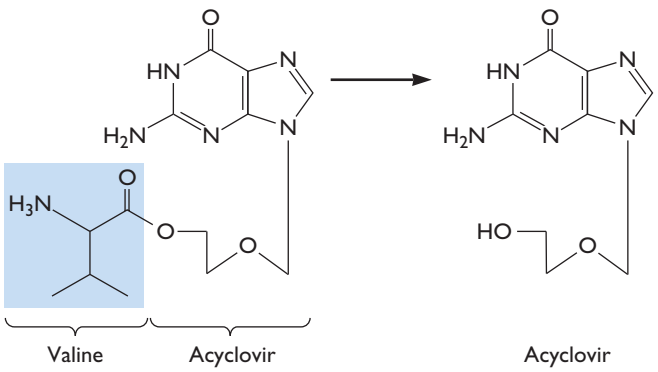


(AIDS) pandemic, even drugs that caused some undesirable side effects can be licensed for human use (e.g., azidothymidine [AZT]).

Drug Formulation and Delivery

The science of formulation and delivery is an essential part of any antiviral discovery program. After introduction into a person, a drug must reach the proper place, at an effective concentration, and for an appropriate time to inhibit virus replication. A compound that cannot enter the bloodstream after ingestion is not likely to be effective. This step alone may not be sufficient, as many compounds bind to albumin or other proteins in the blood and thereby are rendered ineffective. Other compounds may be metabolized as they pass through the liver, and therefore are inactivated and cleared rapidly from the body. Such problems are not easily predicted and often are discovered empirically. Insoluble compounds, or chemicals unable to enter the bloodstream after ingestion (poor bioavailability), can be modifed by the addition of new side chains that may improve absorption from the intestine (see Fig. 9.7 for such a modification of acyclovir). In addition, new delivery vehicles such as liposomes, minipumps, skin patches, or slow-release capsules may improve bioavailability. Other desirable features include stability and cost-effective synthesis in large amounts. Literally tons of precursor materials are needed to manufacture commercial quantities of an antiviral drug.

Figure 9.7 Valacyclovir (Valtrex), an L-valyl ester derivative of acyclovir with improved oral bioavailability. Acyclovir is not taken up efficiently after oral ingestion (poor bioavailability). However, a derivative of acyclovir, valacyclovir, has as much as a fivefold-higher oral bioavailability than acyclovir (levels in serum relative to the dose of drug given orally). The addition of a new side group to acyclovir allows increased passage of drug from the digestive tract to the circulation. These acyclovir derivatives are proprodrugs, which are converted to acyclovir inside the cell by cellular enzymes that cleave off the valine side chain. Adapted from D. R. Harper, *Molecular Virology* (Bios Scientific Publishers, Ltd., Oxford, United Kingdom, 1994), with permission.



Examples of Some Approved Antiviral Drugs

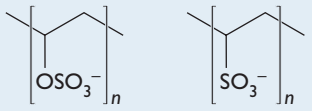
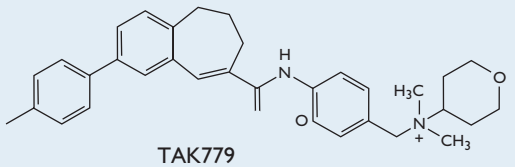
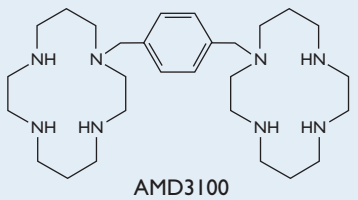
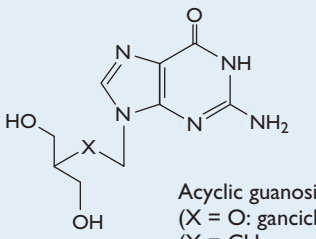
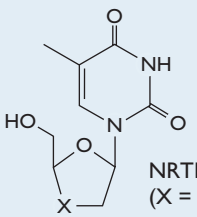
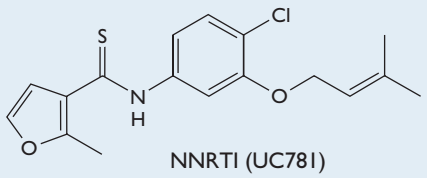
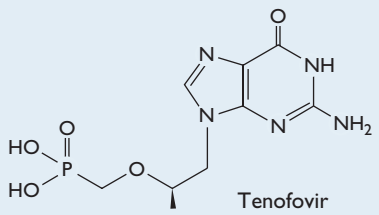
The antiviral drugs approved for use are surprisingly few and belong to a limited number of chemical classes (Fig. 9.8). While many are safe and effective, some are marginally efficacious and have side effects that limit their use. Clearly there is room for improvement. Some of the better known antiviral drugs are discussed below.

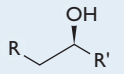
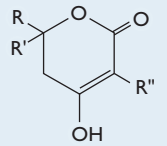
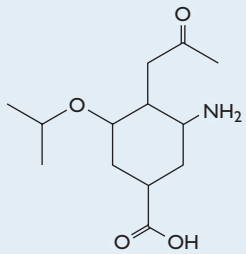
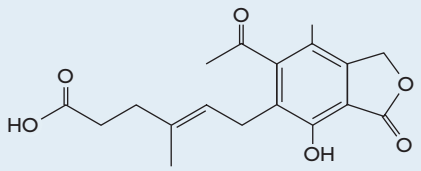
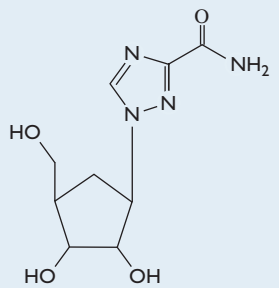
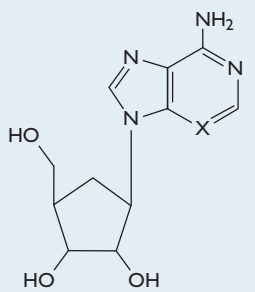
Acyclovir

Acyclovir is an example of a specific, nontoxic drug that is highly efficacious against herpes simplex virus (genital and oral herpes) and, to some extent, varicella-zoster virus (chickenpox and shingles). It was discovered in 1974, but it was not until the mid-1980s that its full potential as an antiherpesviral drug was realized. Acyclovir is a nucleoside analog similar to guanosine, but contains an acyclic sugar group (Fig. 9.9). It is a prodrug, a precursor of the active antiviral compound (Fig. 9.10). Activation of the drug requires the presence of three kinases in the cell to convert acyclovir to a triphosphate derivative, the actual antiviral compound. The first kinase, which converts acyclovir to the monophosphate, is not found in an uninfected cell. Acyclovir therefore has no effect on host DNA replication because it cannot be phosphorylated and incorporated into DNA. Herpes simplex virus and varicella-zoster virus genomes, however, encode a kinase that can phosphorylate the prodrug to produce acyclovir monophosphate. Cellular enzymes complete synthesis of acyclovir triphosphate, which is then incorporated into DNA by the viral DNA polymerase. As acyclovir lacks the 3′-OH group of the sugar ring, the growing DNA chain terminates. The specificity for the herpesviruses depends on a virally encoded thymidine kinase. The herpes simplex virus enzyme normally phosphorylates thymidine to thymidine monophosphate, but also phosphorylates a wide range of other substrates, including acyclovir. Indeed, if herpes simplex virus thymidine kinase is synthesized in an uninfected cell and acyclovir is added, the cell will die because its DNA replication will also be blocked by the chain-terminating base analog. This phenomenon is the basis of several strategies for selective cell killing during gene therapy and manipulation of embryonic stem cells.

Ganciclovir (Cytovene)

Ganciclovir is another derivative of acyclovir that was developed to treat human cytomegalovirus infections (cytomegalovirus is a betaherpesvirus). The cytomegalovirus genome does not carry a thymidine kinase gene, but it does encode kinase functions that phosphorylate gancyclovir. Initial formulations of ganciclovir were given intravenously and were quite toxic. Consequently, ganciclovir was used only for life-threatening human cytomegalovirus infections in AIDS patients and immunosuppressed transplant recipients. Recently, ganciclovir has

Important classes	Structures
Viral adsorption inhibitors	 PVAS PVS
Viral-cell fusion inhibitors	 TAK779  AMD3100
Viral DNA polymerase inhibitor	 Acyclic guanosine analog (X = O: ganciclovir) (X = CH_2: penciclovir)
Reverse transcriptase inhibitors	 NRTI (X = CHN_3, AZT)  NNRTI (UC781)
Acyclic nucleoside phosphonate	 Tenofovir

Important classes	Structures
Protease inhibitors	 Peptidomimetic  Nonpeptidomimetic
Neuraminidase inhibitor	 Oseltamivir
IMP dehydrogenase inhibitors	 Mycophenolic acid  Ribavirin
SAH hydrolase inhibitor	 (X = N or CH)

been developed in an oral formulation that is effective for prophylaxis and long-term use for human cytomegalovirus infections. The oral formulation appears to be much less toxic than the original form delivered intravenously.

Foscarnet (Foscavir, Trisodium Phosphonoformate)

Foscarnet is the only nonnucleoside DNA replication inhibitor of herpesviruses. It is a noncompetitive inhibitor of the pyrophosphate-binding site in herpesvirus DNA polymerase. It also inhibits hepatitis B virus polymerase and the reverse transcriptase of HIV. Foscarnet must be given intravenously. As it causes kidney and bone toxicity, this drug is recommended only for life-threatening infections for which other antiviral drugs are no longer effective.

Ribavirin (Virazole)

Ribavirin, a nucleoside analog (Fig. 9.9), was synthesized in 1972 and was purported to have broad-spectrum activity against many DNA and RNA viruses. However, it is relatively toxic, and its development and indications for use have been controversial. In fact, this drug is not licensed in many countries. Despite its long history, its mechanism of antiviral action is not clear. Ribavirin monophosphate is a competitive inhibitor of cellular inosine monophosphate dehydrogenase, and such inhibition leads to reduced guanosine 5′-triphosphate (GTP) pools in the cell. It also inhibits initiation and elongation by viral RNA-dependent polymerases and interferes with capping of mRNA. Ribavirin is an RNA virus mutagen: once incorporated into a template, it pairs with C or U with equal efficiency. In some studies, its antiviral activity correlates directly with its mutagenic activity (Box 9.3). Even with its unknown mechanism and toxicity, ribavirin is used as an aerosol for treatment of infants suffering from respiratory syncytial virus infection, as well as for treatment of Lassa fever virus and hantavirus infections. Viramidine and levovirin are analogs of ribavirin that are in clinical development for treatment of hepatitis C virus infections.

Lamivudine

Lamivudine is an orally delivered nucleoside analog requiring activation by intracellular kinases to act as a chain terminator (Fig. 9.10). It has been effective in blocking the reverse transcriptases of hepatitis B virus and HIV. The drug is highly bioavailable and has low toxicity, as well as a long half-life, which makes it suitable for treatment of chronic hepatitis B virus infections.

Cidofovir

Cidofovir is an acyclic nucleoside phosphonate that offers potential as a broad-spectrum drug. It has activity against various herpesvirus, papillomavirus, polyomavirus, adenovirus, and poxvirus infections (Fig. 9.10). Other members of this group are adefovir (approved for hepatitis B infections) and tenofovir (approved for HIV infections). These compounds have been formulated for intravenous, topical, and oral applications. Their unique mode of action stands in contrast to that found for the classical acyclic nucleoside analogs. Cidofovir is converted to di- and triphosphate derivatives by host enzymes. The phosphorylated compounds appear to be more selective for viral polymerases than host polymerases. Because the conversion of cidofovir to a monophosphate does not depend on a virus-induced thymidine kinase or a viral protein kinase, essentially all DNA viruses and retroviruses are susceptible.

Amantadine (Symmetrel)

The three-ringed symmetric amine known as amantadine was developed by DuPont chemists almost 40 years ago. It was the first highly specific, potent antiviral drug effective against any virus. At low concentrations, amantadine specifically inhibits influenza A virus. The target of the drug was shown only recently to be the M2 protein, a tetrameric, transmembrane ion channel that transports protons. The specificity of amantadine for influenza A virus and not influenza B virus can now be understood, as influenza B virus genomes do not encode an M2 protein. Influenza A virus mutants resistant to amantadine arise after therapy (Fig. 9.11). All have amino acid changes in the M2 transmembrane sequences predicted to form the ion channel, but it is unknown if the drug binds directly in the channel, or if a mutation that alters the channel affects binding of the drug to another site. In any case, amantadine blocks the channel, so that protons cannot enter the virion, effectively preventing the uncoating of influenza A virus.

Amantadine must be administered in the first 24 to 48 h of infection, and must be given at high doses for at least 10 days. The drug is most effective when given to susceptible patients in anticipation of influenza virus infection. However, at high concentrations side effects are common, particularly those affecting the central nervous system. On follow-up of these side effects, amantadine was found to be useful for relieving symptoms of Parkinson's disease in some patients. Today, more amantadine is sold for central nervous system disease than for antiviral treatment. Rimantadine, a

Figure 9.8 The prototypic compounds (pharmacophores) of important classes of antiviral agents used today. The general class of inhibitors is defined in the yellow columns, and selected structures are shown in the blue columns. AZT, azidothymidine; IMP, inosine monophosphate; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PVAS, polyvinylalcohol sulfate; PVS, polyvinylsulfonate; SAH, *S*-adenosylhomocysteine. TAK779, AMD3100, and UC781 are company designations for the compounds. Adapted from E. De Clercq, *Nat. Rev. Drug Discov.* **1**:13–25, 2002, with permission.

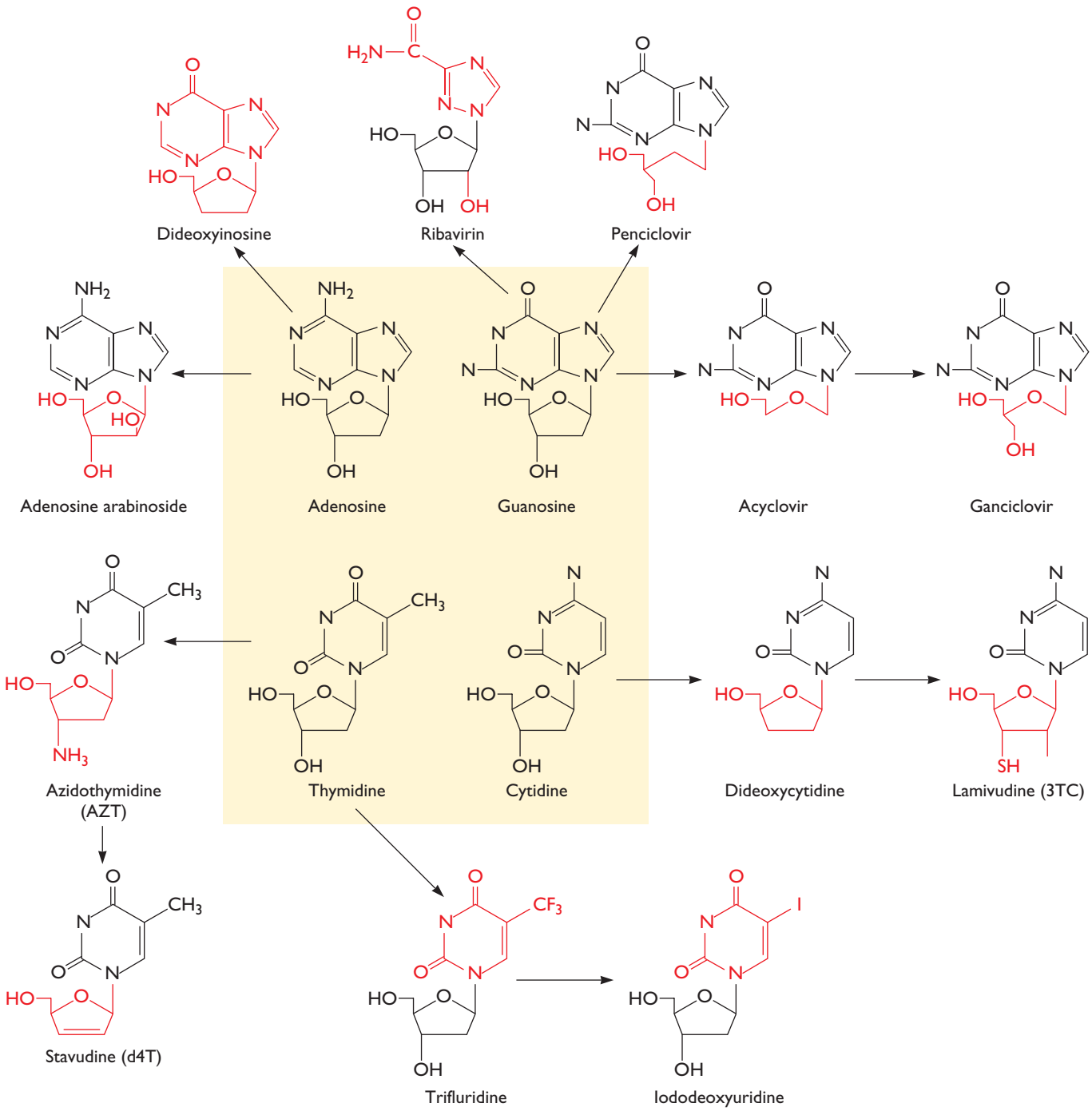


Figure 9.9 Many well-known antiviral compounds are nucleoside and nucleotide analogs. The four natural deoxynucleosides are highlighted in the yellow box. The chemical distinctions between the natural deoxynucleosides and antiviral drug analogs are highlighted in red. Arrows connect related drugs. Adapted from E. De Clercq, *Nat. Rev. Drug Discov.* **1**:13–25, 2002, with permission.

methylated derivative, cannot cross the blood-brain barrier and therefore has fewer central nervous system side effects. For this reasons, the drug often replaces amantadine in the treatment of influenza A virus infections.

An unusual property of amantadine is the concentration dependence of its antiviral activity. The drug has broad antiviral effects at high concentrations, but at low concentrations it is specific for influenza virus A, with no

effect on B or C strains or other viruses. Analysis of resistant mutants provided insight into the apparent complex mechanism of action of amantadine. At concentrations of 100 mM or higher, the compound acts as a weak base and raises the pH of endosomes so that pH-dependent membrane fusion is blocked. Any virus with a pH-dependent fusion mechanism could be affected by high concentrations of amantadine. Resistant mutants of influenza A virus selected under these conditions in cultured cells harbor amino acid substitutions in HA that destabilize the protein and enable fusion at higher pH. Influenza virus mutants selected at concentrations of 5 mM or lower carried mutations in the M2 gene. Specifically, these mutations affected amino acids in the membrane-spanning region of the M2 ion channel protein.

Zanamivir and Oseltamivir

Zanamivir and oseltamivir are inhibitors of the neuraminidase enzyme synthesized by influenza A and B viruses (Box 9.3). Zanamivir is delivered via inhalation, while oseltamivir can be given orally. When used within 48 h of onset of symptoms, the drugs reduce the median time to alleviation of symptoms by about 1 day compared to placebo. When used within 30 h of disease onset, the drugs reduce the duration of symptoms by about 3 days.

The Search for New Antiviral Targets

Entry and Uncoating Inhibitors

The first step of viral replication has long been an attractive target, as virus-receptor interactions offer the promise of high specificity. Early enthusiasm for entry inhibitors came from experiments with monoclonal antibodies that inhibited virion attachment or entry in cultured cells. Passive immunization with these antibodies often can protect animals from challenge, suggesting that small-molecule inhibitors of entry may be useful leads. At present, it is impractical, or not cost-effective, to develop antibodies as antiviral agents; these molecules have been most valuable in identifying proteins required for entry and their mechanisms.

The binding site of antibodies that block entry provides one starting point for screening chemical libraries, or for design of small molecules that block viral entry. It is important that such compounds interfere with virus-host interactions, but not with the normal function of the cellular receptor. Obviously, an important assumption in this strategy is that no other entry routes exist. However, alternative receptors are available for many viruses (e.g., herpesviruses and HIV), and blocking a single virion-receptor interaction may not be effective.

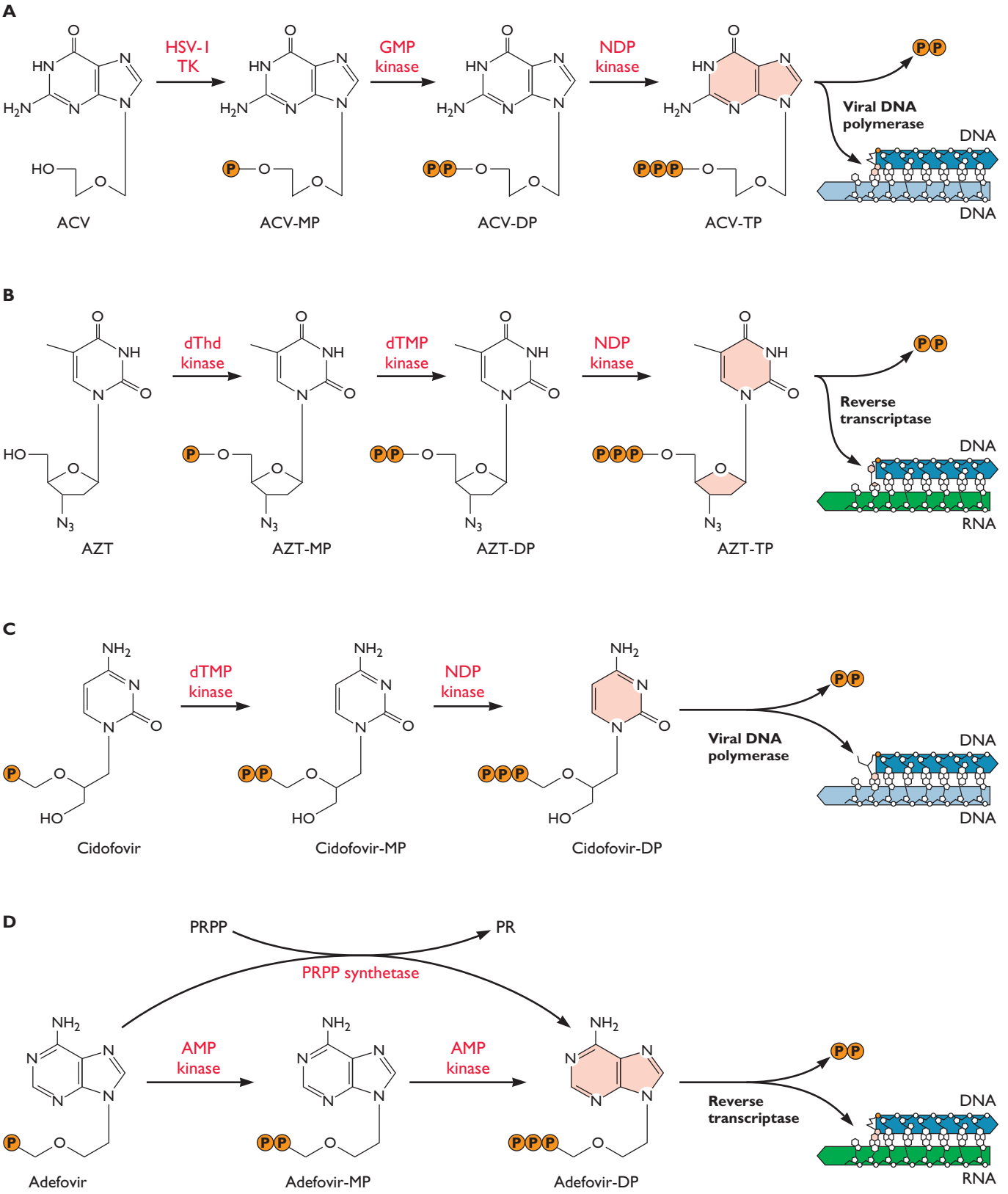
Membrane fusion, the ubiquitous process by which enveloped virions enter cells, is an attractive target for chemotherapeutic intervention because fusion mechanisms are conserved among enveloped viruses. To identify inhibitors of influenza virus fusion, a computer program was first used to predict which small molecules might bind into a pocket of the HA molecule and possibly prevent low-pH-induced conformational change. From the molecules identified in this way, several benzoquinone- and hydroquinone-containing compounds were tested and found to inhibit HA-mediated membrane fusion at low pH. One of these inhibits influenza virus replication. Although it is not known how binding of the compound prevents fusion, it has been suggested that it acts as a "molecular glue" to prevent movement of the fusion peptide. Inhibitors of HIV entry are discussed in more detail below.

Considerable effort is being expended on **microbicides**, creams or ointments that either inactivate or block virions before they can attach and penetrate tissues. The concept is that these treatments either would inactivate virions directly or would bind to many potential receptors on the cell surface, and therefore may act as broad spectrum antivirals. Particular attention is focused on vaginal microbicides to prevent sexually transmitted infections. For example, certain polyanions act as competitive inhibitors of both human immunodeficiency virus and herpes simplex virus binding to cell surface receptors and are easily formulated as inexpensive topical creams. The primary problem is attaining full protection. Many of these compounds are competitive inhibitors of binding, and thus are dependent on the concentration of virions and microbicide.

Proteases

Viral proteases often cleave protein precursors to form functional units or to release structural components during ordered particle assembly (maturational proteases). As discussed in Volume I, Chapter 13, all herpesviruses encode a serine protease that is absolutely required for formation of nucleocapsids. Many features of these enzymes and their substrates are conserved among the members of the family *Herpesviridae*. The structure of the human cytomegalovirus protease is shown in Fig. 9.12. Interest in this enzyme as a target is based on the unusual serine protease fold and the mechanism of catalysis, which bodes well for the safety and efficacy of high-specificity inhibitors.

Hepatitis C virus infects more people than HIV in the United States, and infects millions more individuals around the world. As a result, an intensive antiviral drug discovery effort is in progress. The essential viral protease called NS3 is a major target (Fig. 9.13). Until recently, hepatitis C virus could not be propagated in cultured cells, and so the effect of inhibiting NS3 protease on virus replication was tested initially by using minireplicons in cell-based assays. The crystal structure of NS3 protease has been solved by several companies, as have the structures of NS3 helicase, the NS5B polymerase, and other proteins. Major efforts in structure-based design, as well as mechanism-based


A
HSV-1 TK
GMP kinase
NDP kinase
Viral DNA polymerase
DNA
DNA
ACV
ACV-MP
ACV-DP
ACV-TP
B
dThd kinase
dTMP kinase
NDP kinase
Reverse transcriptase
DNA
RNA
AZT
AZT-MP
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C
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DNA
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PRPP synthetase
AMP kinase
AMP kinase
Reverse transcriptase
DNA
RNA
Adefovir
Adefovir-MP
Adefovir-DP

screening, have identified several NS3 and NS5B inhibitors that currently are in clinical trials.

Virus-Specific Nucleic Acid Synthesis and Processing

Viral replication enzymes are primary targets for antiviral drugs. From genetic analyses, we know that DNA polymerase accessory proteins, such as those that promote processivity or bind to viral origins, are essential for viral replication and also represent attractive targets. The RNA-dependent RNA polymerases of any RNA virus appear to be unique to the virus world. Their varied activities, which include synthesis of primers, cap snatching, and recognition of RNA secondary structure in viral genomes, can be exploited for drug discovery. The unique helicases encoded by many RNA viruses are already in many companies' high-throughput screens, but no lead compounds have yet progressed to the market.

Newly replicated, concatemeric herpesviral DNA is cleaved by viral enzymes into monomeric units during the packaging/assembly process. These processes, which are essential for viral replication, are carried out by specific enzymes. These enzymes represent promising targets for antiviral drugs. For example, 5-bromo-5,6-dichloro-1-β-D-ribofuranosyl benzimidazole binds to the human cytomegalovirus UL89 gene product that is a component of the "terminase" complex responsible for cleaving and packaging replicated concatemeric DNA. Members of the UL89 gene family are highly conserved among all herpesviruses, and have homology to a known ATP-dependent endonuclease encoded in the genome of bacteriophage T4. This class of compounds may therefore be the basis for discovery of broad-based inhibitors of herpesvirus replication. Another class of compounds, the 4-oxo-dihydroquinolines, also have potential as broad-spectrum herpesvirus inhibitors because they interact with a sequence conserved in many herpesvirus polymerases.

Regulatory Proteins

Viral proteins that control transcription often are essential for viral growth and are prime targets for antiviral screens. Fomivirsen is the first licensed compound designed to inhibit the function of a viral regulatory protein. The drug is a phosphorothioate, antisense oligonucleotide and is approved to treat retinitis, a disease caused by human cytomegalovirus. Inhibition depends on binding of the 21-nucleotide antisense molecule to the cytomegalovirus immediate-early 2 mRNA. Host proteins that play central roles in stimulating damaging intrinsic or immune responses are also attractive targets (Box 9.4).

Regulatory RNA Molecules

Micro-RNAs (miRNAs) control the activity of a substantial number of genes and are encoded in both host and viral genomes. Often one class of miRNA regulates the expression of an entire network of genes. One such host miRNA, miR-122, is expressed only in the liver and regulates expression of more than 400 genes including those involved in cholesterol metabolism. Remarkably, when expression of miR-122 is inhibited, not only do levels of cholesterol in circulation drop, but also the liver is protected from hepatitis C virus infection. The mechanism of viral inhibition is not understood. Nevertheless, this finding demonstrates that small RNA molecules may be targets for antiviral compounds.

Antiviral Gene Therapy and Transdominant Inhibitors

In 1988, scientists constructed a cell line that produced an altered VP26 protein from herpes simplex virus type 1. The cell line was subsequently shown to be highly resistant to infection by herpes simplex virus type 1. The mutation in the VP16 gene conferred a dominant-negative phenotype: the altered protein interfered with the function of the

Figure 9.10 Chain termination by antiviral nucleoside analogs. (A) Acyclovir (ACV) is a prodrug that must be phosphorylated in the infected cell. The viral thymidine kinase (herpes simplex virus type 1 [HSV-1] TK), but not the cellular kinase, adds one phosphate (orange circle labeled P) to the 5′-hydroxyl group of acyclovir. The monophosphate is a substrate for cellular enzymes that synthesize acyclovir triphosphate. The triphosphate compound is recognized by the viral DNA polymerase and incorporated into viral DNA. As acyclovir has no 3′-hydroxyl group, the growing DNA chain is terminated, and viral replication ceases. **(B)** AZT targets the HIV reverse transcriptase. It must be phosphorylated by cellular kinases in three steps to the triphosphate compound, which is incorporated into the viral DNA to block reverse transcription. **(C)** Cidofovir [S-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine] is an acyclic nucleoside analog. In contrast to acyclovir and AZT, cidofovir requires only two phosphorylations by cellular kinases to be converted to the active triphosphate chain terminator. **(D)** Adefovir [9-(2-phosphonylmethoxyethyl)adenine] is an acyclic nucleoside analog and also requires only two phosphorylations by cellular AMP kinases. Through the action of phosphoribosyl pyrophosphate synthetase, which forms the triphosphate from the monophosphate in one step, both cidofovir and adefovir bypass the nucleoside-kinase reaction that limits the activity of dideoxynucleoside analogs such as AZT. DP, diphosphate; dThd, (2′-deoxy)-thymidine; MP, monophosphate; NDP, nucleoside 5′-diphosphate; PR, 5-phosphoribose; TP, triphosphate. Adapted from E. De Clercq, Nat. Rev. Drug Discov. 1:13–25, 2002, with permission.

BOX 9.3

EXPERIMENTS

Designer drugs: inhibitors of influenza virus neuraminidase

Influenza virus neuraminidase (NA) protein cleaves terminal sialic acid residues from glycoproteins, glycolipids, and oligosaccharides. It plays an important role in the spread of infection from cell to cell, because in cleaving sialic acid residues, the enzyme releases virions bound to the surfaces of infected cells and facilitates viral diffusion through respiratory tract mucus. Moreover, the enzyme can activate transforming growth factor β by removing sialic acid from the inactive protein. Because the activated growth factor can induce apoptosis, NA may influence the host response to viral infection. NA is a particularly intriguing target for drug hunters.

NA is a tetramer of identical subunits, each of which consists of six four-stranded antiparallel sheets arranged like the blades of a propeller. The enzyme active site is a deep cavity lined by identical amino acids in all strains of influenza A and B viruses that have been characterized. Because of such invariance, compounds designed to fit in this cavity would be expected to inhibit the NA activity of all A and B strains of influenza virus, a highly desirable feature in an influenza antiviral drug. Moreover, as NA inhibitors are predicted to block spread, they may be effective in reducing the transmission of infection to other individuals.

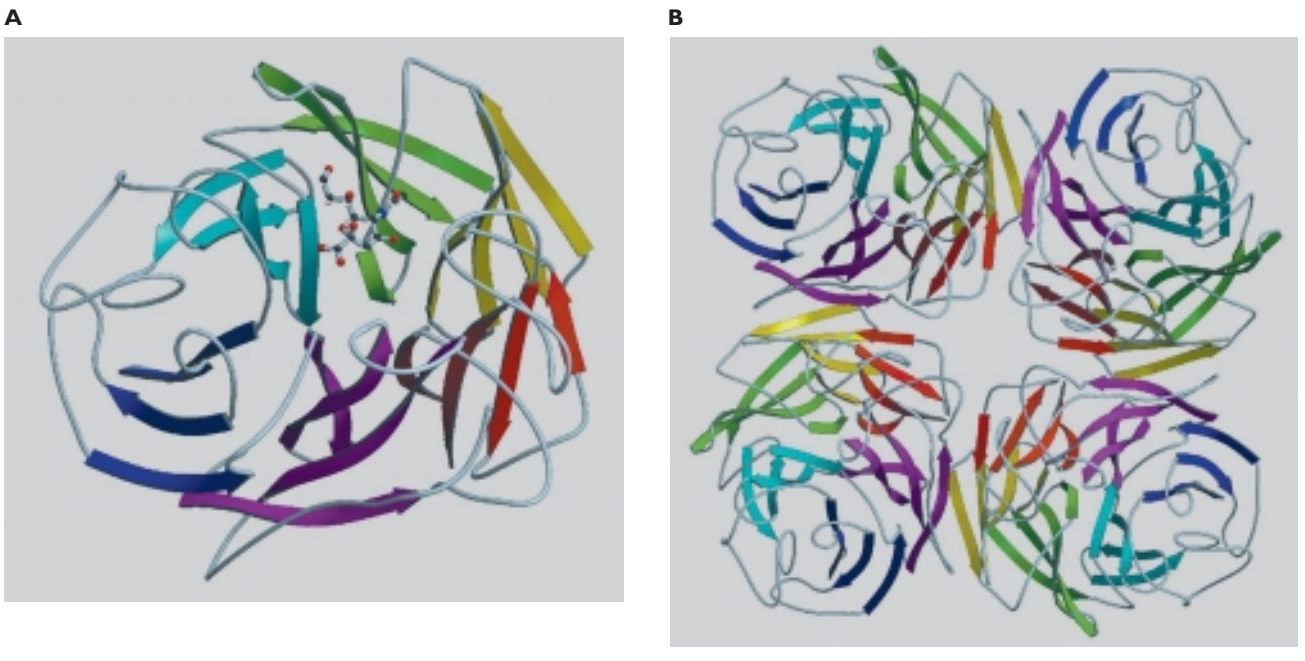
Sialic acid fits into the active site cleft in such a fashion that there is an empty pocket near the hydroxyl at the 4 position on its sugar ring. On the basis of computer-assisted analysis, investigators predicted that replacement of this hydroxyl group with either an amino or a guanidinyl group would fill the empty pocket and therefore would increase the binding affinity by contacting one or more neighboring glutamic acid residues.

Currently, two antiviral drugs, zanamivir and oseltamivir, that inhibit influenza A and B virus NA are licensed for use. Significantly, these drugs are inhibitors of both influenza A and B viruses in cultured cells. They do not inhibit other nonviral NAs, an important requirement for safety and lack of potential side effects.

Varghese, J. N., V. C. Epa, and P. M. Colman. 1995. Three dimensional structure of the complex of 4-guanidino-Neu5Ac2en and influenza virus neuraminidase. *Protein Sci*. **4:**1081–1087.

von Itzstein, M., W. Y. Wu, G. B. Kok, M. S. Pegg, J. C. Dyason, B. Jin, T. Van Phan, M. L. Smythe, H. F. White, S. W. Oliver, P. M. Coleman, J. N. Varghese, D. M. Ryan, J. M. Woods, R. C. Bethell, V. J. Hotham, J. M. Cameron, and C. R. Penn. 1993. Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* **363:**418–423.

Structure of influenza A virus NA. (A) A ribbon diagram of influenza A virus NA with α-sialic acid bound in the active site of the enzyme. The molecule is viewed down the fourfold axis of the tetramer. The molecule is an N2 subtype from A/Tokyo/3/67. The C terminus is on the outside surface near a subunit interface. The six β-sheets of the propeller fold are indicated in colors. **(B)** The packing of the N2 NA tetramer viewed from above and down the symmetry axis. Adapted from J. N. Varghese, p. 459–486, *in* P. Verrapandian (ed.), *Structure-Based Drug Design* (Marcel Dekker, New York, NY, 1997), with permission. Courtesy of J. Varghese.



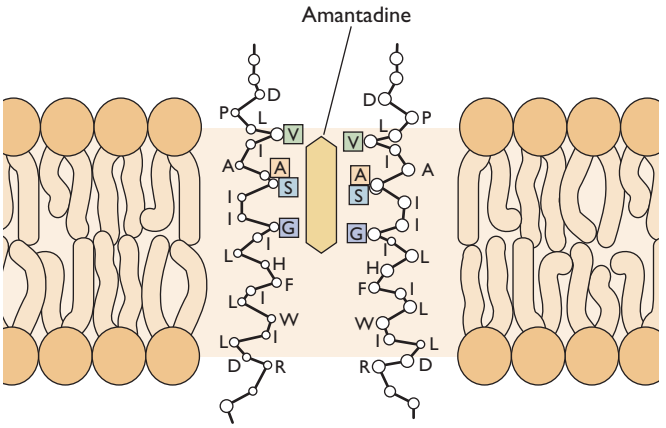


Figure 9.11 Interaction of amantadine with the transmembrane domain of the influenza A virus M2 ion channel. It is thought that at low concentrations (5 μM), amantadine exerts its antiviral effect by blocking the virus-encoded M2 ion channel activity in infected cells. M2 protein is a tetramer with an aqueous pore in the middle of the four subunits. The four locations of single-amino-acid changes in different amantadine-resistant mutants are boxed. A diagram of the putative interaction of amantadine with two diagonally located α-helices of the M2 tetramer in a lipid bilayer is shown. Adapted from A. J. Hay, *Semin. Virol.* **3:**21–30, 1992, with permission. For new data, see D. A. Steinhauer and J. J. Skehel, *Annu. Rev. Genet.* **36:**305–332, 2002.

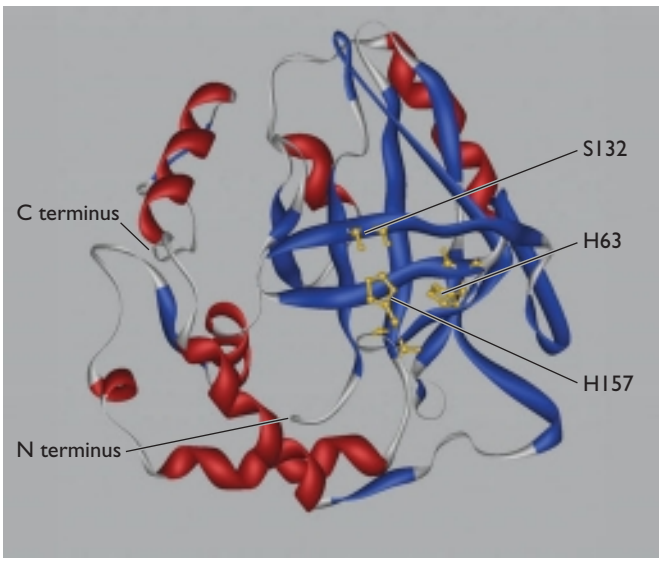
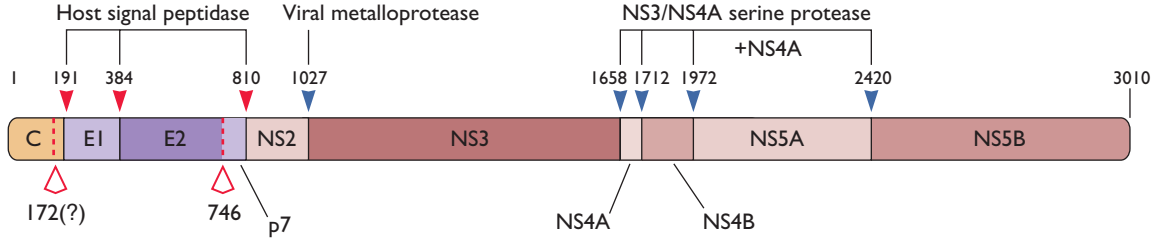


Figure 9.12 Human cytomegalovirus protease. The β-strands are shown as blue ribbons, α-helices are red, and the connecting loops are indicated in white. The active site residues (serine 132, histidine 63, and histidine 157) are indicated in yellow. The amino and carboxy termini are indicated. Adapted from L. Tong et al., *Nature* **383:**272–275, 1996, with permission.

normal VP16 protein. When transgenic mice were produced that synthesized the altered VP16 protein, they too were resistant to infection by herpes simplex virus type 1. Dominant-negative mutations are not new in genetics, but the idea that such an altered protein could be used to protect an animal against infection was novel. A particularly ingenious implementation of this strategy for antiviral drug discovery is described in Box 9.5.

Many natural and laboratory produced gene products that should block intracellular viral growth when present in an infected cell can be imagined, and some of them may yield effective antiviral therapies. For example, RNA-binding proteins, DNA-binding proteins, ribozymes, and antisense oligonucleotides offer interesting possibilities. Some clever ideas are based on hybrid proteins composed of a target domain and a killer domain. The former brings the latter

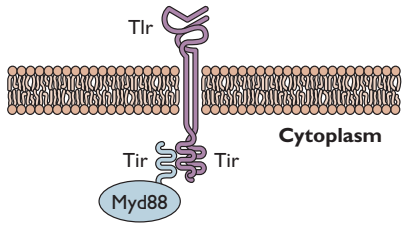
Figure 9.13 The hepatitis C virus polyprotein is cleaved by several proteases. Hepatitis C virus is a human flavivirus with a (+) strand RNA genome. The viral proteins are encoded in one large open reading frame that is translated into a polyprotein. The polyprotein is processed by cellular and viral proteases to release the viral proteins. The numbers above the polyprotein indicate the amino acid marking the start of each viral protein. The last number, 3010, indicates the amino acid number of the C terminus of the polyprotein. The proteases and their cleavage sites are indicated by arrows. Signal peptidase is the host enzyme in the endoplasmic reticulum that cleaves signal peptides from membrane proteins. The red open arrows and dotted lines represent cleavage sites that are inefficiently processed or not well studied. The viral metalloprotease is an autoprotease comprising NS2 and the amino-terminal domain of NS3. The viral serine-type protease is the NS3 protein bound to NS4A and is essential for the assembly of virus particles.



BOX 9.4 DISCUSSION

Intrinsic defense receptors as drug targets

Recent studies demonstrate that the Toll-like receptor protein 3 (Tlr3) plays a central role in the severe "cytokine storm" response to influenza virus infection. This protein is expressed abundantly in bronchial and alveolar epithelial cells, and, remarkably, is overexpressed when these cells are infected with influenza A virus. As a result, the normal danger signal instigated when Tlr3 engages viral RNA is amplified. The central mediators in the inflammatory response are interleukin-8 (IL-8), Rantes, IL-6, and interferon. In addition, expression of the cell surface adhesion protein Icam-1 is increased. The key experiment that identified Tlr3 (and not Tlr7 or Tlr8) was that when Tlr3 knockout mice were challenged with influenza A virus, the knockouts survived and their wild-type littermates died from an overwhelming inflammatory response.



The Tlr proteins may be targets for therapeutic interaction, particularly because of the possibility of broad application. If a small-molecule inhibitor could be designed to block Tlr function, the dangerous expansion of the inflammatory response to some viral infections might be limited.

Guillot, L., R. Le Goffic, S. Bloch, N. Escriou, S. Akira, M. Chignard, and M. Si-Tahar. 2005. Involvement of the Toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza virus. *J. Biol. Chem.* **280**:5571–5580.

to the virus-infected cell. For example, nucleases fused to viral capsid proteins block viral propagation if the viral genome is degraded during virion assembly. Toxins have been fused to antibodies that bind to viral proteins present on the surfaces of infected cells. When the hybrid protein is taken up by receptor-mediated endocytosis, it kills the infected cell.

The effectiveness of dominant-negative mutations and killer or suicide molecules has been validated by using cell culture systems; indeed, the current strategies (now classified under the rubric of gene therapy) are too numerous to mention. While this approach often captures the fancy of molecular virologists, its practical utility in the treatment of disease remains uncertain. Such compounds or approaches provide interesting leads, but they face the same hurdles as more conventional antiviral lead compounds (Table 9.2).

Resistance to Antiviral Drugs

Because viral replication is so efficient and is accompanied by moderate to high mutation frequencies, resistance to any antiviral drug must be anticipated. Drug resistance is of especial concern during the extended therapy required for persistent infections. Indeed, viral mutants resistant to every antiviral drug manufactured to date have been detected in the clinic, a disconcerting fact because our arsenal of antiviral drugs is small. Drug-resistant mutants are dangerous for patients, who cannot be treated for the resistant infection, and are potentially hazardous for the population who might be exposed to them. Nevertheless, a genetic analysis of resistance can provide powerful insight into the antiviral mechanism and may identify new strategies to reduce or circumvent the problem.

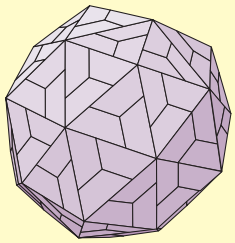
Consider acyclovir-resistant mutants of herpes simplex virus, which arise spontaneously during viral replication and are selected after exposure to the drug. The majority of mutations that confer resistance are in the viral thymidine kinase gene and inactivate kinase function. We can deduce that thymidine kinase is essential for the action of the drug. As the enzyme is not required for herpes simplex virus to grow in cultured cells, such mutants can be identified with ease. A subset of mutations leading to acyclovir resistance are not present in the thymidine kinase gene but, rather, are in the viral DNA polymerase gene. The altered polymerases possess reduced ability to incorporate phosphorylated drug into DNA. Similar patterns of resistance have been reported when other nucleoside analog inhibitors are used against varicella-zoster virus.

The appearance of thymidine kinase mutants after acyclovir therapy is of concern in the clinic. Although these mutants have a markedly reduced ability to invade and replicate in the central nervous system in animal models, they can lead to devastating infections in AIDS patients. Such mutants are still able to cause disseminated disease, and are often resistant to other nucleoside analogs that share the same antiviral mechanism. Fortunately, foscarnet can be used, in some instances, to treat these infections. Acyclovir-resistant DNA polymerase mutants present a special problem because they can also be resistant to foscarnet, leaving essentially no recourse.

BOX 9.5 EXPERIMENTS

Transdominant inhibition of RNA viral replication

The error-prone replication of RNA viral genomes yields a plethora of mutants for selection during every cycle of replication. An infection initiated by a single particle quickly becomes heterogeneous even in a single infected cell. Consequently, a mutant progeny genome with a selective advantage must be able to replicate in the context of hundreds or thousands of other genomes with their own panoply of mutations. One could imagine an altered gene product "helping" or "hindering" other genomes. Similarly, one could imagine mutations that provided a selective advantage to only the genome that carried them. Interestingly, mutant genomes with no selective advantage can sometimes interfere with replication of the entire population.



Four such sets of dominant mutations were identified after a systematic screen of the poliovirus genome. Mutated alleles in capsid- and polymerase-coding regions were dominant, probably because of the oligomeric properties of the proteins. Mutated alleles in an RNA element required for priming RNA synthesis also were dominant, because nonproductive priming intermediates are inhibitory. Certain mutations in the protease 2A coding region also were dominant, presumably because the intramolecular activity of the protease creates a toxic product.

The authors argued that these viral gene products would be excellent drug targets because drug-sensitive viruses should be dominant over drug-resistant variants. In support of this idea, they showed that growth of viral mutants resistant to a capsid-binding antiviral drug was inhibited in the presence of drug-sensitive viruses.

Crowder, S., and K. Kirkegaard. 2005. Transdominant inhibition of RNA viral replication can slow growth of drug-resistant viruses. *Nat. Genet.* **37:**701–709.

Human Immunodeficiency Virus and AIDS

The recognition that HIV was associated with AIDS in the early 1980s provided the impetus for unprecedented focus on antiviral drug discovery. As noted in Chapter 6, the first effective drugs acted on the viral reverse transcriptase. The strategy was based on the well-known ability of nucleoside analogs to block replication of herpesviruses and tumor cells. Researchers quickly deciphered many distinctive steps of the viral life cycle, identifying numerous additional targets (Fig. 9.14). As a result, not only new nucleoside inhibitors but also nonnucleoside inhibitors of reverse transcriptase and potent protease inhibitors became available in the clinic. In 1997, a protocol known as highly active antiretroviral therapy (HAART) was developed in which a combination of these inhibitors is used to treat patients aggressively. Scientists are also identifying more compounds directed to specific steps in the virus life cycle, including inhibitors of virus-cell interaction (attachment, fusion, and entry), integration of viral DNA, and nucleocapsid assembly. As of 2007, more than 20 approved drugs were available to treat HIV infections targeting three different mechanisms (Box 9.6).

Examples of Anti-HIV Drugs

Nucleoside Analogs

AZT, or zidovudine, was the first drug to be licensed (Fig. 9.9). This drug was initially discovered during screens for antitumor cell compounds rather than for antiviral agents. Unlike acyclovir, which is phosphorylated by a viral kinase, AZT is phosphorylated to the active form by cellular enzymes (Fig. 9.10). Like acyclovir, phosphorylated AZT acts as a chain terminator when incorporated into DNA. Phosphorylated AZT is not a good substrate for most cellular polymerases. However, it is incorporated into the DNA copy of the viral genome by the viral reverse transcriptase. The relative selectivity of this drug depends on the fact that reverse transcription takes place in the cytoplasm, where the drug appears first and in the highest concentration. Because AZT monophosphate competes with thymidine monophosphate (TMP) for the formation of nucleoside triphosphate, its presence causes depletion of the intracellular pool of ribosylthymine 5′-triphosphate (TTP). Therefore, AZT is much less selective than acyclovir and has substantial side effects. AZT can be given orally and is absorbed quickly, but is also degraded rapidly by liver glucuronidation enzymes so that its effective half-life in a patient is only about 1 h. Patients must take the drug two or three times daily to maintain an effective antiviral concentration. If the illness is severe, AZT can be administered intravenously. As discussed below, a short half-life is problematic because when an effective concentration of any antiviral drug is not maintained, resistant mutants will be selected. At best, AZT provides modest and transient effects in infected adults, with a concomitant increase in CD4+ T-cell counts. However, it is effective in prophylactic

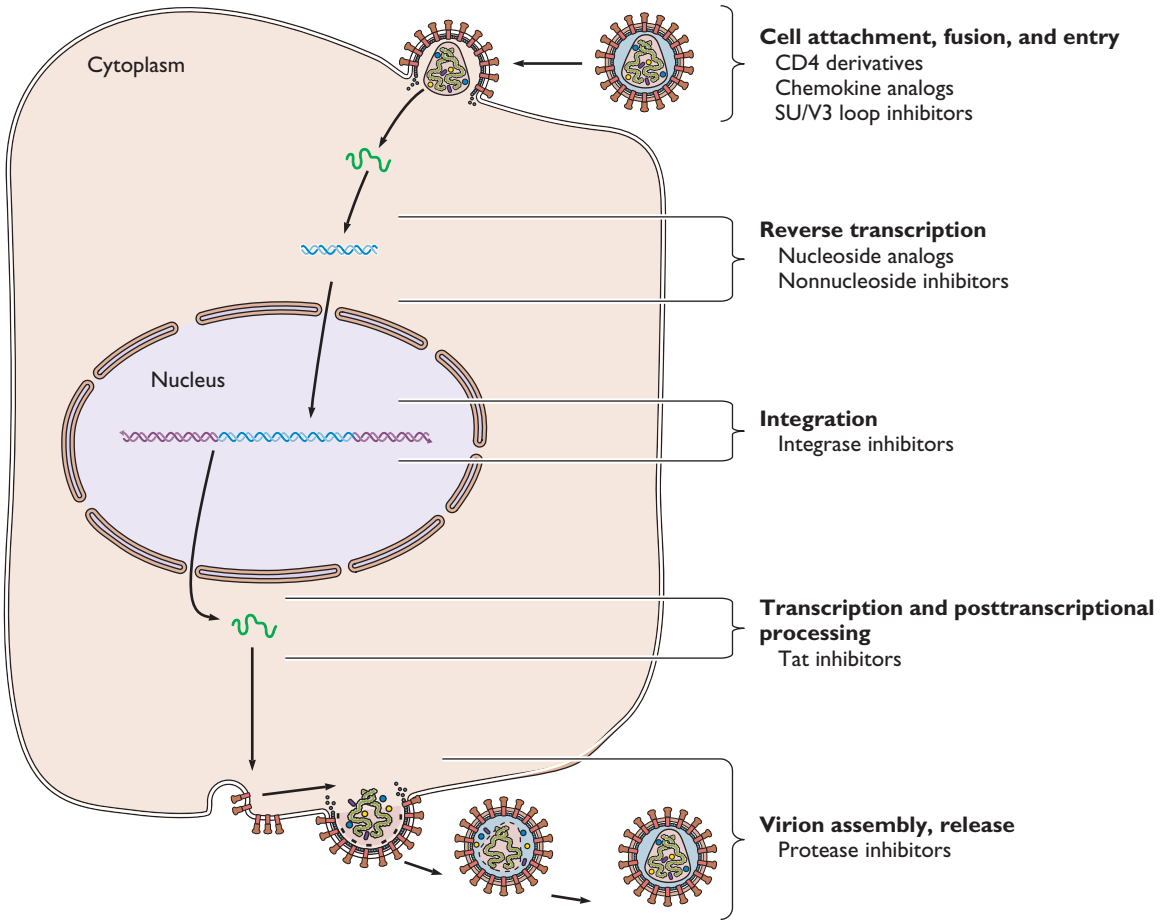


Figure 9.14 Important steps in the replication of human immunodeficiency virus. Five steps in the life cycle of the virus are highlighted as potential targets for antiviral compounds. Known or hypothetical inhibitory compounds are listed under each step. Adapted from D. D. Richman et al., *Clinical Virology* (Churchill Livingstone, New York, NY, 1997), with permission.

treatment for accidental needle sticks, and for treatment of infected pregnant women, when it can reduce considerably the probability of delivering an HIV-infected baby.

AZT toxicity is of great concern when the drug must be given for long periods. AZT treatment can damage bone marrow, resulting in a reduction in the number of neutrophils. Treatment of this problem requires multiple transfusions of red blood cells. Muscle wasting, nausea, and severe headaches are but a few of AZT's other side effects that must be endured. Despite these problems, the drug was used extensively because, until recently, there simply was no alternative.

Considerable effort has been devoted to discovering alternatives to AZT. Particular emphasis has been placed on finding reverse transcriptase inhibitors that are effective against AZT-resistant mutants. Several nucleoside analogs that have therapeutic value are now available (Fig. 9.9).

Nonnucleoside Inhibitors of Reverse Transcriptase

Nonnucleoside inhibitors of viral reverse transcriptase do not bind at the nucleotide-binding site of the enzyme (Fig. 9.15). Examples of these compounds are nevirapine and the tetrahydroimidazobenzodiazepinone (TIBO) class of compounds. Initially, they offered the hope of complementing nucleoside analog inhibitors, but the rapid emergence of resistant mutants dampened enthusiasm. Although they are effective inhibitors of reverse transcriptase, a substitution in any of seven residues that line their binding site on the enzyme confers resistance. Because resistant mutants are selected rapidly, nonnucleoside inhibitors cannot be used by themselves for the treatment of AIDS (monotherapy). However, as with AZT, nevirapine has value for treatment of pregnant women before delivery to prevent infection of the newborn. This use is now the preferred

BOX 9.6 DISCUSSION

A heroic effort: 19 new drugs, 3 targets, 9 companies, and 15 years

We must never forget the daunting task that faced the scientific and medical community in the 1980s when HIV was first identified and every infection was a death sentence. There was no experience with such infections in the clinics, and the drug hunters had nothing in the pipeline that was proven to be effective against retroviruses. In fact, there were few scientists with any experience at all with lentiviruses. Yet as the data in this table demonstrate, a truly heroic effort was mounted over the first 15 years of the pandemic, but it took time, money, and unprecedented cooperation.

Target or mechanism	Generic name	Brand name	Manufacturer	Yr approved
Nucleoside reverse transcriptase inhibitors	Zidovudine (AZT, ZDV)	Retrovir	GlaxoSmithKline	1987
	Didanosine (ddI)	Videx	Bristol-Myers Squibb	1991
	Zalcitabine (ddC)	Hivid	Roche	1992
	Stavudine (d4T)	Zerit	Bristol-Myers Squibb	1994
	Lamivudine (3TC)	Epivir	GlaxoSmithKline	1995
	AZT/3TC	Combivir	GlaxoSmithKline	1997
	Abacavir (ABC)	Ziagen	GlaxoSmithKline	1998
	AZT/3TC/ABC	Trizivir	GlaxoSmithKline	2000
	Tenofovir (TDF)	Viread	Gilead	2001
Nonnucleoside reverse transcriptase inhibitors	Nevirapine	Viramune	Roxane	1996
	Delavirdine	Rescriptor	Agouron	1997
	Efavirenz	Sustiva	Dupont	1998
Protease inhibitors	Saquinavir (hard gel)	Invirase	Roche	1995
	Saquinavir (soft gel)	Fortovase	Roche	1997
	Ritonavir	Norvir	Abbott	1996
	Indinavir	Crixivan	Merck	1996
	Nelfinavir	Viracept	Agouron	1997
	Amprenavir	Agenerase	GlaxoSmithKline	1999
	Lopinavir/ritonavir	Kaletra	Abbott	2000
Summary				
Three enzyme targets	**16 unique compounds**	**19 approved drugs**	**9 companies**	**15 years**

treatment in underdeveloped countries. These drugs have also proved valuable in combination therapy (see below).

Protease Inhibitors

HIV protease, which is encoded in the *pol* gene, is essential for production of mature infectious viral particles. The enzyme cleaves itself from the Gag-Pol precursor polyprotein and then cleaves at seven additional sites in Gag-Pol to yield nine proteins and three enzymes. Active protease has been produced in high yields in many recombinant organisms and has even been synthesized chemically. It was the first HIV enzyme to be crystallized and studied at the atomic level (Fig. 9.5). The active enzyme is a small (only 99-amino-acid) dimeric aspartyl protease, similar to renin and pepsin.

The seven cleavage sites in Gag-Pol are similar but not identical. In the early stages of research to find protease inhibitors, it was essential to understand how the enzyme recognized and cleaved these sites. In pursuing this goal, an important discovery was made: the enzyme can recognize and cleave small peptide substrates in solution. It was subsequently determined that the active site is large enough to accommodate seven amino acids. The parameters of peptide binding and protease activity were determined by screening synthetic peptides containing variations of the seven cleavage sites for their ability to be recognized and cleaved by the enzyme. The first inhibitor leads were peptide mimics (peptidomimetics) modeled after inhibitors of other aspartyl proteases, such as human renin, an enzyme implicated in hypertension (Fig. 9.16). Subsequent screens for mechanism-based and structure-based inhibitors designed *de novo* have yielded several powerful peptidomimetic inhibitors of the protease (Box 9.7).

Integrase

Integrase protein is particularly attractive, because biochemical and structural data are available. In the absence of

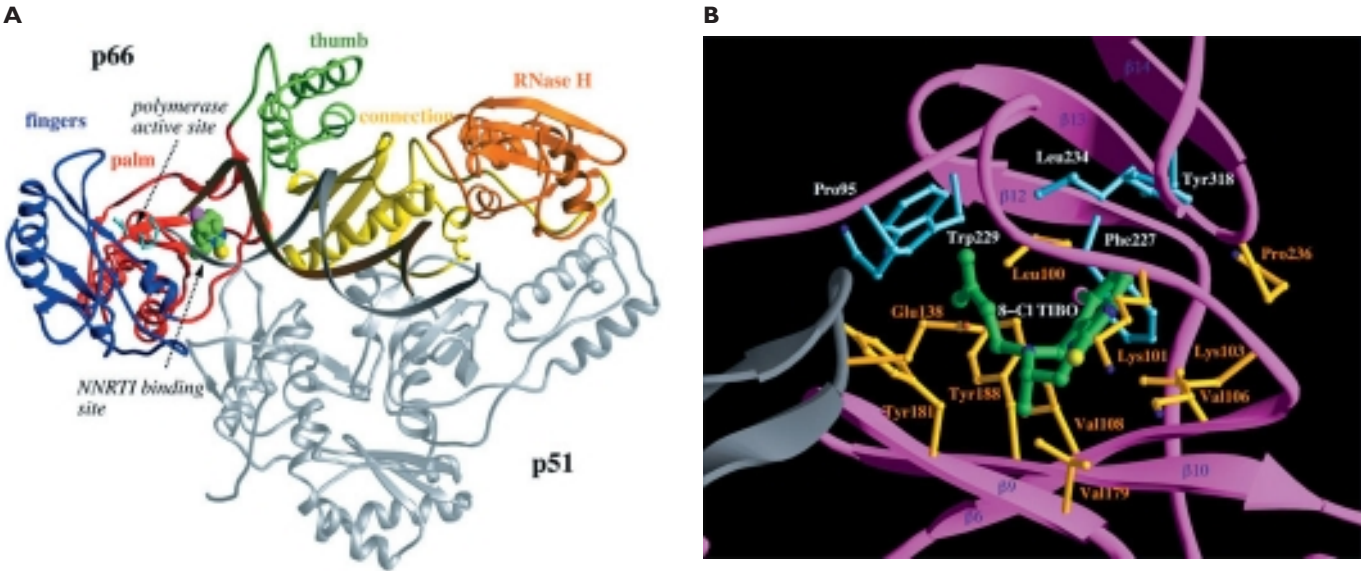


Figure 9.15 Structure of HIV type 1 reverse transcriptase highlighting the polymerase active site and the nonnucleoside reverse transcriptase inhibitor binding site. (A) Structure of the reverse transcriptase p66-p51 heterodimer bound to a double-stranded DNA template-primer, showing the relative locations of the polymerase active site and the site for binding nonnucleoside reverse transcriptase inhibitors (NNRTIs). Data from A. Jacobo-Molina et al., *Proc. Natl. Acad. Sci. USA* **90:**6320–6324, 1993. **(B)** Structure of 8-CL TIBO (a prototype NNRTI; green) bound to reverse transcriptase (green molecule). Amino acid side chains corresponding to sites of drug resistance mutations are shown in orange; residues with cyan side chains are sites at which NNRTI resistance mutations have not been detected. Data from J. Ding et al., *Nat. Struct. Biol.* **2:**407–415, 1995. Courtesy of K. Das and E. Arnold, Center for Advanced Biotechnology and Medicine, Rutgers University.

integrase, viral DNA cannot be inserted into the host genome. As a result, the viral genome is unable to be expressed efficiently and to propagate in dividing cells. Several companies have lead compounds under analysis, and rational design programs based on structures of integrase complexed with inhibitors are in progress. One drug, called raltegravir (Isentress), was approved in late 2007. All current integrase inhibitors target the joining step in the reaction (Volume I, Fig. 7.16). The inhibitors stabilize the DNA-protein intermediate so that the reaction cannot be completed.

Inhibitors of Fusion and Entry

Some neutralizing antibodies that block viral attachment, bind the third variable domain (the so-called V3 loop) of the SU protein (Volume I, Fig. 5.5). A variety of natural and synthetic molecules interfere with V3 loop activity. These compounds include specific antibodies and polysulfated or polyanionic compounds such as dextran sulfate and suramin. These compounds were identified early in the search for antiviral agents, but were discarded because of intolerable side effects such as anticoagulant activity. Although considerable effort was expended in the development of inhibitors of the SU-CD4 receptor interaction, including a "soluble CD4" that theoretically would act as a competitive inhibitor of infection, no effective antiviral agents have been found using this strategy. This lack of success can be attributed, in part, to the high concentration of SU on the virion, as well as to alternative mechanisms for spread in an infected individual.

As often happens, the early research with failed Env inhibitors provided much insight into how virions enter cells, and has focused attention on other targets in the process. For example, it was curious that mutants resistant to neutralizing antibodies have clustered mutations in the V3 loop, yet virus-cell fusion is not affected. The implication was that CD4-V3 interactions were not involved in entry. As described in Volume I, Chapter 5, entry is a multistep process requiring that the target cells synthesize not only CD4, but also any one of several chemokine receptors, such as CCr5 present on macrophages or CXCr4 found on T cells. Chemokine receptors are attractive targets because individuals homozygous for mutations in one such receptor (CCr5) are partially resistant to infection and suffer no apparent ill effects. Recent studies have shown that infected individuals carrying a mutation in the CCr5 chemokine receptor experience a delay of 2 to 4 years in the progression to AIDS. Development of a safe chemokine receptor antagonist that delays the onset of AIDS for a long period, if not indefinitely, may be feasible.

We now know that the V3 loop of SU interacts with the chemokine receptor, exposing previously buried SU sequences required for membrane fusion. These transiently

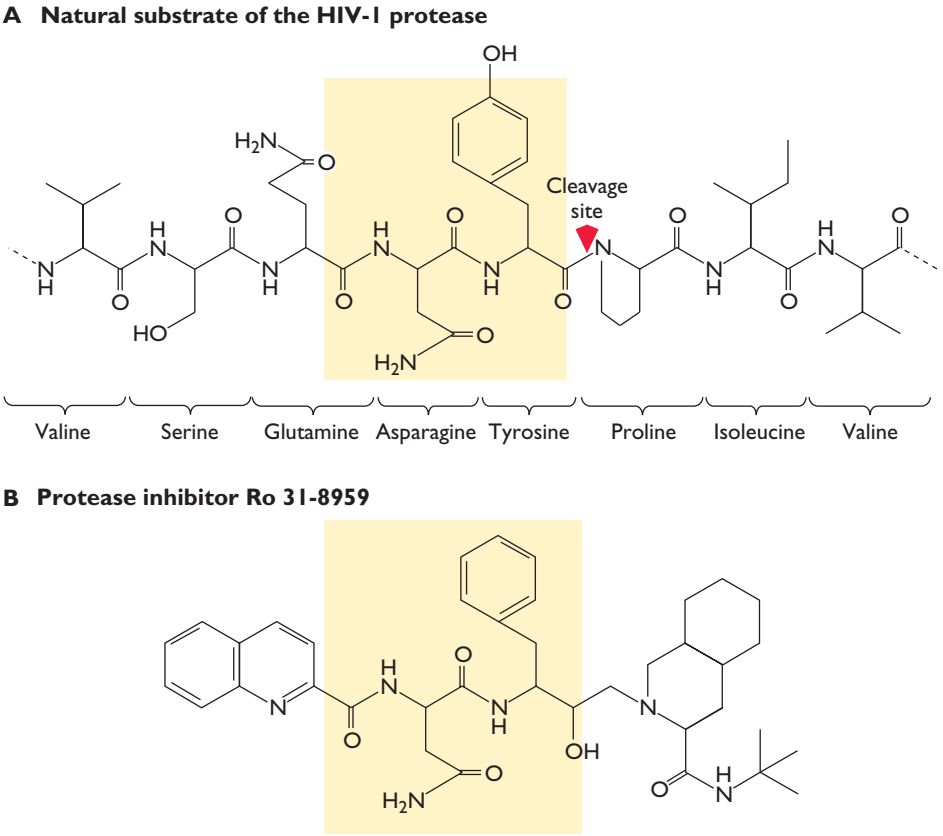


Figure 9.16 Comparison of one natural cleavage site for HIV protease with a peptidomimetic protease inhibitor. (A) The chemical structure of eight amino acids comprising one of the cleavage sites in the Gag-Pol polyprotein. The cleavage site between the tyrosine and proline is indicated by a red arrow. **(B)** The chemical structure of an inhibitory Roche peptide mimic (Ro 31-8959). The dotted box indicates the region of similarity. Adapted from D. R. Harper, *Molecular Virology* (Bios Scientific Publishers Ltd., Oxford, United Kingdom, 1994), with permission.

exposed surfaces are excellent targets for antiviral agents. A 36-amino-acid synthetic peptide, termed T20, derived from the second heptad repeat of SU, binds to the exposed grooves on the surface of a transient triple-stranded coiled-coil and perturbs the transition of SU into the conformation active for fusion. T20 (enfuvirtide) was the first drug approved with this mode of action. enfuvirtide is a peptide and was difficult to develop as a drug. Large-scale synthesis is expensive, and patients must actually prepare the peptide formulation so that they can inject it. Nevertheless, this drug is remarkably effective in reducing HIV titers in the blood.

The Combined Problems of Treating a Persistent Infection and Emergence of Drug Resistance

The Heart of the Problem

The slow pattern of infection exemplified by HIV is characterized by an asymptomatic period that lasts for years to decades after primary infection (see Volume I, Chapters 5 and 6). This asymptomatic phase was initially thought to reflect a quiescent period of no viral replication and unfortunately was called "latency." We now understand that there is extensive viral replication throughout this period and that such relentless replication is the heart of the problem facing effective antiviral therapy.

The Critical Parameters

About 0.1% of an infected individual's $CD4^+$ T cells are replicating HIV at any time during the asymptomatic period, and at least 10% of the total $CD4^+$ population contains viral nucleic acid. The steady-state level of HIV RNA detected in blood depends primarily on the rate of virion production in $CD4^+$ T cells. Ultimately, in the untreated individual, viral RNA concentrations increase, $CD4^+$ T-cell numbers decrease, and death is inevitable. The late stage of infection is characterized by high levels of replication (often more than 10^9 particles produced per day), a high turnover of viral particles (50% turnover in less than a day), and

BOX 9.7 EXPERIMENTS

Highly specific, designed inhibitors may have unpredicted activities

The discovery and development of structure-based inhibitors of HIV protease have been pronounced a triumph of rational drug design. Structural biology and molecular virology came together to provide the protease inhibitors that anchor today's highly active antiretroviral therapy. However, patients receiving protease inhibitors often respond in unexpected ways, and the extent of immunological recovery with treatment is being debated. One study showed that the protease inhibitor ritonavir unexpectedly inhibits the chymotrypsin-like activity of the proteasome. As a result, the protease inhibitor blocks the formation and subsequent presentation of peptides to cytotoxic T lymphocytes by major histocompatiblity complex class I proteins. In another study, the saquinavir protease inhibitors were found to inhibit Zmpste24, a protease involved in conversion of farnesylprenlamin A to lamin A, a structural component of the nuclear lamina.

The challenge is to determine if these secondary activities help or hinder AIDS therapy. As discussed in Chapter 4, CTLs not only kill virus-infected cells, but also are responsible for significant immunopathology in persistent infections. Perhaps ritonavir blocks such immunopathology. On the other hand, reduction in immunosurveillance by cytotoxic T lymphocytes potentiates persistent infections. In this case, the secondary activity of ritonavir may presage long-term problems. We now know that ritonavir and saquinavir inhibit proteasome activity, while indinavir and nelfinavir do not. The inhibition of Zmpste24 by the saquinavir class of compounds may be involved in the debilitating partial lipodystrophy side effect (redistribution of adipose tissue from the face, arms, and legs to the trunk). Genetic data indicate that individuals with missense mutations in *LmnA*, the gene encoding prelaminA and lamin C, have a significant loss of adipose tissues.

Therefore, it will be of some interest to monitor the antiviral effects, lymphocyte functions, and accumulation of prelamin A in patients under treatment with these different protease inhibitors. Tailoring HIV protease inhibitors to limit their action to the intended target is an important goal. As noted by the investigators who found these surprising activities, the human genome carries approximately 400 genes encoding proteases. About 70 of these proteases are targets for new drugs, and the unexpected side effects of antiviral protease inhibitors may be useful in finding new therapies.

Andre, P., M. Groettrup, P. Klenerman, R. DeGiuli, B. L. Booth, Jr., V. Cerundolo, M. Bonneville, F. Jotereau, R. M. Zinkernagel, and V. Lotteau. 1998. An inhibitor of human immunodeficiency virus-1 protease modulates proteasome activity, antigen presentation, and T-cell responses. *Proc. Natl. Acad. Sci. USA* **95:**13120–13124.

Coffinier, C., S. Hudon, E. Farber, S. Chang, C. Hrycyna, S. Young, and L. Fong. 2007. HIV protease inhibitors block the zinc metalloproteinase ZMPSTE24 and lead to an accumulation of prelamin A in cells. *Proc. Natl. Acad. Sci. USA* **104:**13432–13437.

massive loss of $CD4^+$ T cells. The quantity of HIV RNA and the number of $CD4^+$ T cells are therefore the most useful monitors of clinical status and effectiveness of antiviral therapy. More than 500 $CD4^+$ lymphocytes per ml is considered a normal value; fewer than 200 is the definition of AIDS. Viral load is estimated from the number of copies of viral RNA per milliliter of serum or plasma. The lowest level detectable with current methodologies is about 50 copies, and values greater than 10^6 have been recorded.

The First Drug-Resistant Mutants

Viral mutants that grew with impunity in the presence of AZT appeared almost immediately after the drug was approved for general use. The genomes of the mutants harbor single-base-pair changes at one of at least four sites in the reverse transcriptase gene. Reverse transcriptase enzymes bearing these substitutions no longer bind phosphorylated AZT, but they retain enzymatic activity. Mutants resistant to other nucleoside analogs, as well as to protease inhibitors, also arose with disheartening frequency. Resistance to protease inhibitors is found only after acquisition of several distinct amino acid substitutions, but mutants resistant to these drugs have been isolated from patients. Such drug-resistant mutants were transmitted to new hosts and threatened to undermine the entire antiviral effort.

Because HIV replicates extensively during the asymptomatic period, mutations accumulate. On average, every new viral genome can be expected to carry at least one new mutation, and a single patient will harbor many viral genomes in various tissues (Fig. 9.17). If an antiviral drug is given long after the primary infection, selection of drug-resistant mutants is inevitable (Fig. 9.18). Even genomes with detrimental mutations are maintained in the population, if their defects can be complemented. Drug resistance does not have to be absolute, because any increase in fitness, no matter how subtle, can result in large changes in the virus population. The reality is that we do not know the principal factors controlling viral load in an infected patient. Future drug and vaccine trials providing quantitative data on immune responses will go far to solve this problem.

Antiviral Therapy Has the Potential To Promote or Prevent the Emergence of Resistant Viruses

Mutations appear only when the viral genome is replicated. Accordingly, when replication is blocked, no

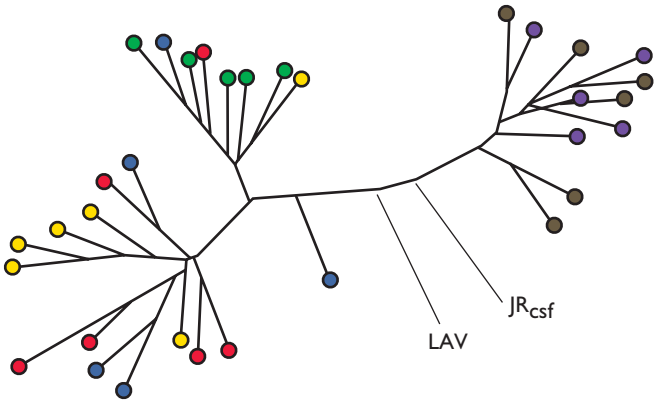


Figure 9.17 HIV-1 evolution within a single patient. An unrooted phylogenetic tree shows relationships among 33 viral variants coexisting within a single infected patient. Two prototype viruses, LAV and JR$_{csf}$, are shown in the center of the tree. Colors denote tissue sources for virus isolation: blue, lymph nodes; red, peripheral blood; yellow, spleen; green, lung; purple, spinal cord; brown, dorsal root ganglion. Adapted from M. Ait-Khaled et al., *AIDS* **9:**675–683, 1995, with permission.

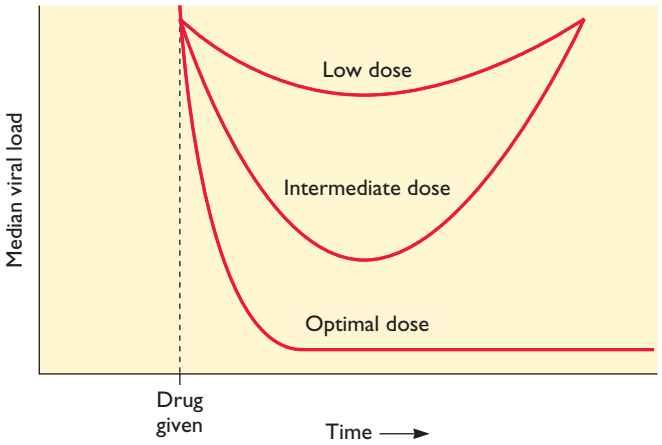


Figure 9.18 Viral load depends on the dose of antiviral drug. If virus replication is allowed in the presence of an antiviral drug, mutants resistant to that drug will be selected. This phenomenon is illustrated by plotting median virus load in relative units on the *y* axis as a function of time after exposure to a drug on the *x* axis as indicated (Drug given). In the top curve (Low dose), the concentration of antiviral drug is insufficient to block virus replication, and the viral load is reduced transiently, if at all. Viruses that replicate may be enriched for resistant mutants. In the middle curve (Intermediate dose), the concentration of antiviral drug appears to be successful in lowering the viral load initially, indicating that some replication was blocked. In this example, replication was not blocked completely, and resistant viruses overwhelmed the patient. In the bottom curve (Optimal dose), the concentration of the antiviral drug is such that all virus replication is blocked. As a consequence, no drug-resistant mutants can arise, and the viral load drops dramatically. Redrawn from J. H. Condra and E. A. Emini, *Sci. Med.* **4:**14–23, 1997, with permission.

drug-resistant mutants can arise. If an individual harboring a small number of viral genomes with no relevant preexisting mutations is given sufficient drug to block all viral replication, the infection will be held in check (Fig. 9.18). In contrast, if the same antiviral drug is given after the viral population has expanded, or if the drug concentration is insufficient to block replication entirely, genomes that harbor mutations will survive and will continue to replicate and evolve. When the viral genome numbers are small, the infection may still be cleared by the host's immune system before resistant mutants take over. If resistance to an antiviral drug requires multiple mutations (e.g., resistance to protease inhibitors), the chance that all mutations preexist in a single genome is much lower than if only a single mutation is required. But if replication is allowed in the presence of the inhibitor, resistant mutants will accumulate.

Cross-Resistance to Similar Inhibitors

HIV mutants resistant to different nucleoside analogs often carry different amino acid substitutions in the reverse transcriptase. Furthermore, a mutation conferring resistance to one inhibitor may suppress resistance to another (Table 9.4). Consequently, combinations of nucleoside analogs were tested with the expectation that double-resistance mutants would be rare, perhaps nonviable, or at least severely crippled. While initially promising, many combinations failed miserably, with mutants resistant to both drugs appearing after less than a year of therapy. Furthermore, the barrier to resistance to many pairwise combinations of nucleoside and nonnucleoside inhibitors was higher than that for any single drug, but not high enough.

Experience with protease substrate analog inhibitors has been similar; resistance to two inhibitors emerges almost as fast as resistance to either one alone. As current protease inhibitors are all peptide mimics that bind to the substrate pocket of the enzyme, a change in residues lining this pocket can affect the binding of more than one inhibitor.

It is clear that treatment of a patient with one antiviral drug at a time is of limited clinical value. Furthermore, the use of a single drug must be contemplated carefully because this same drug may be used for combination therapy in the future.

Combination Therapy

The Use of Two or More Antiviral Drugs To Combat the Resistance Problem

The use of two or more treatments simultaneously is well known in tuberculosis therapy and cancer treatment. Combining two mechanistically different treatments often leads to more effective killing of the bacterium or tumor cells, thereby circumventing the appearance of cells

Table 9.4 Unpredicted drug resistance and susceptibility patterns

Compound	Substitution conferring resistance	Drug sensitivity phenotypes (amino acid substitution)
Zidovudine	T215F in reverse transcriptase	Didanosine resistance (L74V) restores zidovudine susceptibility
		Lamivudine resistance (M184V) restores zidovudine susceptibility
		Nevirapine and loviride resistance (Y181C) restores zidovudine susceptibility
		Foscarnet resistance (W88G) restores zidovudine susceptibility
VX-478	M46I + I47V + I50V in protease	Saquinavir resistance (G48V + I50V + I84L); restores VX-478 susceptibility
		Indinavir resistance (V32I, A71V); restores VX-479 resistance
Delavirdine	P236L in reverse transcriptase	Increased susceptibility to nevirapine; R82913 (TIBO); and L-697,661 (pyridinone)
Foscarnet	E89K + L92I + S56A + Q161L, H208Y in reverse transcriptase	Increased susceptibility to zidovudine, nevirapine, and R82150 (TIBO)

resistant to one treatment or the other. This principle also applies to antiviral therapy. In theory, if resistance to one drug occurs once in every 10^3 genomes, and resistance to a second occurs once in every 10^4, then the likelihood that a genome carrying both mutations will arise is the product of the two probabilities, or one in every 10^7.

Problems and Promise

Combination therapy does not always clear an infection. One obvious reason for resurgence of virion production is the appearance of drug-resistant mutants if therapy is given late in the progression of disease, or if the combination is not sufficiently potent. Combination therapy can be demanding for physician and patient. For example, if other infections are being treated, as they almost always are in AIDS patients, then many pills a day may be required. Other problems arise because storing and keeping track of different medications are daunting tasks for an ill patient. To compound the problems, every drug has side effects, and some are severe. For example, the gastrointestinal problems that accompany many protease inhibitors are particularly stressful for patients. Some side effects, such as the wasting of limbs and face with fat accumulation in the gut (lipodystrophy), may appear only after months of continuous use of current antiprotease drugs (Box 9.7). Because of these problems, some patients simply do not take their medication. The most insidious failure lies in wait when the patient begins to feel better and stops taking the pills. Viral replication resumes when the inhibitors are removed. Replication means mutation, and in such cases combination therapy may be ineffective if ever reinstated. Finally, combination therapy is very expensive, currently costing thousands of dollars per year for some regimens. In addition to the costs of medication, tests for viral load and CD4 counts must be performed regularly to monitor therapy. Such tests also are costly. Clearly, combination therapy is not accessible to everyone.

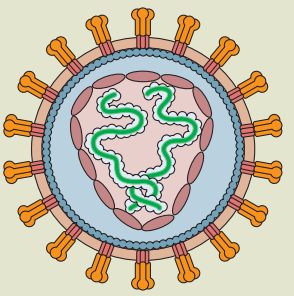
Despite these seemingly formidable issues, combination therapy has been effective, particularly for long-term control of infection. Many think that the clinical success of combination therapy is truly remarkable and represents one of the high points in the battle against AIDS (Box 9.8).

BOX 9.8 DISCUSSION

The first triple-drug combination, once-a-day pill

Twelve years ago, it was common for an HIV infected individual to take more than 20 pills a day to treat not only his or her HIV infection, but also the variety of other infections and complications inherent in AIDS. As more potent inhibitors were discovered, patients could take fewer pills to control their infection. More importantly, combination therapy developed as a therapeutic concept to deal with drug-resistant viruses. In 2006, the first triple drug, once-a-day pill for HIV therapy was approved. Atripla consists of three active antiviral drugs: a nucleotide reverse transcriptase inhibitor (tenofovir), a nucleoside reverse transcriptase inhibitor (emtricitabine), and a nonnucleotide reverse transcriptase inhibitor (efavirenz). It took over 20 years to develop this combination pill and required the cooperation of two pharmaceutical companies. This achievement is a landmark in the battle against HIV.

De Clercq, E. 2006. From adefovir to Atripla™ via tenofovir, Viread™ and Truvada™. *Fut. Virol.* **1**:709–715.



Strategic Treatment Interruption

The underlying premise of strategic treatment interruption is that combination drug treatment will stop replication and, as a consequence, the immune system will recover. The hope is that cycles of drug therapy, bolstered by drug-free periods, will enable the patient's own immune defenses to clear the infection. There are many reasons why such a hypothesis is attractive. A primary one is that "drug holidays" can be very attractive to patients. Initial studies with monkeys were encouraging when drug therapy was given very early in the acute infection. At these early times, the population of viral genomes in infected individuals is probably not as diverse, because it has not been subjected to many rounds of selection by the immune system. Consequently, replication could be held in check by the recovering immune system. However, studies with infected humans have not been as promising. Most AIDS patients have been infected for years, and the genetic diversity of viral genomes in their bodies is enormous. Indeed, the emergence of drug-resistant mutants, the small number of antiviral drugs available to treat resistant infections, and the appearance of cytotoxic T-lymphocyte and antibody escape mutants present serious problems for widespread use of strategic treatment interruption.

Challenges and Lessons Learned

Of utmost importance is that drugs must be potent. Such potency, which must be achieved to avoid selection of resistant mutants, can be accomplished only with combinations of drugs. Potency must be attained despite unpredictable patient adherence, individual differences in drug metabolism, and inevitable toxicity. A potent drug is of no use if the patient will not take it because the pills are too large, or too numerous or cause side effects.

At the moment, drug therapy is our only proven weapon. Therefore, it is prudent to avoid therapies in which resistance can be conferred by a single mutation in the viral genome. As a corollary, therapies that require multiple mutations for drug resistance to emerge should be used. Combinations of drugs will be most effective if the patient has not been treated previously with any drug.

With aggressive use of potent antiviral drugs, HIV replication can be suppressed, but the infection **cannot** be cured. Even when viral RNA has been undetectable for years during drug therapy, as soon as the drug is removed, replication begins again. We simply have no way to eliminate every last viral genome from the body of an infected individual. As years go by during this insidious, slow infection, the diversity of viral genomes increases as a result of mutation, recombination, and selection by drugs and an active immune system. While we understand in principle how this diversity arises, we have little insight about how to eliminate it.

The hope of any therapy is that as the cells of the immune system die and are replaced, proviral copies will be reduced to a point at which the infection cannot be sustained. Even if a cure is wishful thinking, combination therapies, where available, have converted HIV infection to a chronic treatable disease rather than a death sentence.

Perspectives

The world's surprisingly small arsenal of antiviral drugs is directed against a minor subset of viral diseases, notably those caused by HIV and the herpesviruses. Few drugs in the arsenal are effective against some of the most deadly viral diseases, which are caused by RNA viruses and poxviruses. There are many reasons for this state of affairs. One formidable problem is that we are unable to diagnose acute viral infections accurately and within sufficient time for effective intervention with antiviral drugs. Another arises from economic considerations. Many debilitating viral infections affect people in the developing world, a population that lacks the means to pay as well as the infrastructure to deliver therapy.

Persistent infections such as those caused by HIV, herpes simplex virus, the hepatitis viruses, and the papillomaviruses present a different set of challenges. At present, these infections are controlled by drugs, but not cured. Patients often must take the drug, or more likely a combination of drugs, for the rest of their lives, a difficult and expensive proposition. New approaches have been undertaken, and many promising lead compounds and therapies for treatment, and even cure, of persistent infections are being investigated. For example, it may be possible to reduce viral load by antiviral drugs and then promote clearance of the remaining infection by treating with drugs that bolster immune responses.

Regardless of what comes out of the antiviral drug pipeline, selection of resistant mutants is inevitable. This certainty is a specter that continues to haunt antiviral research and public health. As the search for effective therapies to treat HIV infection demonstrates, we still have much to learn.

Fortunately for our species, antiviral drugs and vaccines are not the only means to prevent viral infections. Effective public health measures, proper nutrition, and simple personal hygiene remain fundamental contributors to the prevention of viral infections. Indeed, in some underdeveloped countries, these nontechnical solutions are the most effective and often the only defense against viral diseases. The principles underlying the high- and low-technology approaches are remarkably similar: the sources and avenues of viral spread must be eliminated. Availability of clean drinking water, adequate sewage disposal, insect control, good medical practice, and an uncontaminated blood

supply can be of paramount importance in reducing the incidence of viral infection. Simple personal actions such as washing hands, seeking protection from insects and rodents, and using condoms can be remarkably effective, low-cost preventative measures. Because malnourished individuals have reduced innate and acquired immune defenses, proper nutrition can be a major though unappreciated defense.

Unfortunately, even these fundamental practices and policies cannot be put in place without basic infrastructure and social policies that promote public awareness of infectious disease, understanding of potential hazards and risks, appreciation of the importance of early detection, and reporting of the incidence of infections.

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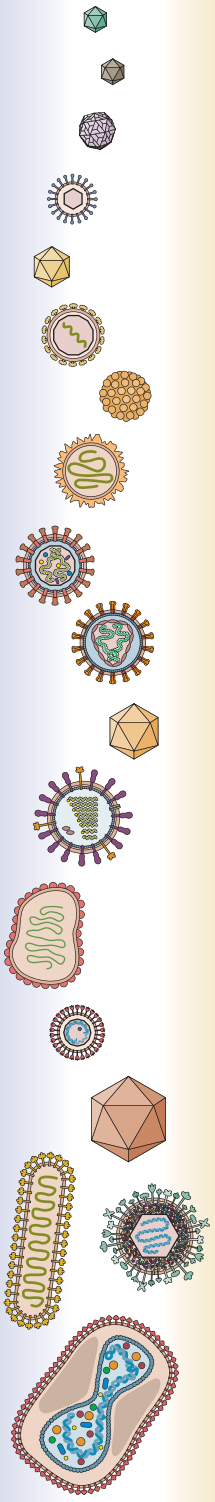


10

Evolution and Emergence

Anything produced by evolution is bound to be a bit of a mess.
SYDNEY BRENNER

Around here, it takes all the running you can do just to stay in the same place.
LEWIS CARROLL
The Red Queen to Alice in *Alice In Wonderland*

Virus Evolution

The word "evolution" conjures up images of fossils, dusty rocks, and ancestral phylogenetic trees covering eons. Modern virology shatters this staid image and reminds us that evolution not only is contemporary (we are made aware of it every day), but also carries profound implications for the future. Indeed, as host populations grow and adapt, virus populations that can infect them are selected. It also works the other way: viral infections can be significant selective forces in the evolution of host populations.

The public is made aware of virus evolution by frequent, often sensationalized reports in the press of new viral diseases such as severe acute respiratory syndrome (SARS), killer viruses like Ebola and Marburg, and real pandemics like the acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus. In addition to these more spectacular events, everyone experiences regular bouts with influenza and common cold viruses. Scientists are challenged regularly to devise new antiviral drugs, vaccines, diagnostics, and treatments for these emerging diseases. In truth, we are only beginning to develop an understanding of the selective forces that drive virus evolution, the emergence of new and old infections, and the corresponding implications for our own survival.

The Classic Theory of Host-Parasite Interactions

To put the process of evolution of viruses in perspective, it is important to have a basic understanding of host-parasite interactions. A fundamental principle of virus biology and evolution is that viral genomes must spread from host to host to maintain the viral population. An infection will spread if each infected individual infects, on average, more than one new individual before dying or clearing the infection. The infection rate is related to the population size: infections can spread only if population density exceeds a minimal value.

In 1983, these concepts were incorporated into a comprehensive theory of host-parasite interactions that is well known in ecological circles, but not

always appreciated among molecular virologists. Briefly, this work suggests that spread of viral infection can be described in quantitative terms such as transmission rate, host survival/immunity, and virulence. The original work assumed well-mixed, homogeneous host populations in which each individual has the same probability of becoming infected. This theory states that selection maximizes R_0, the average number of secondary infections resulting from one infected host in an otherwise uninfected population. If R_0 is less than 1, it is impossible to sustain an epidemic. In fact, if R_0 is less than 1, it may be possible to eradicate the disease. If R_0 is slightly greater than 1, an epidemic is possible, but random fluctuations in early stages of infection in a susceptible population can lead to extinction or explosion of the infection. If R_0 is high, an epidemic is almost certain. A large R_0 value is typical of a disease that features "super-spreaders" (e.g., the individual who transmitted SARS coronavirus so successfully to others in the Hong Kong Hotel Metropole). A low R_0 value is typical of an infection in which the exposure time is reduced, the yield of infectious virus is low, or the duration of replication is short. In the standard model, the infection rate is predicted to be proportional to population size and virulence is related to transmissibility.

The original host-parasite theory remains robust, but additional terms and constraints must be added to the classic equations as we learn more about viral infections in the wild (Box 10.1, see also Fig. 5.2). For example, disease dynamics are oscillatory, but are not simple predator-prey cycles (e.g., foxes and rabbits). Actions of the immune system affect the appearance of immune-resistant viral mutants with differences in virulence and transmissibility. These events all are manifestations of complex variables that are difficult to evaluate. Not only are viral populations more complicated than first thought, but also the structure of host populations affects the evolution of viral agents. For example, as humans alter ecosystems and expand in numbers, viruses and hosts are mixing to an extent never before experienced. As a result, more viral diseases are appearing, precisely as predicted. It is possible to understand the basics of how these changes occur from first principles, as we discuss below.

How Do Viral Populations Evolve?

The harsh realities presented by a large, genetically variable host population dispersed in ever-changing environments appear to present insurmountable barriers to the survival of inanimate, submicroscopic, obligate intracellular pathogens. Obviously, such thinking must be flawed, as viruses abound everywhere. The primary reason for the remarkable success of viruses is that despite a minimal set of genes, viral populations display spectacular diversity. It is such diversity, manifested in the large collection of genomic permutations that are present in viral populations at any given time, that provides constant opportunities for survival. The sources of this diversity are **mutation, recombination and reassortment,** and **selection.** The constant change of a viral population in the face of selective pressures is the definition of **virus evolution**.

Positive and negative selection for particular preexisting mutants can occur at any step in a viral life cycle. The selective forces acting on viral populations are imposed not only by the environment, but also by the limitations of the information encoded in the viral genome. The requirement to spread within an infected host, as well as among individuals in the population, exposes virions to a variety of host antiviral defenses. The population density, the social behavior, and the health of potential hosts represent but a few of the powerful selective forces determining the survival of viral populations.

BOX 10.1 BACKGROUND

Virulence, selection, and viral evolution

Is virulence a positive or negative trait for selection? One idea is that increased virulence reduces transmissibility (hosts die faster, reducing exposure to uninfected hosts). Debilitating disease may actually reduce transmission because the infected individual may not interact with other susceptible hosts.

If everything were simple, one might expect that all viruses would evolve to be maximally infectious and completely avirulent. A different view appears when real-life infections are studied. Persistent infections often lie dormant for years with respect to symptoms and then kill the host at the end stage of disease. Virulent viruses for one species may be maintained as asymptomatic infections in another. The interplay of contextual terms such as severity of disease and transmissibility is complicated. In fact, for some diseases, a strong case may be made that increased virulence actually increases R_0 and is strongly selected for in natural viral infections.

Boots, M., and M. Mealor. 2007. Local interactions select for lower pathogen infectivity. *Science* **315:**1284–1286.

Weiss, R. 2002. Virulence and pathogenesis. *Trends Microbiol.* **10:**314–317.

We define virus evolution in terms of a population, **not** an individual viral particle. Viral populations comprise diverse arrays of mutants that are produced in prodigious quantities. In most infections, thousands of progeny are produced after a single cycle of replication in one cell, and when copying is error prone, almost every new genome can differ from every other. Accordingly, it is confusing, and even misleading, to think of an individual particle as representing an average virion for that population. Virologists are population biologists whether they know it or not. As Stephen Jay Gould put it, the median is **not** the message when it comes to evolution. The diversity of the population provides surprising avenues for survival, yet every individual is a potential winner. Occasionally, even the most rare genotype in a current population may be the most common after a single selective event.

It is important to make a distinction between viral strategies that produce large numbers of progeny (high reproductive output or ***r*-replication strategies**) and those that result in a lower reproductive output but better competition for resources (***K*-replication strategies**) (see Chapter 3). The former are characterized by short reproductive cycles with production of many progeny and are effective when resources are in short supply. The *K*-replication strategy produces persistent or latent infections with little pathogenesis. Fewer progeny are produced, but they have a high probability of surviving. Viruses that replicated via *K*-replication strategies survive as long their hosts survive.

The notations *r* and *K* come from the following equation:

$$dN/dt = rN(1 - N/K)$$

where *r* is the growth rate, *N* is the population size, and *K* is the carrying capacity in the current environment (see also Fig. 5.2).

The trajectory of evolution has long been a subject of debate. Scientists and philosophers have considered many questions such as the following. Is there a predictable direction for evolution? If so, what is the path? Are there really evolutionary dead ends? Virology provides a productive area for research into these questions. A primary lesson is that we must avoid judging outcomes as "good or bad." While making such judgments is a common human activity, anthropomorphic assessments and the comfort of analogies must be avoided, particularly when virus evolution is considered. Furthermore, from the first principle that there is no goal but survival, we can deduce that evolution does not move a viral genome from "simple" to "complex," or along some trajectory aimed at "perfection." Rather, change is effected by elimination of the ill adapted of the moment, not by the prospect of building something better for the future.

Virus-Infected Cells Produce Large Numbers of Progeny

The *r*-replication strategy (high reproductive output) is common among viruses. A single cell infected with poliovirus yields about 10,000 virus particles, and, in theory, three or four cycles of replication at this rate could produce enough virions to infect every cell in a human. Such overreplication does not happen for a variety of reasons, including a vigorous host defense and tropism for certain tissues. Nevertheless, high rates of replication over short periods and the resulting accumulation of large quantities of infectious particles are hallmarks of many common acute and persistent viral infections. This feature is illustrated in Table 10.1 for human immunodeficiency virus and hepatitis B virus. These high rates of particle production can continue for years. In the case of human immunodeficiency virus, the time from release of an infectious virion to infection and lysis of another target cell is estimated to be 2.6 days during the later stages of infection. What is more striking is the 50 to 90% turnover rate (replication minus elimination by host defenses) of this virus in plasma. Such measurements reveal the prodigious, relentless production of new virus particles in the face of a vigorous host

Table 10.1 *In vivo* dynamics of human immunodeficiency virus and hepatitis B virus

Characteristic	Value in:	
	Hepatities B virus	Human immunodeficiency virus
Virus in plasma		
Half-life	24 h	6 h
Daily turnover	50%	90%
Total production in blood	$>10^{11}$	$>10^{9}$
Virus in infected cell		
Half-life	10–100 days	2 days
Daily turnover	1–7%	30%

defense. The interface of host defense and virus replication is fertile ground for selection and evolution.

Large Numbers of Mutants Arise When Viral Genomes Replicate

Two simple principles are that evolution is possible only when mutants arise in a population, and that mutations are introduced during copying of any nucleic acid molecule. When viral genomes replicate, mutations invariably accumulate in their progeny (Box 10.2).

RNA virus evolution. Most viral RNA genomes are replicated with considerably less fidelity than those comprising DNA (see Volume 1, Chapters 6 and 7). The average error frequencies reported for copying of RNA genomes are about one misincorporation in 10^4 or 10^5 nucleotides polymerized, more than 10^6 times greater than the rate for a host genome. Given a typical RNA viral genome of 10 kb, a mutation frequency of 1 in 10^4 corresponds to an average of 1 mutation in every replicated genome. Not all viral RNA genomes have the same mutation rate: there is some evidence that the replication machinery of viruses with larger RNA genomes (e.g., the 30,000-base human SARS coronavirus genome) may operate with higher replication fidelity than the polymerase complexes of smaller RNA viral genomes.

DNA Virus Evolution

The error rate of viral DNA replication is estimated to be about 300-fold lower than that for most RNA genomes described above. Many RNA polymerases lack error-correcting mechanisms, while most DNA polymerases can excise and replace misincorporated nucleotides (Volume 1, Chapter 9). Experimental data indicate that replication of small single-stranded DNA virus genomes (e.g., *Parvoviridae* and *Circoviridae*) is more error prone than is replication of the double-stranded DNA genomes of larger viruses. The best evidence for this conclusion comes from studies of canine parvovirus, first observed to cause disease in dogs in 1978, that has become a ubiquitous pathogen worldwide. When mutation rates were estimated by sequencing multiple isolates of canine parvovirus over the time of the pandemic, the rate of nucleotide substitution was closer to that of RNA viruses than to that of double-stranded DNA viruses. The mechanism for this increased misincorporation of nucleotides remains to be elucidated.

The Quasispecies Concept

A 1978 paper described a detailed analysis of an RNA bacteriophage population (phage Qβ). The authors made a startling conclusion:

> A Qβ phage population is in a dynamic equilibrium with viral mutants arising at a high rate on the one hand, and being strongly selected against on the other. The genome of Qβ cannot be described as a defined unique structure, but rather as a weighted average of a large number of different individual sequences.
>
> E. Domingo, D. Sabo, T. Taniguchi, and C. Weissmann, *Cell* **13:**735–744, 1978

This conclusion has been validated for many virus populations. Indeed, we now understand that virus populations

BOX 10.2 DISCUSSION

Error rates are difficult to quantify

Estimates of mutation rates must be viewed with caution. Absolute error rates (measured as the number of misincorporations per nucleotide polymerized) for any nucleic acid polymerase are difficult, if not impossible, to determine. A variety of technical issues must be considered, including sampling problems and potential artifacts of experimental design. Estimates of error rate can vary substantially, depending on the experimental method by which they are assessed. For example, PCR technology is commonly used to sample viral genomes, but the polymerase used may itself introduce copying errors that must be factored into the analysis.

Another popular method makes use of reporter genes (e.g., the *lacZ* gene, which encodes β-galactosidase). These genes can be inserted into a viral genome so that errors in the reporter gene can be scored by inspection or analysis of virus plaques. The error rate for the viral genome is then extrapolated from that determined for the reporter gene. While this method is relatively simple, it can yield misleading data, because errors of incorporation are not uniformly distributed as each genome is copied and are often dependent upon the particular sequence analyzed. For example, the reverse transcriptase from human immunodeficiency virus is inaccurate when measured *in vitro*, with an average error rate per nucleotide incorporated of 1 in 1,700. However, certain positions in this genome can be hot spots for mutation at which the error rate can be as high as 1 per 70 polymerized nucleotides.

Drake, J. 1992. Mutation rates. *Bioessays* **14:**137–140.

Drake, J. 1991. A constant rate of spontaneous mutation in DNA-based microbes. *Proc. Natl. Acad. Sci. USA* **88:**7160–7164.

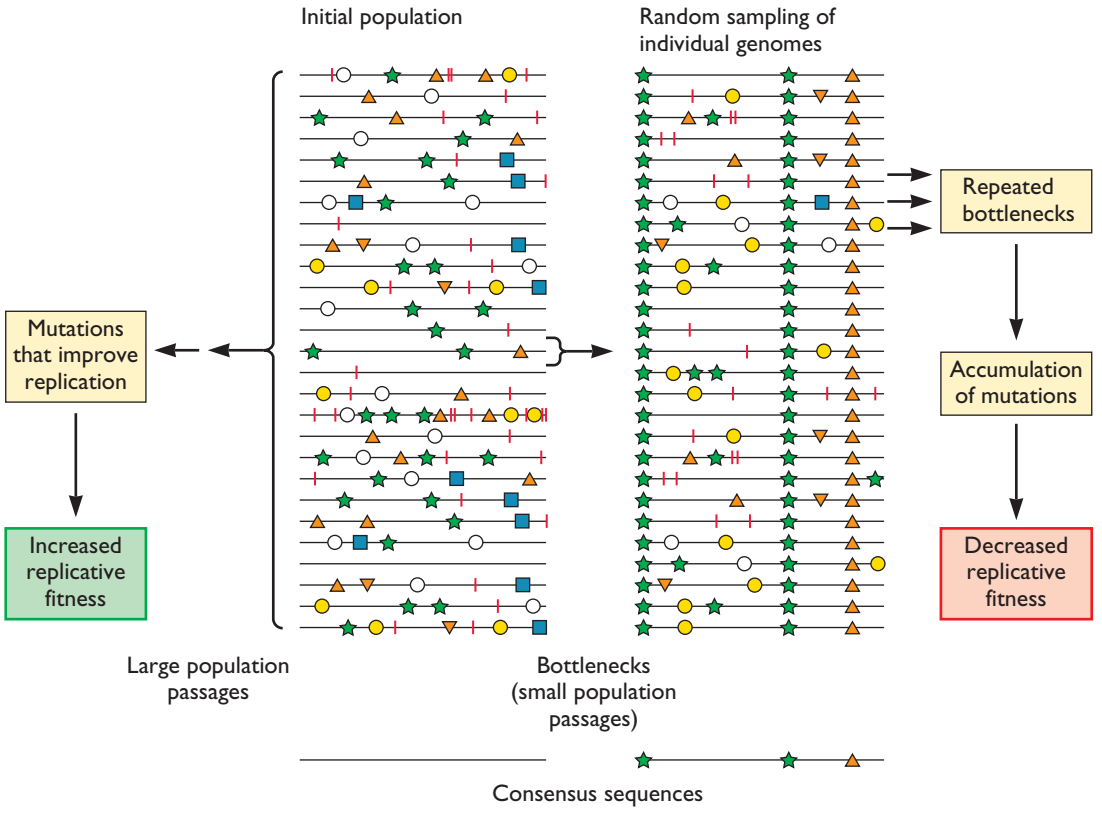
Drake, J. 1993. Rates of spontaneous mutation among RNA viruses. *Proc. Natl. Acad. Sci. USA* **90:** 4171–4175.

Hwang, Y. T., and C. B. C. Hwang. 2003. Exonuclease-deficient polymerase mutant of herpes simplex virus type 1 induces altered spectra of mutations. *J. Virol.* **77:**2946–2955.

exist as dynamic distributions of nonidentical but related replicons, often called **quasispecies**, a concept developed by Manfred Eigen. The classical definition of a species (an interbreeding population of individuals) has little meaning when considering viruses. For example, most viral infections are initiated not by a single virion, but rather by a population of particles. The large number of progeny produced after such infections are complex products of intense selective forces that operate **inside an infected host.** The relatively few virions that successfully infect another host have been subjected to an entirely new set of **external** selective forces. Therefore, a steady-state, equilibrium population of a given viral quasispecies must comprise vast numbers of particles. Such an equilibrium cannot be attained in the small populations typically found after isolated infections in nature or in the laboratory. Consequently, when small populations of virus particles replicate, extreme fluctuations in genotype and phenotype are possible (Fig. 10.1).

For a given RNA virus population, the genome sequences cluster around a consensus or average sequence, but virtually every genome can be different from every other. A rare genome with a particular mutation may survive a selection event, and this mutation will be found in all progeny genomes. However, the linked but unselected mutations in that genome get a free ride. Consequently, the product of

Figure 10.1 Viral quasispecies, population size, bottlenecks, and fitness. Genomes are indicated by the horizontal lines. Mutations are indicated by different symbols. When an RNA virus replicates, mutations accumulate in the progeny genomes. A population of genomes, each member containing a characteristic set of mutations, is shown in the center. The consensus sequence for this population is shown as a single line at the bottom. Note that there are **no** mutations in the consensus sequence, despite their presence in every genome in the population (every genome is different). A population of genomes that emerges after passage of one genome through one bottleneck is depicted on the right. The consensus sequence for this population is shown as a single line at the bottom. Note that in this example, three mutations selected to survive the bottleneck are found in every member of the population, and these appear in the consensus sequence. If the large population is propagated without passage through bottlenecks (situation on the left), repeated passage enriches for mutant genomes that **improve replication and increase the fitness** of the population. If the population continues to propagate through serial bottlenecks, mutations accumulate that result in **reduced fitness**. Adapted from E. Domingo et al., p. 144, *in* E. Domingo, R. Webster, and J. Holland (ed.), *Origin and Evolution of Viruses* (Academic Press, Inc., San Diego, CA, 1999), with permission.



selection after replication is a new, diverse population that shares only the selected mutation. The ramifications of this phenomenon in virus evolution and pathogenesis are only now being appreciated.

Quasispecies theory predicts that viral quasispecies are not simply a collection of diverse mutants, but rather a group of interactive variants that characterize the particular population. Diversity of the population, therefore, is critical for survival. It has been possible to test the idea that viral populations, **not** individual mutants, are the target of selection by limiting diversity. Previously, we made the assertion that creation of diversity is the primary reason for virus survival. This conclusion follows in part from the identification of certain polymerase substitutions that reduce the frequency of incorporation errors during growth in cultured cells. Such mutants are called **antimutators**. Certain spontaneous mutants of human immunodeficiency virus that are resistant to the reverse transcriptase inhibitor lamivudine exhibit a 3.2-fold reduction in error frequency. The seemingly modest increase in fidelity was associated with a significant growth disadvantage in infected individuals. Poliovirus replication is notoriously error prone, producing a remarkably diverse population. Certain ribavirin-resistant poliovirus mutants have increased fidelity of about sixfold (~0.3 mutation per genome compared to ~2 mutations per genome for the parental virus). Importantly, the mutant was much less pathogenic in animals than was the wild-type virus: reduced diversity led to attenuation and loss of neurotropism. Further studies showed that in a diverse quasispecies, isolated viral mutants complemented each other, providing proof that it is the population, not the individual, that is evolving. As the wild-type viruses have maintained high mutation rates, we can infer that lower rates are neither advantageous nor selected in nature.

Sequence Conservation in Changing Genomes

Not all is in flux during viral genome replication. Despite high mutation rates, the so-called *cis*-acting sequences of RNA viruses change very little during propagation. These sequences are required for genome replication, messenger RNA (mRNA) synthesis, and genome packaging. They are often the binding sites for one or more viral or cellular proteins. Any genome with mutations in such sequences, or in the gene that encodes the corresponding binding protein, is likely to be less fit, or may not replicate at all. Changes must occur in both partners for restoration of function. The tight, functional coupling of binding protein and target sequence may be a marked constraint for evolution. In some instances, these sequences are stable enough to represent lineage markers for molecular phylogeny.

The Error Threshold, Lethal Mutagenesis, and Extinction

The capacity to sustain prodigious numbers of mutations is a powerful advantage. Yet, at some point, selection and survival must balance genetic fidelity and mutation rate. Many mutations are detrimental, and if the mutation rate is high, accumulating mutations can lead to a phenomenon called **lethal mutagenesis** when the population is driven to extinction. Intuitively, mutation rates higher than one error in 1,000 nucleotides incorporated must begin to challenge the very existence of the viral genome, as precious genetic information can be irreversibly lost. The **error threshold** is a mathematical parameter that measures the complexity of the information that must be maintained to ensure survival of the population. RNA viruses tend to evolve close to their error threshold (Box 10.3), while DNA viruses have evolved to exist far below it. We can infer these remarkable properties from experiments with mutagens. After treatment of cells infected by an RNA virus (such as vesicular stomatitis virus or poliovirus) with a base analog such as 5-azacytidine, virus titers drop dramatically. The error frequency per surviving genome increases only two- to threefold at best. In contrast, a similar experiment performed with a DNA virus, such as herpes simplex virus or simian virus 40, reveals an increase in single-site mutations of several orders of magnitude among survivors.

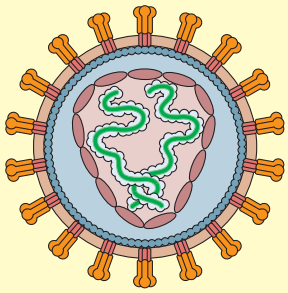
The error threshold concept is more complicated than it might first appear. In fact, one line of argument indicates that theory predicting error catastrophe, while mathematically rigorous, cannot represent a viral infection realistically. If this is so, other explanations for the antiviral behavior of mutagens must be considered. In addition, many important biological parameters contribute to virus survival, including a complex property called **fitness**, the replicative adaptability of an organism to its environment. Fitness depends on the context of the experiment and what outcome is measured. In the laboratory setting, fitness may be measured by comparison of growth rates or virus yields. Fitness is far more difficult to measure under more natural conditions, such as infection of complex organisms that live in large, interacting populations. Another essential component, equally difficult to measure, is the stability or predictability of the environment as it affects propagation of a virus. Host population dynamics and seasonal variation are but two examples of the many complicated environmental variables that exist. Finally, given the diversity in any viral population, determining the fitness of one population versus that of another depends on the mathematics of population genetics, a subject beyond the scope of this text.

BOX 10.3 EXPERIMENTS

Lethal mutagenesis: pushing human immunodeficiency virus over the "error threshold"?

At the end stage of AIDS, individuals infected with human immunodeficiency virus produce more than 1010 particles per day, and, on average, each genome contains one mutation. Diverse viral populations accumulate in the various tissues of every infected individual. Most of the virions appear to be nonviable, suggesting that the population exists at its error threshold and cannot tolerate many additional mutations. What would happen if one could intentionally push replicating genomes over the error threshold with a mutagenic nucleoside analog?

In 1990, scientists first reported that it is difficult to increase the mutation rate for two RNA viruses by using mutagens. Nine years later, another group selected antiviral nucleoside analogs that lacked toxicity for human cells, but were incorporated into human immunodeficiency virus DNA by the viral reverse transcriptase. These are not chain-terminating compounds, but rather they promote base misincorporations when copied. One compound, 5-hydroxydeoxycytidine, was promising



in that it was not toxic to human cells, but was efficiently incorporated into replicating viral genomes.

The experiments comprised serial passage of virions in human cells in the presence of the nucleoside analog. In seven of nine experiments, there was a precipitous loss of viral replication after 9 to 24 serial passages in the presence of the compound. Sequence analysis showed that the progeny genomes accumulated G-to-A substitutions. Loss of viral replication was not observed in 28 control infections in which virions were serially passaged without the analog.

Similar results have been obtained after infection of cultured cells infected with a variety of RNA viruses.

Bull, J., R. Sanjuan, and C. Wilke. 2007. Theory of lethal mutagenesis for viruses. *J. Virol.* **81:**2930–2939.

Crotty, S., C. E. Cameron, and R. Andino. 2001. RNA virus error catastrophe: direct molecular test by using ribavirin. *Proc. Natl. Acad. Sci. USA* **98:**6895–6900.

Eigen, M. 2002. Error catastrophe and antiviral strategy. *Proc. Natl. Acad. Sci. USA* **99:**13374–13376.

Holland, J. J., E. Domingo, J. C. de la Torre, and D. A. Steinhauer. 1990. Mutation frequencies at defined single codon sites in vesicular stomatitis virus and poliovirus can be increased only slightly by chemical mutagenesis. *J. Virol.* **64:**3960–3962.

Loeb, L. A., J. M. Essigmann, F. Kazazi, J. Zhang, K. D. Rose, and J. I. Mullins. 1999. Lethal mutagenesis of HIV with mutagenic nucleoside analogs. *Proc. Natl. Acad. Sci. USA* **96:** 1492–1497.

Pariente, N., S. Sierra, P. R. Lowenstein, and E. Domingo. 2001. Efficient virus extinction by combinations of a mutagen and antiviral inhibitors. *J. Virol.* **75:**9723–9730.

Summers, J., and S. Litwin. 2006. Examining the theory of error catastrophe. *J. Virol.* **81:**20–26.

Genetic Shift and Drift

In Chapter 5, we discussed the process of antigenic variation and its contribution to modulating the immune response. Selection of mutants resistant to elimination by antibodies or cytotoxic T lymphocytes is inevitable when sufficient virus replication occurs in an immunocompetent individual. The terms **genetic drift** and **genetic shift** describe distinct mechanisms of diversity production. Diversity arising from copying errors and immune selection (drift) is contrasted with diversity arising after recombination or reassortment of genomes and genome segments (shift). Drift is possible every time a genome replicates. Shift can occur only under certain circumstances and is relatively rare. The episodic pandemics of influenza (Appendix B) provided strong evidence for this conclusion. For example, there are only six established instances of genetic shift for the influenza virus hemagglutinin gene since 1889. The combination of rapid drift and slow shift contributes to yet more diversity in populations (Boxes 10.4 and 10.5). When retrovirus infection results in integration of multiple proviral genomes in a single cell, genetic shift may be a frequent event, with ramifications that are only now being appreciated.

Genetic Bottlenecks

Unlike lethal mutagenesis, which is deterministic and can cause extinction of large populations, the **genetic bottleneck** represents extreme selective pressure on small populations that results in loss of diversity, accumulation of nonselected mutations, or both (Fig. 10.1). A simple experiment illustrating this principle is easily done in the laboratory. A single RNA virus plaque formed on a monolayer of cultured cells is picked and expanded. Next, a single plaque is picked from the expanded stock, and the process is repeated many times. The bottleneck is the consequence of restricting further viral replication to the progeny found in a single plaque that contains a few thousand virions derived from a single infected cell. The perhaps surprising result is that after about 20 or 30 cycles of single-plaque amplification, many virus populations are barely able to propagate. They are markedly less fit than the original population (Table 10.2). The

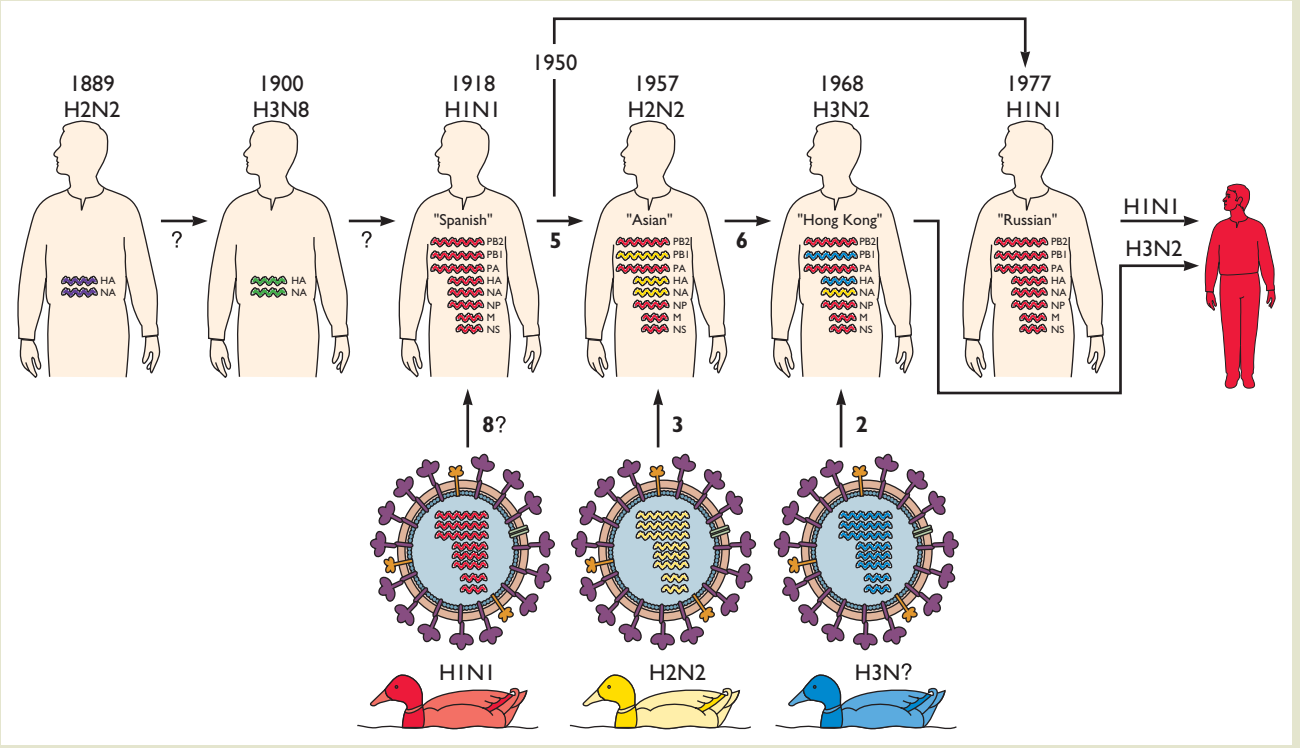
BOX 10.4 DISCUSSION

Postulated evolution of human influenza A viruses from 1889 to 1977

The major influenza pandemics are characterized by viral reassortants. The emerging reassortant carries H and N genes that had not been in circulation in humans for some time, and consequently immunity is low or nonexistent. Six times since 1889, an influenza virus H subtype that had not been seen for years entered the human population. Three influenza virus H subtypes display a cyclic appearance, with the sequential introduction of H2 in 1889, H3 in 1900, H1 in 1918, H2 again in 1957, H3 again in 1968, and H1 again in 1977. With each H subtype introduction, the world experienced an influenza pandemic characterized by a new combination of H and N.

The figure depicts the appearance and transmission of distinct serotypes of influenza A virus in humans. In 1889 and 1900, only the H and N serology can be deduced from historical data. The bottom part shows the nature of the avian influenza viruses that reassort with human viruses. The color of the genome segments represents a particular viral genotype. Segments of the predominant influenza virus genome and its gene products are indicated in each human silhouette for each year. The number next to the arrow indicates how many segments of the viral genome are known to have been transmitted.

Adapted from R. G. Webster and Y. Kawaoka, *Semin. Virol.* **5**:103–111, 1994, with permission.



environment is constant, and the only apparent selection is that imposed by the ability of the population of viruses from a single plaque to replicate. Why does fitness plummet?

The answer lies in a phenomenon dubbed **Muller's ratchet**: small, asexual populations decline in fitness over time if the mutation rate is high. The genomes of replicating RNA viruses accumulate many mutations. As noted previously, they survive close to their error threshold. By restricting population growth to serial single founders (the bottleneck) under otherwise nonselective conditions, so many mutations accumulate that fitness decreases.

The ratchet metaphor should be clear: a ratchet on a gear allows the gear to move forward, but not backward. After each round of replication, mutations accumulate but are not removed. Each round of error-prone replication works like a ratchet, "clicking" relentlessly as mutations accumulate at every replication cycle. Each mutation has the potential to erode the fitness of subsequent populations. Simple studies such as the serial plaque transfer experiment indicate

BOX 10.5 DISCUSSION

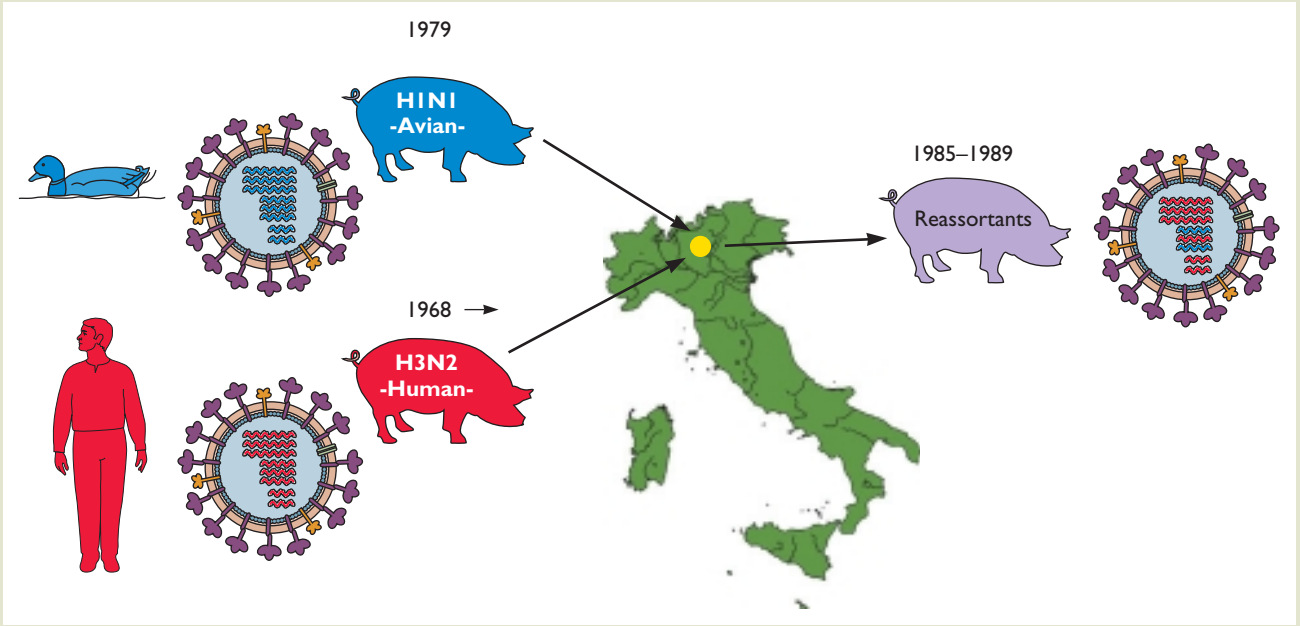
Reassortment of influenza virus genome segments

Pandemic influenza results from shifts in H and N serotypes due to the exchange of genome segments by mammalian and avian influenza viruses. Virologists have demonstrated that certain combinations of H and N are better selected in avian hosts than in humans. An important observation was that both avian and human viruses replicate well in certain species such as pigs, no matter what the H-N composition. Indeed, the lining of the throats of pigs contains receptors for both human and avian influenza viruses, providing an environment in which both can flourish. As a result, the pig is a good nonselective host for mixed infection of avian and human viruses, in which reassortment of H and N segments can occur, creating new viruses that can reinfect the human population.

One might think that this combination of human, bird, and pig infections must be extremely rare. However, the dense human populations in Southeast Asia that come in daily contact with domesticated pigs, ducks, and fowl remind us that these interactions are likely to be frequent. Indeed, epidemiologists can show that the 1957 and 1968 pandemic influenza A virus strains originated in China and that the human H and N serotypes are circulating in wildfowl populations.

Studies of Italian pigs provide evidence for reassortment between avian and human influenza viruses. The figure shows how the avian H1N1 viruses in European pigs reassorted with H3N2 human viruses. The color of the segments of the influenza genome indicates the origin of the avian and human viruses. The host of origin of the influenza virus genes was determined by sequencing and phylogenetic analysis. These studies support the hypothesis that pigs can serve as an intermediate host in the emergence of new pandemic influenza viruses.

Adapted from R. G. Webster and Y. Kawaoka, *Semin. Virol.* **5:**103–111, 1994, with permission. For more information, see J. S. Peiris et al., *J. Virol.* **75:**9679–9686, 2001.



that Muller's ratchet can be avoided if a more diverse viral population is replicated by serial passage. One such study showed that pools of virus from 30 individual plaques were required in serial transfer to maintain the culture's original fitness. This observation can be explained as follows: more diversity in the replicating population facilitates the construction of a mutation-free genome by recombination or reassortment, removing or compensating for mutations that affect propagation adversely. Even if such a recombinant is rare, it has a powerful selective advantage in this experimental paradigm. Indeed, its progeny ultimately will predominate in the population. The message is simple but powerful: diversity of a viral population is important for the survival of individual members; remove diversity, and the population suffers.

While the particular bottleneck of single-plaque passage is obviously artificial, infection by a small virus population and subsequent amplification are often found in nature. Examples include the small droplets of suspended virus particles during transmission as an aerosol, the activation

Table 10.2 Fitness decline compared to initial virus clone after passage through a bottleneck[a]

Virus	No. of bottleneck passages	% Decrease in fitness (avg)
ϕ6 (bacteriophage)	40	22
Vesicular stomatitis virus	20	18
Foot-and-mouth disease virus	30	60
Human immunodeficiency virus	15	94
MS2 (bacteriophage)	20	17

[a]A. Moya, S. Elena, A. Bracho, R. Miralles, and E. Barrio. *Proc. Natl. Acad. Sci. USA* **97:**6967–6973, 2000.

of a latent virus from a limited population of cells, and the small volume of inoculum introduced in infection by insect bites. An important question is, how do viruses that spread in nature by these routes escape Muller's ratchet? They do so by exchanging genetic information.

Exchange of Genetic Information

Genetic information is exchanged by recombination or by reassortment of genome segments (Volume I, Chapters 6, 7, and 9). In one step, recombination creates new combinations of many mutations that may be essential for survival under selective pressures. As discussed above, this process allows the construction of viable genomes from debilitated ones and can avoid Muller's ratchet. Recombination occurs when the polymerase changes templates (copy choice) during replication, or when nucleic acid segments are broken and rejoined. The former mechanism is common among RNA viruses, whereas the latter is more typical of double-stranded DNA virus recombination. Another mechanism for exchange of genetic information is reassortment among genomic segments when cells are coinfected with segmented RNA viruses. It is an important source of variation, as exemplified by orthomyxoviruses and reoviruses (Boxes 10.4 and 10.5).

Insertion of nonviral nucleic acid into a viral genome (sequence-independent recombination) is well documented and makes a central contribution to virus evolution. Incorporation of cellular sequences can lead to defective genomes, or to more pathogenic viruses. Examples of such recombination include the appearance of a cytopathic virus in an otherwise nonpathogenic infection by the pestivirus bovine viral diarrhea virus (see Volume I, Chapter 6) or the sudden appearance of oncogenic retroviruses in nononcogenic retroviruses. The acquisition of activated oncogenes from the cellular genome is the hallmark of acutely transforming retroviruses such as Rous sarcoma virus (Chapter 7). Poxvirus and gammaherpesvirus genomes carry virulence genes with sequence homology to host immune defense genes. These genes are usually found near the ends of the genome. One explanation for their location is that the process of DNA packaging (gammaherpesviruses) or initiation of DNA replication (poxviruses) stimulates virus-host recombination when viral DNA is cleaved.

Information can be exchanged in a variety of unexpected ways during viral infections. For example, a host can be infected or coinfected by many different viruses during its lifetime (Box 10.6). In fact, serial and concurrent infections are commonplace. Both have a profound effect on virus evolution. In the simplest case, propagation of a virus quasispecies in an infected individual allows coinfection of single cells, phenotypic mixing, and genetic

BOX 10.6 EXPERIMENTS

Virus evolution by host switching and recombination

Viruses can be transmitted to completely new host species that have not experienced any prior infection. Usually host defenses stop the infection before any replication and adaptation can take place. On rare occasions, a novel population of viruses arises in the new host. Past interspecies infections can occasionally be detected by sequence analyses. The data provide a glimpse of the rather amazing and unpredictable paths of virus evolution.

Circoviruses infect vertebrates and have small, circular, single-stranded genomes (Volume I, Chapter 3). Nanoviruses have the same genome structure, but infect plants. The genes encoding the Rep protein of these viruses appear to be hybrids: they share significant sequence similarity in the 5′ coding sequences. They also exhibit homology in the 3′ sequences with an RNA-binding protein encoded by caliciviruses (RNA viruses that infect vertebrates).

The scientists who analyzed the DNA sequences suggested that two remarkable events occurred during the evolution of circoviruses and nanoviruses. At some time, a nanovirus was transferred from a plant to a vertebrate, perhaps when a vertebrate was exposed to sap from an infected plant. The virus adapted to vertebrates, and the circovirus family was established. As all known caliciviruses infect vertebrates, the recombination between circovirus and calicivirus sequences would have occurred after the host switch of nanoviruses to vertebrates.

Gibbs, M. J., and G. F. Weiller. 1999. Evidence that a plant virus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus. *Proc. Natl. Acad. Sci. USA* **96:**8022–8027.

complementation. As a result, recessive mutations are not immediately eliminated, despite the haploid nature of most viral genomes. Of course, such coinfection also provides an opportunity for physical exchange of genetic information.

Viral infections occur as host defenses are modulated or bypassed. Infections that suppress the immune response have a marked effect on concurrent or subsequent infections of the same host by very different viruses. Indeed, human immunodeficiency virus and measles virus infections lead to serious disease and death of their hosts by facilitating subsequent infections by diverse pathogens. Animals infected with poxviruses are more susceptible to infection by a variety of pathogens, because infected cells secrete soluble inhibitors of interferons and other cytokines required for innate defense. Conversely, activation of host defenses by one viral infection can impair subsequent infection by a different virus. The existence of such multifactorial interchange among diverse viruses reinforces the idea that selection acts not only on one population of viruses, but also on interacting populations of different viruses (Box 10.7).

Two General Pathways for Virus Evolution

An overarching principle is that viruses and their hosts exist along a continuum of *r*- and *K*-selection. *r*-selected viruses have high yields and short generation times and often kill their hosts, while *K*-selected viruses coexist with their hosts for long periods. Since viruses are absolutely dependent on their hosts for replication, viral evolution tends to take one of two general pathways. In one, viral populations coevolve with their hosts so that they share a common fate; as the host prospers, so does the viral population. However, given no other host, a bottleneck now exists: the entire viral population can be eliminated with antiviral measures or by loss of the host. In the other pathway, viral populations occupy broader niches and infect multiple host species. When one host species is compromised, the viral population can replicate in another. In general, as discussed below, the first pathway is typical of DNA viruses, whereas the second is common for RNA viruses.

The Origin of Viruses

Where did viruses come from? Certainly, the mists of time and lack of a fossil record cloud their origins. We can be sure of one basic fact: viruses cannot exist without living cells. Soon after the discovery of viruses, many articulated the idea that viral genomes may be very ancient and even predecessors to modern cells. Despite early interest, the origin of the first viruses remains a matter of conjecture and debate. Technological advances in nucleic acid chemistry, sequencing, and genomics/computer analyses have provided a wealth of data engendering some provocative speculation.

BOX 10.7 DISCUSSION

Evolution by nonhomologous recombination and horizontal gene transfer

In the early 1970s, scientists working with bacteriophage lambda and related viruses formed heteroduplexes between various pairs of viral DNA and visualized them in the electron microscope. The images were striking and revealed that the genomes of this group of lambdoid phages were mosaics; that is, the genomes contained blocks of genes (modules) that were shuffled during evolution by recombination. Further analyses of bacteriophages that had picked up host genes by nonhomologous recombination revealed that horizontal gene transfer among bacteria by bacteriophages was a central feature evolution of both. With large-scale genome sequencing, scientists know that bacteriophage genomes have ancestral connections to viruses of eukaryotes and archaea.

Murray, N., and A. Gann. 2007. What has phage lambda ever done for us? *Curr. Biol.* **17:**R305–R312.

Electron microscope image of phage lambda (negative stain). Courtesy of Robert Duda, University of Pittsburgh, Pittsburgh, PA (http://www.pitt.edu/~duda).

One challenge to viral genomics is that the "tree of life" as conceived by biologists does not include viral genomes. Their consensus is that the viruses are not alive and thus cannot be considered organisms. Obviously, such thinking is short sighted. Virions indeed are inanimate; they are complicated biomolecules incapable of replicating on their own. However, the infected cell has novel properties, clearly distinct from those of uninfected cells.

Theories about the origins of viruses center around three nonexclusive ideas. The **regressive theory** suggests that viruses are derived from intracellular parasites that have lost all but the most essential genes, those encoding products required for replication and maintenance. The **cellular origin theory** proposes that viruses arose from cellular components that gained the ability to replicate autonomously within the host cell. The **independent-entity theory** postulates that viruses coevolved with cells from the origin of life itself. As there is no fossil record and there are few viral stocks more than 80 years old, we cannot test the three hypotheses for the primordial origins of viruses. Nevertheless, we can gain some insight into viral evolution by examining contemporary viruses. One cannot help but conclude that nothing looks like the world of viral genomes (the virosphere). Viruses abound in the three domains of life (bacteria, eukaryotes, and archaea) and define three distinct arms of the virosphere. However, viral genomes in each arm share homologous features, providing a tantalizing hint that the viral genomes indeed are ancient and that they pre-date the last universal cellular ancestor. For example, the DNA viruses of green algae may be the oldest eukaryotic viruses, because sequence analysis places their genomes near the root of most eukaryotic sequences (Box 10.8).

A common but puzzling fact is that as more viral genomes are sequenced, more and more genes with no obvious homology to genes of known hosts are found. In addition, the very large viral DNA genomes often have **sequence coherence;** that is, these genomes do not appear to be mosaics (e.g., mimivirus [Box 10.9; see also Box 10.7]). The homogeneity of genomes within a family and the lack of any obvious homology among families are difficult to explain using the model that they arose by the sequential acquisition of exogenous genes by a primordial, precursor viral genome.

The existence of RNA and DNA viral genomes presents an interesting conundrum for any theory of virus origin. Which genome type came first? Some speculate that DNA genomes were a viral invention that was shared later with cells harboring RNA genomes. Given that viruses are obligate intracellular parasites, they are in a position to drive significant evolutionary change. The bringing together of two distinct genomes in a common cell is thought to have driven major evolutionary leaps such as the acquisition of mitochondria by eukaryotic cells. It has been proposed that the eukaryotic nucleus arose from an infection of a primordial cell (perhaps with an RNA genome) with DNA viruses that replicate in the cytoplasm (Fig. 10.2). One critical issue inherent in such a model is the source of the deoxyribonucleoside triphosphates required for DNA replication. Can an organism with RNA biology produce these essential nucleotides?

To account for the shared gene pool for DNA viruses in the three domains of life, at least three independent transfers of DNA to the last universal cellular ancestor would be required to give rise to the modern viruses. The origin of RNA viruses remains even more speculative. They could be escapees from an ancient RNA world, but their distribution among the eukaryotes and bacteria is decidedly nonuniform. For example, at this time, no RNA viral genomes have been found in archaea. Given the extreme environments populated by these organisms, perhaps RNA genomes just cannot survive (or perhaps investigators have methodological problems). On the other hand, maybe the ancestral RNA protoviruses never infected the common archaeal ancestor. A better guess might be that we just have not looked hard enough.

DNA Virus Relationships Deduced by Nucleic Acid Sequence Analysis

We know the sequences of more than 1,600 viral genomes (roughly equal numbers of RNA and DNA genomes are in the databases). A "virochip" has been constructed with oligonucleotides representing the distinctive features of all 1,600 genomes. It is now possible to sample the ecosystem to determine the nature and diversity of viral genomes without having to propagate the viruses in the laboratory. Viral ecology is now described by the results of large-scale sequencing efforts. One type of study purifies all capsids from water samples and sequences all the DNA released from these capsids. This type of **metagenomic analysis** has revealed remarkable diversity. In fact, the vast majority of viral sequences determined so far by these technologies represent unknown viral genomes.

The origins of herpesviruses. Nucleic acid sequence analyses have identified many relationships among different viral genomes, providing considerable insight into the origin of viruses. The herpesvirus family exhibits a unique complex virion structure found in no other virus family (see Volume I, Appendix). The three main subfamilies of the family *Herpesviridae* (*Alphaherpesvirinae, Betaherpesvirinae,* and *Gammaherpesvirinae*) are easily distinguished by genome sequence analysis even though the original taxonomic separation of these families was based on general, often arbitrary, biological properties. Researchers have related the timescale of herpesviral genome evolution

BOX 10.8 DISCUSSION

Chlorella viruses: clues to viral and eukaryotic origins?

Paramecium bursaria chlorella virus (PBCV-1) is one of the oldest eukaryotic viruses. This remarkable virus is a large, icosahedral, plaque-forming, double-stranded DNA virus that replicates in certain unicellular, eukaryotic chlorella-like green algae (family *Phycodnaviridae*, genus *Chlorovirus*). In nature, the chlorella host is a hereditary endosymbiont of the ciliated protozoan *Paramecium bursaria*. The alga host can be grown in the laboratory in liquid and on solid media. Chloroviruses have been found in freshwater sources throughout the world, and many genetically distinct isolates usually can be found within the same sample. The titer of the viruses within a single water source can reach as high as 40,000 plaque-forming units (PFU) per ml.

The 330,744-bp PBCV-1 genome is predicted to harbor ~375 protein-encoding genes and 10 transfer RNA genes. The predicted products of ~50% of these genes resemble proteins of known function. Besides their large genome, the chloroviruses have other unusual features, including multiple DNA methyltransferases and DNA site-specific endonucleases, the entire machinery to glycosylate viral glycoproteins, at least two types of introns (a self-splicing intron in a transcription regulatory gene and a splicesome-processed intron in the viral DNA polymerase gene), a potassium ion channel, and the smallest known topoisomerase. Phylogenetic analyses based on DNA sequences and protein motifs indicate that these viral sequences lie near the root of most eukaryotic sequences. The implication is that the earliest eukaryotes were exchanging information with ancient members of the *Phycodnaviridae*.

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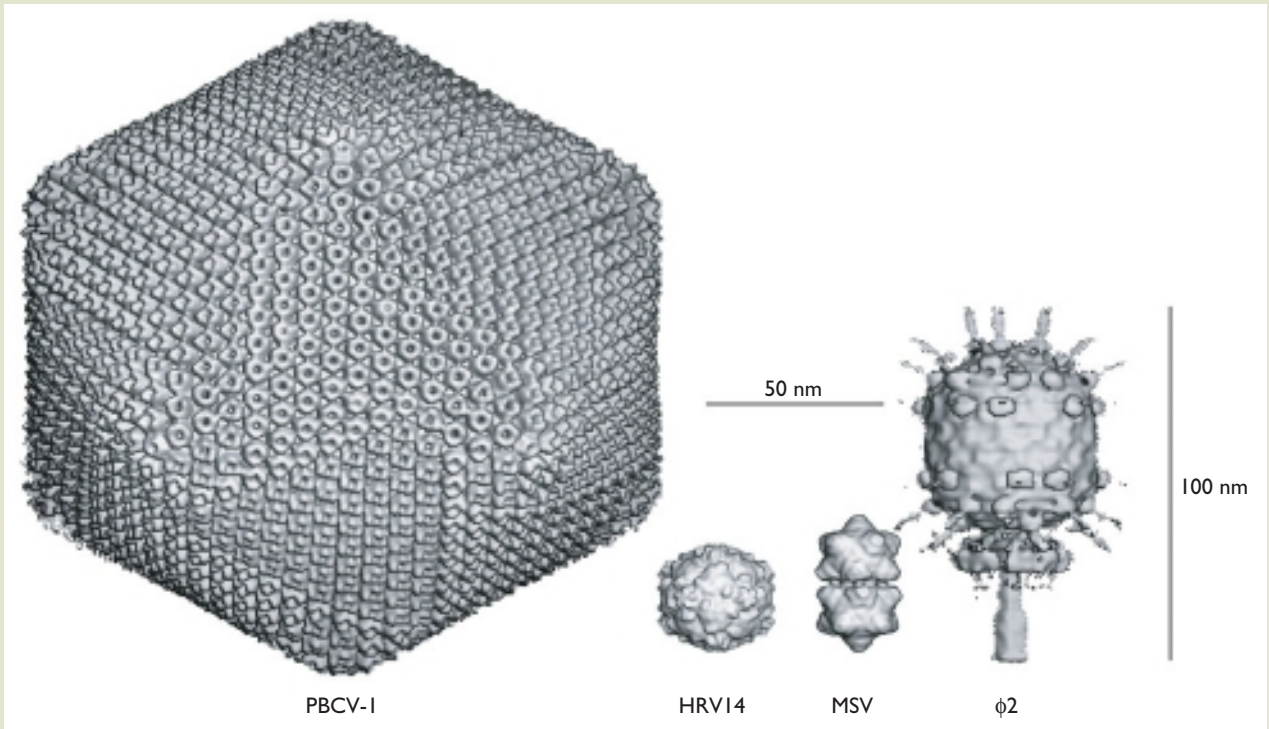
Villarreal, L. P., and V. R. DeFilippis. 2000. A hypothesis for DNA viruses as the origin of eukaryotic replication proteins. *J. Virol.* **74:**7079–7084.

Cryo-electron microscopic images courtesy of Tim Baker (University of California, San Diego [http://cryoem.ucsd.edu/]) and J. Van Etten (University of Nebraska).



to that of the hosts (based on fossil records). For most herpesviruses, points of sequence divergence coincide with well-established points of host divergence. The conclusion is that an early herpesvirus infected an ancient host progenitor, and subsequent viruses developed by cospeciation with their hosts. Consistent with this conclusion, the genomes of **all** *Alphaherpesvirinae, Betaherpesvirinae,* and *Gammaherpesvirinae* that have been sequenced contain a core block of genes, often organized in similar clusters in the genome.

BOX 10.9 BACKGROUND

Mimivirus: the largest known virus particle and genome

A 1992 pneumonia outbreak in Bradford, England, led to the isolation of the world's largest virus. Investigators attempted to isolate *Legionella*-like pathogens of amoebae from hospital cooling towers and isolated what appeared to be a small gram-positive bacterium. All attempts to identify it using universal bacterial 16S ribosomal RNA (rRNA) PCR amplification failed. Transmission electron microscopy of infected *Acanthamoeba polyphaga* revealed 400-nm icosahedral virus particles in the cytoplasm. The virus was named "mimivirus" because it mimicked a microbe. This giant virus of amoebae challenges many preconceived notions about the nature of viruses and their origin.

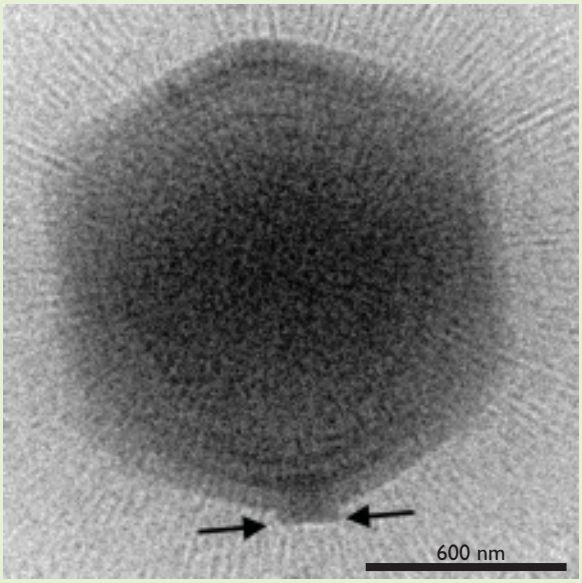
Virion: 750 nm in diameter

Genome: 1.2×10^6 base pairs (bp)

Genes: 911 protein-coding genes

Still a virus: Despite having a number of genes predicted to be involved in protein synthesis, the genome does not encode a complete translation system.

Amazing sequence conservation: While it is difficult if not impossible to do genetics with mimivirus, bioinformatics has been used to probe the mimivirus DNA sequence. Mimivirologists have pointed out some rather provocative features that have implications for mimivirus evolution. For example, an AAAATTGA motif is found in more than half the mimivirus genes. This motif is proposed to be the structural motif of the TATA box core promoter element of unicellular eukaryotes, particularly the amoeba hosts of mimivirus. The motif is specific to the mimivirus lineage and may correspond to an ancestral promoter structure predating the radiation of the eukaryotes.



Raoult, D., S. Audic, C. Robert, C. Abergel, P. Renesto, H. Ogata, B. LaScola, M. K., Suzan, and J. Claverie. The 1.2Mb genome sequence of Mimivirus. *Science* **306:**1344–1350.

Suhre, K. I., S. Audic, and J.-M. Claverie. 2005. Mimivirus gene promoters exhibit an unprecedented conservation among all eukaryotes. *Proc. Natl. Acad. Sci. USA* **102:**14689–14693.

Our current best estimate is that the three major groups of herpesviruses arose approximately 180 million to 220 million years ago. These three subfamilies of viruses must therefore have been in existence before mammals spread over the Earth 60 million to 80 million years ago. Perhaps surprisingly, fish, oyster, and amphibian herpesviruses have virtually identical virion architecture, but little or no sequence homology to the *Alphaherpesvirinae, Betaherpesvirinae,* and *Gammaherpesvirinae*. They are related only tenuously to the mammalian and avian herpesviruses by common virion architecture and must represent a very early branch of this ancient family.

Origins of papillomaviruses and polyomaviruses. Coevolution with a host also is a characteristic of small DNA viruses, the parvoviruses, polyomaviruses, and papillomaviruses. Here, the evidence for coevolution comes not from comparison of host and viral genes, but rather from finding close association of a given viral DNA sequence with a particular host group. The linkage of host to virus was particularly striking when human papillomavirus types 16 and 18 were compared: the distribution of distinct viral genomes is congruent with the racial and geographic distribution of the human population. Another example of the same phenomenon is provided by JC virus, a ubiquitous human polyomavirus associated with a rare, fatal brain infection of oligodendrocytes. This virus exists as five or more genotypes identified in the United States, Africa, and parts of Europe and Asia. Recent polymerase chain reaction (PCR) analyses of these subtypes indicate that JC virus not only coevolved with humans, but also did so within specific human subgroups. Probably the most striking finding was

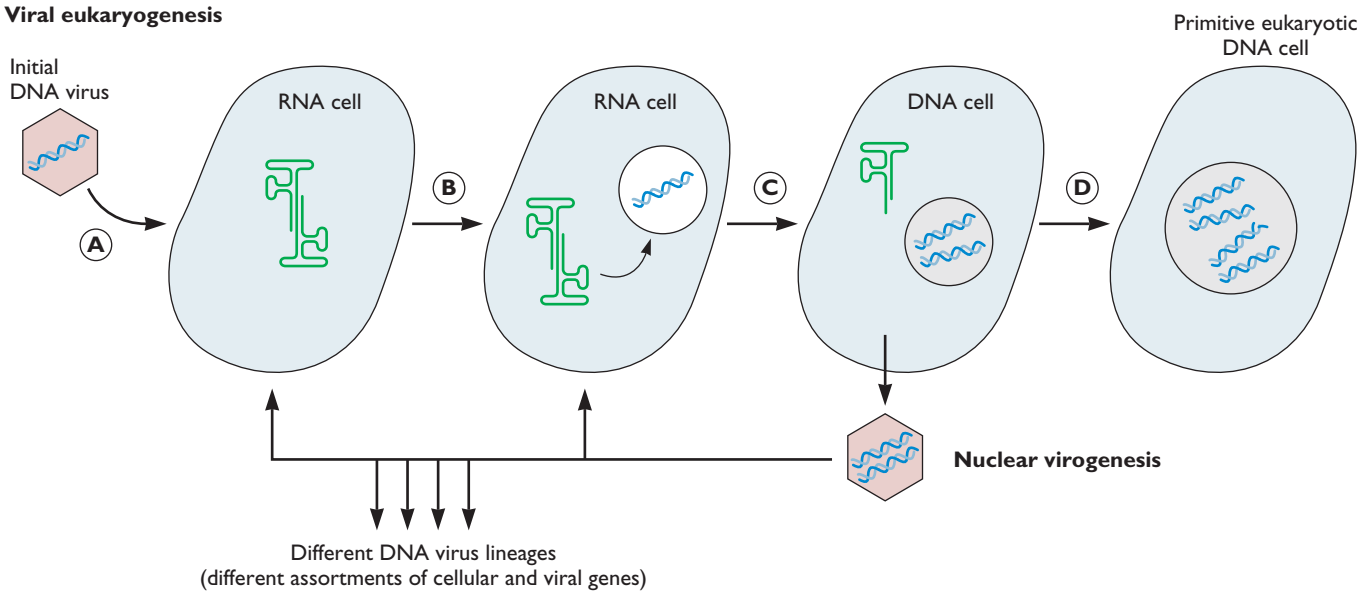


Figure 10.2 Primordial encounter of a DNA virus with an RNA cell: hypothetical origin of the nucleus. (A) An early DNA virus (perhaps a bacteriophage ancestor) engages a cell with an RNA genome. **(B)** The DNA virus is sequestered within a vesicle in the "cytoplasm" and replicates in this compartment. **(C)** Cellular genes are recruited to the enlarging nucleus; new DNA chemistry provides selective advantages. **(D)** This unstable situation may produce novel virions better adapted to infection of and cytoplasmic replication in cells with RNA genomes as well as the evolution of a stable eukaryotic cell with a nucleus and DNA replication machinery. See J.-M. Claverie, *Genome Biol*. **7:**110–114, 2006, and C. Zimmer, *Science* **312:**870–872, 2006.

that the JC virus of a particular group provides a convenient marker for human migrations from Asia to the Americas in both prehistoric and modern times.

How can virus evolution be linked to specific human populations in a manner akin to vertical transmission of a host gene? We can begin to appreciate this perhaps counterintuitive phenomenon from the unusual biology of human papillomaviruses (see also Box 10.10). Infection of the basal keratinocytes of adult skin leads to viral replication, as the cells differentiate. Virus particles are assembled only as cells undergo terminal differentiation near the skin surface. Mothers infect newborns with high efficiency, because of close contact or reactivation of persistent virus during pregnancy or birth. The infection therefore appears to spread vertically, in preference to the more standard horizontal spread between hosts. This mode of transmission was the predominant mechanism for papillomavirus and polyomavirus. It stands in contrast to that observed for most acutely infecting viruses, which are spread by aerosols, contaminated water, or food.

Origins of smallpox virus. An analysis of smallpox virus genomes provides unexpected insights into the evolution of this scourge of humanity. The genome sequences of 45 epidemiologically different smallpox virus isolates are remarkably similar. The genome sequences can be organized into three clades, which cluster according to the origin of the isolates (West Africa, South America, and Asia, respectively), but gene content is remarkably constant. Lack of diversity among diverse isolates indicates a recent introduction into humans. Interestingly, the only member of the large *Poxviridae* family with credible sequence homology to smallpox virus was a gerbil poxvirus. Perhaps human smallpox virus arose after a zoonotic infection from infected gerbils.

RNA Virus Relationships Deduced by Genome Sequence Analysis

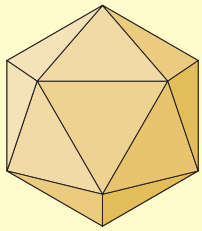
The relationships among RNA viruses can also be deduced from sequence analyses, but the high rates at which mutations accumulate impose some difficulties. Moreover, genomes of RNA viruses are often small and carry few if any nonessential genes that might be useful for comparative studies. And, in contrast to the large DNA viruses, viral RNA genomes contain few, if any, genes in common with a host that might be used to correlate virus and host evolution. Nevertheless, when nucleotide sequences of many (+) and (−) strand RNA viral genomes are compared, blocks of genes that encode proteins with

BOX 10.10 EXPERIMENTS

The modular nature of papillomavirus genomes

Papillomaviruses infect vertebrate stratified squamous epithelia. Some are associated with certain benign lesions and some are associated with cancers. Sequence analysis of host and viral genomes has revealed a close association of host and viral genomes. In general, papillomavirus genomes have been classified according to the sequence of the L1 capsid protein gene and a rather elaborate phylogenetic scheme as been proposed.

Garcia-Vallve et al. measured the sequence divergence of all the viral open reading frames and found five well-defined regions in the viral genome with apparently different evolutionary histories. They suggest that the primordial papillomavirus genome (protovirus) comprised



the E1, E2, L1, and L2 open reading frames, while the E5, E6, and E7 genes were acquired later. Given that all the early (E) viral gene products interact with host proteins that participate in cell cycle control and DNA replication, it is possible to speculate about the host for the protovirus whose progeny now infect essentially all warm-blooded vertebrates. The authors suggest that the low-divergence genes (E1, E2, L1, and L2) define the protopapillomavirus genome that infected an early land-dwelling vertebrate. The E6 and E7 genes have more diversity, consistent with their later addition to the protovirus genome. When the mammalian lineage appeared 150 million years ago, the protoviral genomes were exposed to various selective pressures, resulting in a rapid diversification of the sequences. The E5 open reading frame was the most recent addition to the protoviral genome about 65 million years ago.

Garcia-Vallve, S., A. Alonso, and I. Bravo. 2005. Papillomaviruses: different genes have different histories. *Trends Microbiol.* **13:**514–521.

similar functions can be defined. Common coding strategies can also be deduced. These groups are often called "supergroups" because the similarities suggest a common ancestry (Fig. 10.3 and 10.4). Alternatively, similarities may result from convergent evolution with no implications of shared lineages.

If one examines the sequences of many (–) strand RNA genomes, the first obvious common feature is the limited number of encoded proteins (as few as 4 and not more than 13). These proteins can be placed in one of three functional classes: core proteins that interact with the RNA genome, envelope glycoproteins that are required for attachment and entry of virus particles, and a polymerase required for replication and mRNA synthesis (Fig. 10.3).

The (+) strand RNA viruses (excluding the retroviruses) are the largest and most diverse subdivision of viruses: the genome sequences of more than 100 viruses representing at least 30 distinct groups are available for analysis. The number of proteins encoded by (+) strand RNA viruses ranges from 3 to more than 12, and, like the (–) strand RNA virus proteins, they can be divided into three groups by function: those required for RNA replication, encapsidation, and accessory functions. This comparison has resulted in the identification of three virus supergroups (Fig. 10.4). A unifying feature is that the RNA polymerase gene appears to be the most highly conserved, implying that it arose once in the evolution of these viruses. As each of the supergroups contains members that infect a broad variety of animals and plants, an ancestor present before their separation might have provided the primordial RNA polymerase gene. Alternatively, the ancestral (+) strand virus could have radiated horizontally among plants, animals, and bacteria (RNA viral genomes are not found in archaea).

It is instructive to consider the postulated evolution of supergroup 3, which contains the alphaviruses. Earlier work had indicated that they arose less than 5,000 years ago, but certain assumptions were made about the rate of RNA genome divergence and the constancy of virus evolution in different hosts and environments. More recent analyses comparing protein and RNA sequences indicate that the assumption of uniform nucleotide substitution rates was invalid; nucleotide changes are far from uniform. With new data from E1 protein and gene sequences, a phylogenetic tree can be constructed, but no time estimates for the appearance of the alphavirus progenitor are possible (Fig. 10.5). The data do suggest some interesting hypotheses about radiation of the species. The Old World viruses (e.g., the Semliki Forest virus complex) and the New World viruses (e.g., Venezuelan, eastern, and western equine encephalitis viruses) are clearly distinct. The mosquito-borne alphaviruses could have arisen in either the Old World or the New World, but at least two transoceanic introductions are required to account for their current distribution.

Predictive Powers of Sequence Analyses

Phylogenetic dendrograms relating nucleic acid sequences depict the relationships as if founder and intermediary sequences were on a trajectory to the present

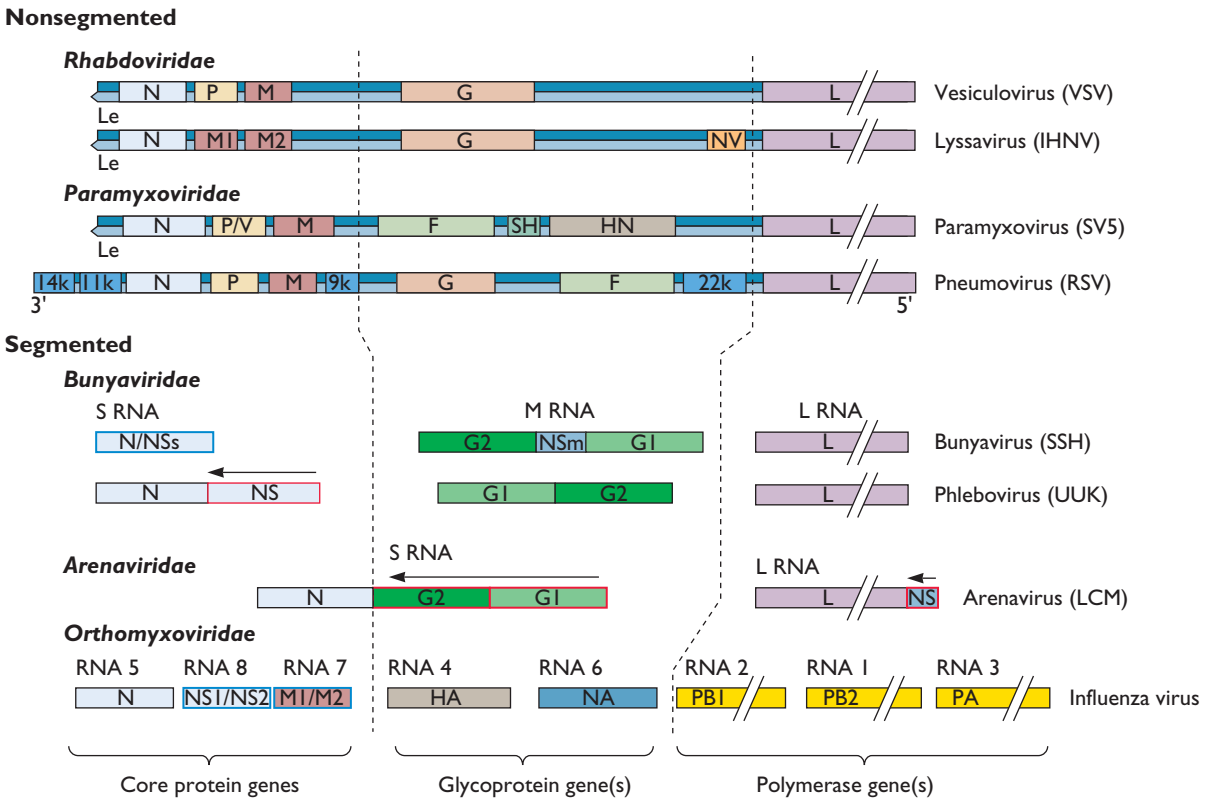


Figure 10.3 The genetic maps of selected (–) strand RNA viral genomes. Maps of the genes of *Rhabdoviridae*, *Paramyxoviridae*, *Bunyaviridae*, *Arenaviridae*, and *Orthomyxoviridae* are aligned to illustrate the similarity of gene products. The individual gene segments of the Orthomyxoviridae are arranged according to functional similarity to the two other groups of segmented viruses. Within a given genome, the genes are approximately to scale. For segemented genomes, blue-outlined genes are those in which multiple proteins are synthesized from different open reading frames. Red-outlined genes are expressed by the ambisense strategy. Virus abbreviations: VSV, vesicular stomatitis virus; IHNV, infectious hematopoietic necrosis virus; RSV, respiratory syncytial virus; SV5, simian virus 5; SSH, snowshoe hare virus; UUK, Uukuniemi virus; LCM, lymphocytic choriomeningitis virus. Le is a nontranslated leader sequence. Gene product abbreviations: N, nucleoprotein; P, phosphoprotein; M (M1 and M2), matrix proteins; G (G1 and G2), membrane glycoproteins; F, fusion glycoprotein; HN, hemagglutinin/neuraminidase glycoprotein; L, replicase; NA, neuraminidase glycoprotein; HA, hemagglutinin glycoprotein; NS (NV, SH, NSs, and NSm), nonstructural proteins; PB1, PB2, and PA, components of the influenza virus replicase. Figure derived from J. H. Strauss and E. G. Strauss, *Microbiol. Rev.* **58:**491–562, 1994, with permission.

sequences. This deduction is a gross oversimplification. Certainly, only extant genomes can be sequenced. Any intermediate that was lost during evolution will not contribute to the dendrogram. In addition, any recombination or gene exchange by coinfection with similar viral genomes will scramble ordered lineages (see also Box 10.7). A fair question is, can we predict the future trajectory of the dendrogram? What will comprise future branches of a given lineage? We can never answer these straightforward questions for two reasons: we cannot describe the diversity of any given virus population in an ecosystem, and we cannot we predict the selective pressures that will be imposed.

Origins of Viral Groups Suggested by Sequence Analysis

Japanese encephalitis virus. This flavivirus is the most important cause of epidemic encephalitis in the world. The virus was first isolated in 1935 and has subsequently been found across most of Asia. The origins of the virus are uncertain, but one idea has been entertained. Sequence analysis of all known Japanese encephalitis virus isolates indicates an origin a few centuries ago in the Indonesia-Malaysia region. Evolutionary biologists have long recognized that this area of the world is a unique environment with remarkable examples of divergent evolution of plants and animals. The number of insect species and their

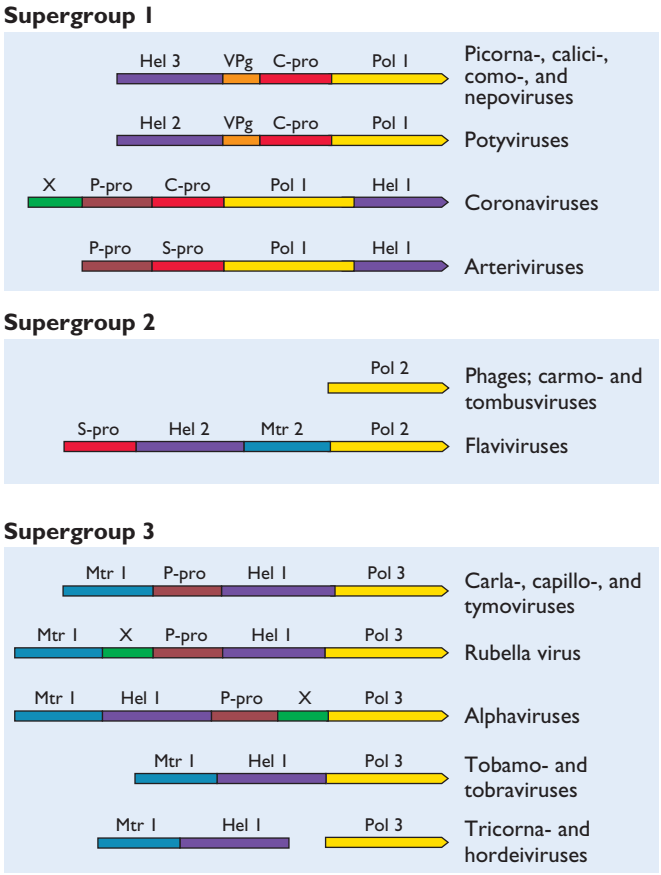


Figure 10.4 RNA virus genomes and evolution. Organization of (+) strand RNA genomes. The genomes of (+) strand RNA viruses comprise several genes for replicative functions that have been mixed and matched in selected combinations over time. These functions include a helicase (purple), a genome-linked protein (orange), a chymotrypsinlike protease (red), a polymerase (yellow), a papainlike protease (brown), a methyltransferase (dark blue), and a region of unknown function, X (green). Differences in the polymerase gene define the three supergroups. In this figure, the genes are not shown to scale and the structural proteins have been omitted for clarity. Derived from J. H. Strauss and E. G. Strauss, *Microbiol. Rev.* **58:**491–562, 1994, with permission.

viruses is enormous. The remarkable but unexplained variation among Murray Valley encephalitis virus isolates in New Guinea compared with those from Australia indicates that tropical Southeast Asia has a virus ecology worthy of detailed investigation.

Influenza virus. The ecology and biology of influenza have provided much food for thought about virus evolution. The same viral population can infect many different species. Each host species imposes new selections for replication and spread of the infection. As a result, the influenza virus gene pool is immense, with a dynamic ebb and flow of genetic information as infection spreads among many different animals. Large-scale sequencing has provided a view of the state of the viral gene pool at various points in time and space, as infections are transmitted from human to animal, animal to human, and human to human. In one analysis alone, a consortium of scientists sequenced more than 200 human influenza virus genomes and collected almost 3 million bases of sequence. One salient finding was that a given influenza virus population in circulation contains multiple lineages at any time. In addition, alternative minor lineages exchange information with the dominant lineage. As selection pressures change, the numbers of distinct immune escape mutants rise and fall, as do the numbers of mutants with alterations in receptor-binding affinity. These studies even provide answers about gene function. A newly discovered open reading frame called PB1-F2 is preserved in almost all of the sequenced genomes, putting to rest the idea that it was not a functional gene.

Important clues to the epidemiology of influenza virus came from the sequencing and analysis of more than 1,300 influenza A virus isolates from various geographic locations. It was clear that the viral genome changes by frequent gene reassortment and occasional bottlenecks of strong selective "sweeps." More importantly, the study suggests that new antigenic subtypes have different dynamics but that all follow a classical "sink-source" model of viral ecology. In this model, antigenic variants emerge at intervals from a persisting reservoir in the tropics (the source) and spread to temperate regions, where they have only a transient existence before disappearing (the sink). Similar large-scale sequencing of avian influenza viruses is under way to expand our knowledge of the dynamic and rapid evolution of influenza virus genomes.

Measles virus. Sequence analyses indicates that measles virus, a human virus, is closely related to rinderpest virus, a bovine pathogen. It is thought to have evolved from a zoonotic ancestral rinderpest virus when humans first began to domesticate cattle. The best estimates indicate that measles virus is a relatively new human pathogen and probably became established in the Middle East about 5,000 years ago, when human populations began to congregate in cities. Measles virus spread around the world by colonization and migration, reaching the Americas in the 16th century (with disastrous effects on the native Americans).

The Protovirus Theory for Retroviruses

The origins of retroviruses may be more accessible than any other virus group. Their unique life cycle centers on the enzymes reverse transcriptase and integrase. These enzymes ensure that the RNA genome in virions is converted to a

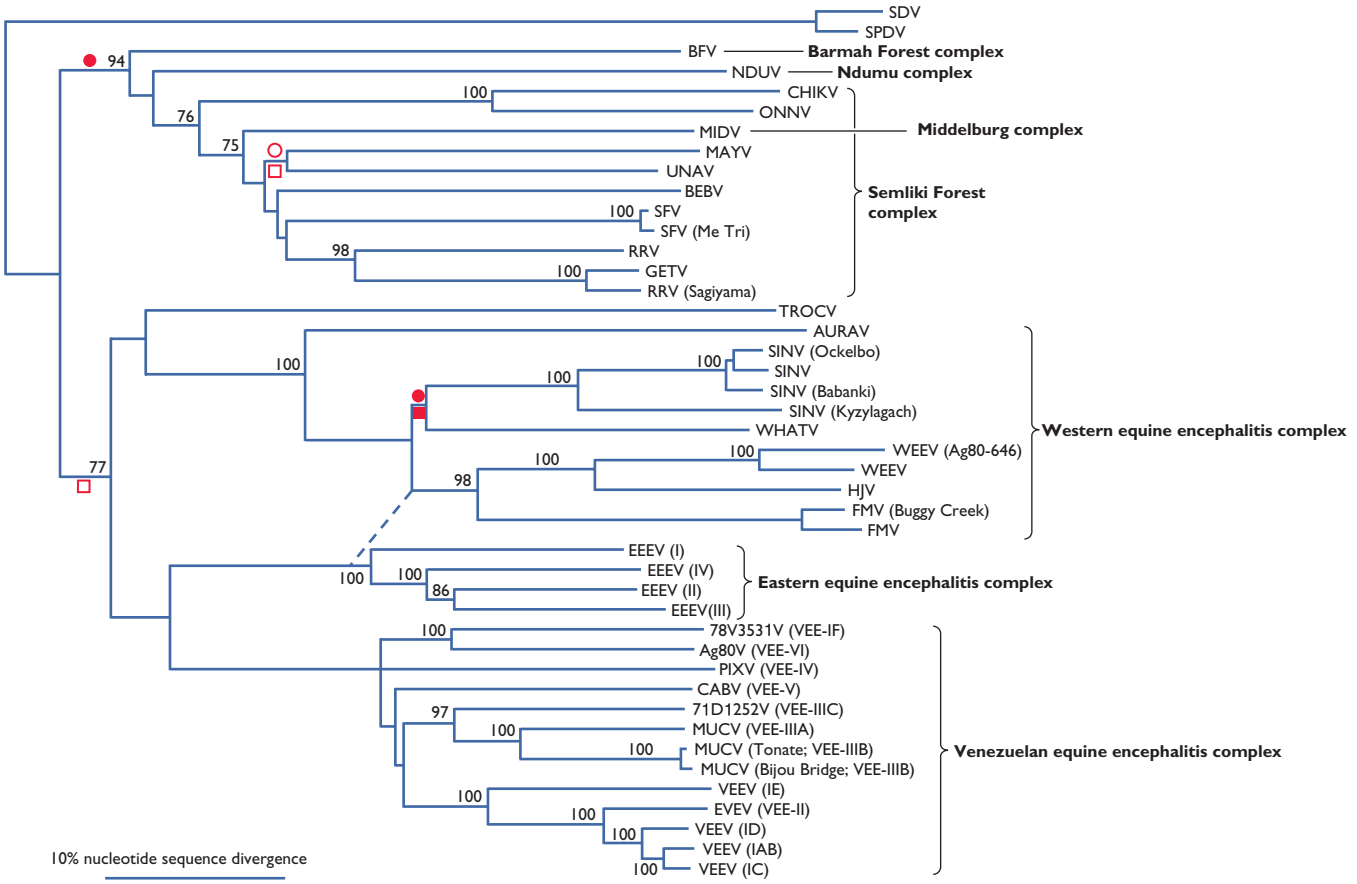


Figure 10.5 Dendrogram depicting a proposed phylogenetic tree of modern alphaviruses based on nucleic acid sequence comparisons of the E1 gene. (Top) Semliki Forest complex; **(middle)** western equine encephalitis complex; **(bottom)** Venezuelan equine encephalitis complex. The western equine encephalitis virus (WEEV) subgroup arose by a recombination event between a member of the eastern equine encephalitis virus (EEEV) lineage and the Sindbis virus lineage (dashed line). The bar indicates 10% nucleotide divergence. The open red circle adjacent to a branch indicates a hypothetical Old World-to-New World introduction, and the closed red circle indicates New World-to-Old World introduction, assuming a New World origin. The open red square indicates Old World-to-New World introduction, and the closed red square indicates New World-to-Old World introduction, assuming an Old World origin of the non-fish alphavirus clade. Redrawn from A. M. Powers et al., *J. Virol.* **75:**10118–10131, 2001, with permission.

DNA copy permanently integrated in the host DNA genome (the provirus). Howard Temin, who shared the Nobel Prize for the discovery of reverse transcriptase, first proposed the "protovirus theory" for the origin of this virus family. This theory posits that a cellular reverse transcriptase-like enzyme copied segments of cellular RNA into DNA molecules that were then inserted into the genome to form retroelements. These DNA segments in turn acquired more sequences, including those encoding integrase, ribonuclease H (RNase H) domains, regulatory sequences, and structural genes (Fig. 10.6). This theory predicts that evidence for this process might exist in the genomes of mammals and other species. Indeed, many of the predicted intermediates are found in abundance, including pseudogenes, retrotransposons, and a variety of endogenous retroviruses (see Volume I, Chapter 7). In humans, such endogenous retroviruses are surprisingly abundant, comprising several percent of the human genome, and may represent footprints of ancient infection of germ cells.

Contemporary Virus Evolution

Although we cannot describe the origins of viruses, we should be able to define modern precursors of new ones by studying current virus ecology. Even with powerful technology, the task is daunting. It is probably safe to assume that every living thing is infected with viruses, and that we have only scratched the surface to identify them all. Nevertheless, studying the processes that result in the

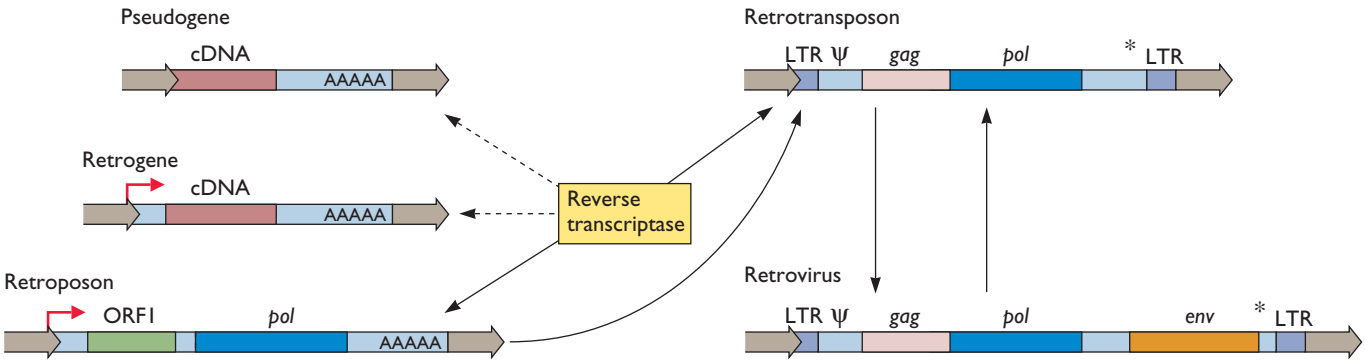


Figure 10.6 The action of a primordial reverse transcriptase may drive evolution of retroelements. A speculative scheme shows the evolution of various retroelements through the action of a primordial reverse transcriptase. Dashed arrows depict the emergence of genetic elements by reverse transcription; the complementary DNA copies are shown integrated into the host genome (gray). Solid arrows show the acquisition of new elements by recombination. cDNA, double-stranded DNA copied by reverse transcriptase; ORF, open reading frame; *gag*, capsid protein gene; *pol*, polymerase gene coding for reverse transcriptase, RNase H, integrase, and other enzymes; *env*, envelope protein gene; LTR, long terminal repeat; red arrow, promoter, initiation of transcription; AAAAA, poly(A) tail; y, packaging signal; *, polypurine tract. Direct repeats of cellular DNA are indicated in gray. Adapted from R. J. Loewer et al., *Proc. Natl. Acad. Sci. USA* **93:**5177–5184, 1996, with permission.

emergence of new viruses seems likely to prove more helpful in divining the present and future evolution of viruses than in explaining their origins.

As viruses are not likely to arise *de novo*, modern and future viruses must arise from progenitors that already exist. Hence, even human immunodeficiency virus, which appeared within the past few decades, has many relatives in nature and descended from one, or a combination, of them. Therefore, the sources of new viruses are strictly limited to two possibilities: a mutant virus already existing in an infected host can be selected, or a virus can enter a naive population from an entirely different infected species. These two simple possibilities notwithstanding, the interactions of host and virus required to establish a stable relationship are remarkably complex.

The Fundamental Properties of Viruses Constrain and Drive Evolution

We can recognize a herpesvirus, an adenovirus, a retrovirus, or an influenza virus genome by sequence analysis, despite many rounds of replication, mutation, and selection. This fact is emphasized by sequence analyses of human immunodeficiency virus strains isolated from patients around the world. As much as 10% of the total viral genome can vary from isolate to isolate, yet viral isolates fall into consistent subgroups called **clades.** Each clade differs from the others in amino acid sequence by at least 20% for Env proteins and 15% for Gag proteins. Differences within a clade can be as much as 8 to 10%, emphasizing the rather arbitrary delineation. As discussed in Chapter 6, these viruses have replicated in widely dispersed geographic locations and have very different histories, yet each sequence is clearly recognizable as the human immunodeficiency virus genome.

The important message is that viral populations frequently maintain quite stable master or consensus sequences, despite opportunities for extreme variation (Fig. 10.1). Diversity exists—indeed is necessary for virus survival—but the consensus sequence remains, despite many years of selection and growth. How is stability maintained in the face of mutation, recombination, and selection? One answer is that all viruses share fundamental characteristics that define and constrain them. Those that can function within the constraints survive. Comprehending the evolution of viruses requires an understanding of these shared properties.

Constraining Viral Evolution

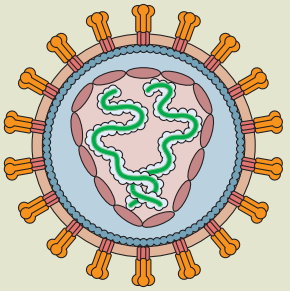
The very characteristics that enable us to define and classify viruses are the primary barriers to major genetic change; that is, extreme alterations in the viral consensus genome obviously do not survive selection. Certainly, some changes are simply impossible. One obvious constraint is the viral genome itself: DNA genomes cannot mutate to become RNA genomes and vice versa. Once a replication and expression strategy has evolved, there can be no turning back, because solutions to replication or the decoding of viral information are limited. Every step in viral replication requires interactions with host cell machinery. Consequently, any change in a viral component without a compensating change in the interacting host component may compromise replication. Similarly, inappropriate

BOX 10.11 DISCUSSION

A constraint on evolution? Selection for survival inside a host

A thought-provoking finding from studies of human immunodeficiency virus infections indicates that additional constraints on virus evolution must be considered. Virions that initiate infections typically are macrophage tropic and engage the CCr5 chemokine receptor. At the end stage of disease, the infected individual is producing billions of virions that survive in the face of host defenses and antiviral therapy. Invariably, these virions are T-cell tropic and engage the CXCr4 chemokine receptor. The diversity in this final population is a result of evolution **inside** a single individual.

Amazingly, when virions from end-stage disease infect a new host, the first replicating viral genomes that can be detected are macrophage tropic, and engage the CCr5 receptor. The progeny genomes have passed through a bottleneck, and only a few of the diverse variants are passed on. The processes that select these variants from the previous T-cell-tropic population are not well understood. However, one conclusion is clear: the virions that ultimately devastate the immune system after years of replication and selection within a host are not the most fit for infection of new hosts.



synthesis, concentration, or location of a viral component is likely to be detrimental.

A second constraint is the physical nature of the capsid required for transmission of the genome. For example, icosahedral capsids have a defined internal space that fixes the size of packaged nucleic acids. Once the genes encoding assembly of an icosahedral capsid are selected, genome size is essentially fixed; only very limited duplication or acquisition of sequences is allowed without compensating deletion of other sequences. A final constraint is that selection occurs during host-to-host spread of infection, as well as during spread of infection within a single individual (Box 10.11). All viral genomes encode products capable of modulating a broad spectrum of host defenses, including physical barriers to viral access and the vertebrate immune system. A mutant that is too efficient in bypassing host defenses will kill its host and suffer the same fate as one that does not replicate efficiently enough: it will be eliminated. These general constraints define the viruses we see today, as well as the further evolution of new viruses.

Finite Strategies To Replicate Viral Genomes

In Volume I, Chapter 4, we describe seven genome replication strategies that are likely to represent all possible solutions. We also outline a small number of expression strategies for protein production from these genomes. That the provenance of all viruses can be described by a short list of replication and expression strategies is extraordinary. Understanding how protein function and gene expression strategies evolved represents a new research frontier for which we have few data to guide us. Some initial studies have been provocative. For example, RNA virus replication complexes described for different families have fundamental similarities (Box 10.12). Localization of genomes to membrane sites or to assembling capsids leads to precise temporal and spatial organization of viral compartments important for gene expression, replication, and particle assembly. Are these overtly similar mechanisms products of convergent evolution and coincidence, or do they imply a common evolutionary origin for this abundant group of viral genomes? One thought is that similar mechanisms were selected because they sequestered viral nucleic acid from the cytoplasmic intrinsic defense proteins such as RigI/Mda5, Pkr, and Tlr proteins.

Evolution of New Viruses

Even in the seemingly constrained context of a given virus, the number of all possible viable mutants is astronomical, if not inconceivable (Box 10.13). In fact, the number of possible mutants is so large that all the possibilities can never be tested in nature. Sequence comparisons of several viral RNA genomes have demonstrated that well over half of all nucleotides can accommodate mutations. This property means that, for a 10-kb viral RNA genome, more than $4^{5,000}$ sequence permutations define all possible mutants. If one considers deletions, recombination, and reassortment, the numbers become even larger. Considering that there are roughly 4^{135} atoms in the visible universe, this is a large number indeed. Even with the high rates of replication and mutation characteristic of viruses, we can be sure that only a minuscule fraction of all possible viral genomes have arisen since life began: even the most efficiently replicating virus will fail to spawn all the possible permutations for a run through the gauntlet

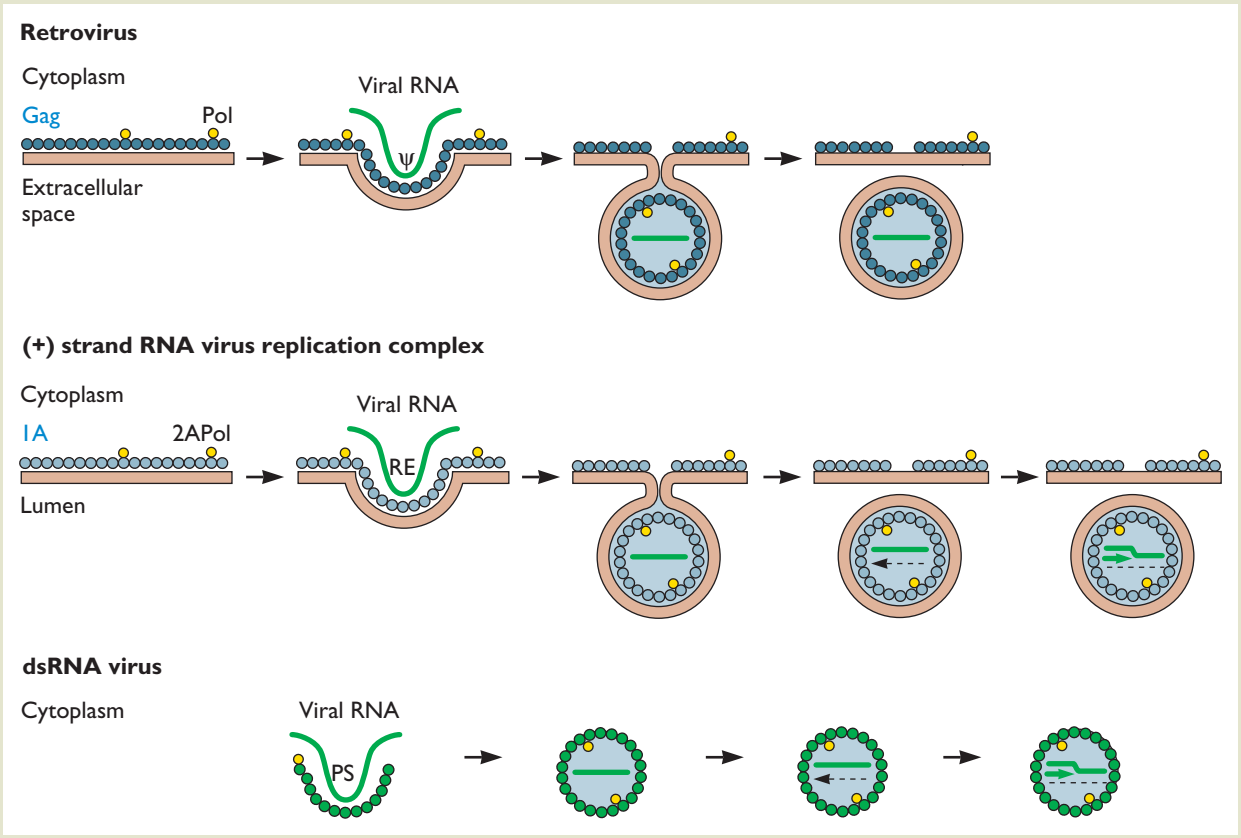
BOX 10.12 DISCUSSION

Parallels in replication of (+) strand and double-stranded RNA genomes

The mRNA templates of viruses with double-stranded RNA (dsRNA) and (+) strand RNA genomes (including retroviruses) are sequestered in a multisubunit protein core that directs synthesis of the RNA or DNA intermediate from which more viral mRNA is made. Similarities in how the mRNA template and core proteins are assembled suggest that all three virus groups may share evolutionary history, despite a complete lack of genome sequence homology. It is possible that this ancient replicative strategy provides RNA genomes with increased template specificity and retention of negative-strand products in the core or vesicle for template use. In addition, by sequestering RNA in vesicles or capsids, host defenses such as RNA interference, dsRNA-activated protein kinase, and RNase L are avoided.

Schwartz, M., J. Chen, J. Janda, M. Sullivan, J. den Boon, and P. Ahlquist. 2002. A positive-strand RNA virus replication complex parallels form and function of retrovirus capsids. *Mol. Cell* **9**:505–514.

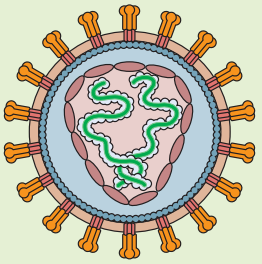
Similarities in replication and budding reactions. In the case of retroviruses, specific sequences on the RNA genome bind to Gag proteins that define the budding site. Gag proteins encapsidate viral RNA and reverse transcriptase with plasma membrane. Similarly, (+) strand RNA genomes are replicated on intracellular membrane vesicles that form in response to a viral protein that binds to membranes. Polymerase complexes and viral RNA templates are recruited to these vesicles. Replication of dsRNA genomes occurs in compartments formed by assembling capsid proteins that sequester single-stranded genome templates via specific protein-RNA interactions. Blue circles are Gag proteins (retrovirus), 1A protein for (+) strand RNA virus, and inner capsid protein for dsRNA virus. The polymerase protein (Pol or 2APol) interacts with Gag or 1A, respectively. The polymerase is part of the assembling capsid of dsRNA viruses. The RNA genome is indicated in green, and the binding sites for interaction with Gag, 1A, or capsid protein are ψ, RE, or PS, as indicated for each virus. The final reaction for retroviruses is the release of an enveloped particle with the genome and polymerase; in the case of (+) strand RNA viruses, the product is not an enveloped virion but, rather, an involuted vesicle or the surface of a membrane vesicle where mRNA synthesis, (–) strand genome template synthesis, and (+) strand genome synthesis occur. In the case of dsRNA viruses, the product is a capsid compartment within which mRNA synthesis and complementary strand genome replication occur.



BOX 10.13 BACKGROUND

The world's supply of human immunodeficiency virus genomes provides remarkable opportunity for selection

Tens of millions of humans are infected by human immunodeficiency virus. Before the end stage of disease, each infected individual produces billions of viral genomes per day. As a result, more than 10^{16} genomes are produced each day on the planet. Almost every genome has a mutation, and every infected human harbors viral genomes with multiple changes resulting from recombination and selection. Practically speaking, these large numbers provide an amazing pool of diversity. For example, mutants resistant to **every combination** of anti-reverse transcriptase and protease drugs in use, or in the pipeline, arise thousands of times each day, simply by chance.



of selection in nature. The conclusions are inescapable: virus evolution is relentless, new mutants will always arise, and the possibilities are literally unimaginable. Those who seek to predict the trajectory of virus evolution face enormous challenges, as mutation rates are probabilistic and viral quasispecies are indeterminate. Nevertheless, we can be confident that all future viruses will arise from those now extant: they will be mutants, recombinants, and reassortants.

Emerging Viruses

As far as we know, humans have suffered for millions of years from infectious diseases. However, since the rise of agriculture (the past 11,000 years), new infectious agents have invaded human populations primarily because these infections (e.g., measles and smallpox) can be sustained only in large, dense populations that were unknown before agriculture and commerce. The source of these emerging infectious agents is a popular topic of research, debate, and concern.

We define an **emerging virus** as the causative agent of a new or hitherto unrecognized infection in a population. Occasionally, emerging infections are manifestations of expanded host range with an increase in disease that was not previously obvious. More generally, emerging infections of humans reflect transmission of a virus from a wild or domesticated animal with attendant human disease (**zoonotic infections**). Occasionally, a cross-species infection will establish a new virus in a population (e.g., human immunodeficiency virus moving from chimpanzees to humans). On the other hand, a similar cross-species infection will emerge in certain human populations, but the infection cannot be sustained (e.g., Ebola and Marburg viruses moving from bats to humans).

While the term "emerging virus" became part of the popular press in the 1990s (usually with dire implications ["killer viruses on the loose"]), emerging viruses are not new to virologists, public health officials, and epidemiologists. These infections have long been recognized as an important manifestation of virus evolution. The most important factors driving the emergence of infectious diseases include unprecedented human population growth and large-scale change occurring in all ecosystems brought about by human occupation of almost every corner of the planet. The convergence of these factors drives viral emergence (Fig. 10.7). In recent years, emerging infections not only have been increasing in absolute frequency, but also are more easily detected because of advances in technology and better communication of disease outbreaks. Indeed, global communication has brought some emerging viral infections to center stage on the local news. Unfortunately, the lay public often know the name of the virus, but cannot pinpoint the location of the outbreak on a map or articulate its significance. Anyone with access to television, radio, the Internet, or newspapers knows something about SARS, West Nile virus, Ebola virus, and certainly H5N1 avian influenza virus. Some examples of less well known emerging virus outbreaks are given in Table 10.3. Despite the many different viruses and geographical locations of these outbreaks, some common parameters do exist. These parameters define the rules of engagement for viruses and their potential hosts.

The Spectrum of Host-Virus Interactions

The spectrum of possible interactions among hosts and viruses may appear too complex for analysis. However, it is constructive to simplify the variables and consider only the general domains of these interactions. To illustrate this principle, we define four hypothetical interactions: **stable, evolving, dead-end,** and **resistant** (Fig. 10.8). These definitions are arbitrary snapshots of the extremes of dynamic host-virus interactions designed to emphasize essential concepts. The arrows suggest the hypothetical

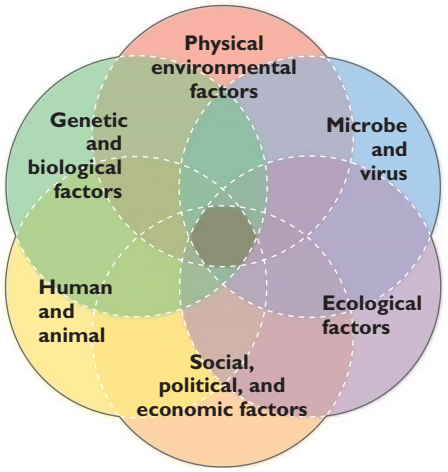


Figure 10.7 The convergence model for emerging viral infections. The provenance of six factors leads to convergence and the potential emergence of an infectious disease. The overlapping territories are obvious, but the dark center represents maximum convergence of these factors plus other unpredictable interactions. The point is that in the ecology of virus-host interactions, many factors are interlocking and interconnected. From the Institute of Medicine study *Microbial Threats to Health, Emergence, Detection and Response*, 2003.

transmission of infection; they stress the continuity of viral interactions in nature. In addition, it is important to understand that these definitions apply to large populations and **not** to a single virus-host interaction. In this hypothetical set of interactions, emerging viral infections are defined as human infections that derive from stable host-virus interactions preexisting in nonhuman hosts.

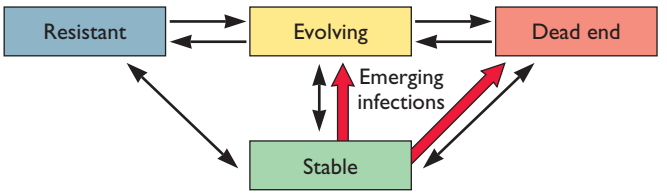


Figure 10.8 The general interactions of hosts and viruses. Four hypothetical host-virus interactions are indicated in the boxes. The **stable interaction** maintains the virus in the ecosystem. The **evolving interaction** describes the passage of a virus from "experienced" populations to naive populations in the same or other host species. The **dead-end interaction** represents one-way passage of a virus to different species. The host usually dies, or if it survives, the virus is not transmitted efficiently to the new host species. The **resistant host interaction** represents situations in which the host completely blocks infection. The arrows indicate possible transmission of infection from one situation to another or the possible transformation of one interaction into another. The red filled arrows indicate the sources of zoonotic (emerging) infections.

Stable Interactions

Stable host-virus interactions are those in which both participants survive and multiply. Such interactions are essential for the continued existence of the virus, and may influence host survival as well. This state is optimal for a host-parasite relationship, but the interaction need be neither benign nor permanent in an outbred population. Infected individuals can become ill, recover, develop immunity, or die, yet in the long run, both populations survive. While this situation is often described as an equilibrium, the term is misleading. The interactions

Table 10.3 Some examples of viruses that cause zoonotic infections

Virus	Family	Emergence factors
Dengue virus	*Flaviviridae*	Urban population density; open water storage favors mosquito breeding (e.g., millions of used tires)
Ebola virus	*Filoviridae*	Human contact with unknown natural host in Africa; importation of monkeys in Europe and the United States
Hantaan virus	*Bunyaviridae*	Human contact with rodents as a result of agricultural techniques
Human immunodeficiency virus	*Retroviridae*	Transfusions and blood products; sexual transmission; needle transfer during drug abuse
Human T-lymphotropic virus	*Retroviridae*	Transfusions and blood products; contaminated needles; social factors
Influenza virus	*Orthomyxoviridae*	Integrated pig-duck agriculture; mobile population
Junin virus	*Arenaviridae*	Agriculture techniques favor human contact with rodents
Noroviruses	*Caliciviridae*	New methods for detection; infectious diarrhea
Machupo virus	*Arenaviridae*	Agriculture techniques favor human contact with rodents
Marburg virus	*Filoviridae*	Unknown; importation of monkeys in Europe
Rift Valley virus	*Bunyaviridae*	Dams, irrigation
Sin Nombre virus	*Bunyaviridae*	Natural increase of deer mice and subsequent human/rodent contact
West Nile virus	*Flaviviridae*	Unknown introduction into United States

are dynamic and fragile, and certainly are rarely reversible. Viral populations may come to be more or less virulent if such a change enables them to be maintained in the population, while hosts may evolve mechanisms that attenuate the more debilitating effects of the viruses that infect them.

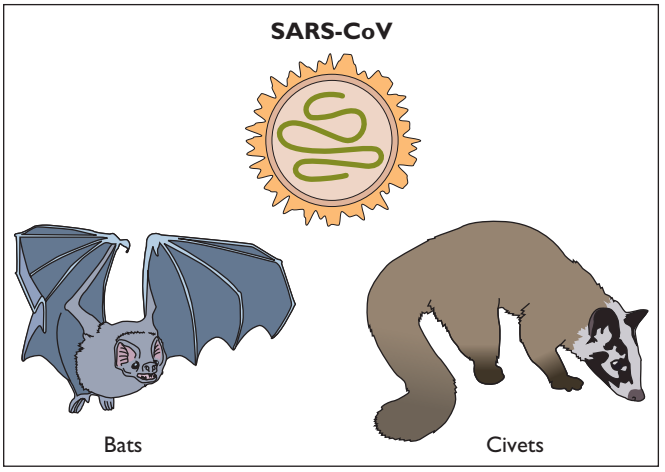
Some stable interactions are effectively permanent. For example, humans are the sole natural host for a several viruses, including measles virus, herpes simplex virus, human cytomegalovirus, and smallpox virus. Similarly, simian cytomegalovirus, monkeypox virus, and simian immunodeficiency virus infect only certain species of monkeys. Stable interactions can also include infection of more than one host species with the same virus. For example, influenza A virus, flaviviruses, and togaviruses, are capable of propagating in a variety of species. Indeed, many of the flaviviruses and togaviruses replicate efficiently in some insects as well as in mammals and birds. In these instances, the host that maintains the virus may not be apparent or obvious. Influenza A virus infects humans, wild birds, and pigs, but birds may be its natural host. This conclusion is discussed below.

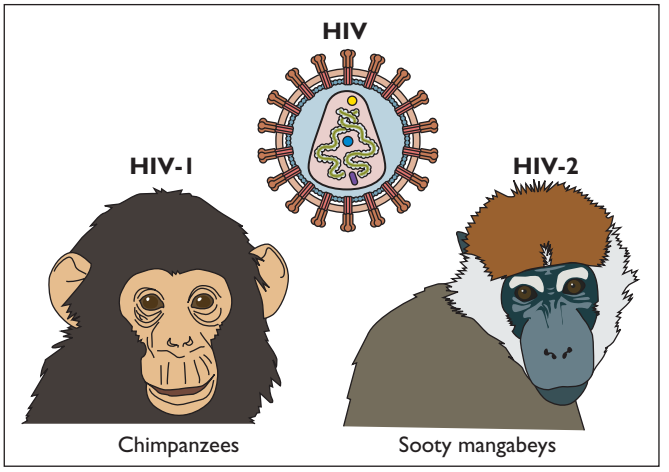
Establishment of a stable host-virus interaction is not necessarily the optimal solution for survival. The trajectory of evolution is unpredictable: what is successful today may be suicidal at another time. Once a virus population becomes completely dependent on one, and only one, host, it has entered a potential bottleneck that may constrain its further evolution. If the host becomes extinct for whatever reason, the virus is also likely to be exterminated. If humans disappeared, many virus populations, including poliovirus, measles virus, and several herpesviruses, would cease to exist. Eradication of natural smallpox virus was possible because humans are the only hosts and worldwide immunization was achieved.

The Evolving Host-Virus Relationship

This relationship emphasizes that stability is unlikely to be established instantly (Fig. 10.9). We highlight this interaction to illuminate the dynamic consequences of a virus spreading among populations of the same or perhaps closely related species. The hallmarks of this interaction are instability and unpredictability. These properties are to be expected, as selective forces are applied to both host and virus, and are magnified when host populations are small. For example, some host subpopulations may experience high infection rates, while others are unaffected. The outcome of infection may range from relatively benign symptoms to death. Such an interaction is typified by the introduction of smallpox and measles to natives of the Americas by Old World colonists and slave traders. Europeans previously had experienced the same horror when these diseases spread to Europe from Asia. Other opportunities to enter the evolving host-virus interaction may arise if the virus in a stable interaction acquires a new property that increases its virulence or spread, or if the host population suffers a far-reaching catastrophe that reduces resistance (e.g., famine or mass population changes during wars). The introduction of West Nile virus into the Western Hemisphere in 1999 provides a contemporary example of an evolving host-virus interaction (Box 10.14).

Figure 10.9 Sources of three well-known emerging infections. (Left) SARS human coronavirus (SARS-CoV) is endemic in bats. Bats can spread infection to certain wild mammals that can, in turn, spread infection to humans. **(Right)** The progenitors of human immunodeficiency virus types 1 and 2 cause natural infections of chimpanzees and sooty mangabeys, respectively.



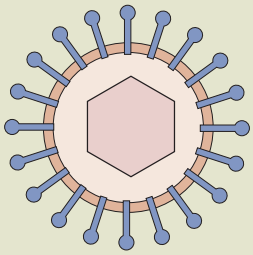


BOX 10.14 DISCUSSION

An evolving virus infection: the West Nile virus outbreak

In 1999, virologists had the opportunity to observe a virus population establish a new geographic niche. The West Nile virus, an Old World flavivirus discovered in 1937 in the West Nile district of Uganda, had never been isolated in the Western Hemisphere. In August 1999, six people were admitted to Flushing Hospital in Queens, NY, with similar symptoms of high fever, altered mental status, and headache. They were subsequently discovered to be infected with West Nile virus. The virus has now spread from the Atlantic to the Pacific, as well as to Canadian provinces and territories. In the summer of 2002, it reached epidemic status, causing encephalitis in hundreds of cases.

The New York isolate of West Nile virus is nearly identical to a virus isolated in 1998 from a domestic goose in Israel during an outbreak of the disease. The close relationship between these two isolates suggests that the virus was brought to New York City from Israel in the summer of 1999. How it crossed the Atlantic will probably never be known for sure, but it might have been via an infected bird, mosquito, human, horse, or other vertebrate host. These events mark the first introduction in recent history of an Old World flavivirus into the New World. A fascinating, and yet unanswered, question is why the infection was established in New York City. The summer of 1999 was particularly



hot and dry. Similar conditions spawn outbreaks of West Nile virus encephalitis in Africa, the Middle East, and the Mediterranean basin of Europe. Such conditions may promote mosquito breeding in polluted, standing water.

The infection spreads via many species of mosquitoes and is now circulating in wild birds. The virus or virus-specific antibodies have been found in more than 157 species of birds, but crows and jays appear to be particularly sensitive. Many zoos are reporting deaths of their exotic birds from West Nile virus infections. The virus has been found to infect at least 37 kinds of mosquitoes and 18 other vertebrates.

Humans and other animals acquire infections by mosquito bites after the insect has fed on infected birds. Human infections may be spread by transfusion from an infected donor, a possibility with far-reaching implications for our blood supply. Horses develop a lethal encephalitis, hundreds of cases of which have been reported. A vaccine for horses is available.

About 20% of infected humans experience flu-like symptoms, but only 1 in 150 of these individuals develop meningitis, encephalitis, or poliomyelitis-like symptoms. West Nile virus infection claimed a total of 564 lives in the United States in the 5 years from 1999 to 2003. In 2007, the Centers for Disease Control and Prevention reported 906 cases of West Nile virus infection with 26 fatalities.

By 2007, the North American epidemic was resolving as the virus became established. Sequence analysis indicates that viral populations were more diverse at the start of the epidemic than they are now. Until we understand the complex ecology of this viral infection, the consequences for public health are difficult to predict.

For an update on West Nile Virus, see http://www.cdc.gov/ncidod/dvbid/westnile/index.htm.

Brinton, M. A. 2002. The molecular biology of West Nile virus: a new invader of the Western Hemisphere. *Annu. Rev. Microbiol.* **56:**371–402.

Briese, T., and K. Bernard. 2005. West Nile virus—an old virus learning new tricks? J. *Neurovirol.* **11:**469–475.

Despommier, D. 2001. *West Nile Story*. Apple Trees Productions LLC, New York, NY.

Snapinn, K. W., E. C. Holmes, D. S. Young, K. A. Bernard, L. D. Kramer, and G. D. Ebel. 2007. Declining growth rate of West Nile virus in North America. *J. Virol.* **81:**2531–2534.

The Dead-End Interaction

A commonly encountered host-virus relationship is described rather vividly as a **dead-end interaction**. It has much in common with the evolving host-virus interaction, as both represent departures from a stable relationship, often with lethal consequences. A dead-end interaction is a frequent outcome of cross-species infection (but not of intraspecies infections). A **zoonosis** is a disease or infection that is naturally transmitted between vertebrate animals and humans. Unanticipated viral zoonoses often are classic cases of emerging viral infections. In many cases, the host is killed so quickly that there is little or no subsequent transmission of the virus to others. In other cases, the newly infected host is incapable of transmitting the infection to other individuals of the same species

The dead-end interaction is often observed with the many viruses carried by arthropods like ticks and mosquitoes that cycle in the wild between insects and a vertebrate host in a stable relationship. Occasionally the infected insect bites a new species (e.g., humans) and transmits the virus (Fig. 10.10). As a consequence, the infection results in severe pathogenic effects in the infected human, but is not transmitted further; i.e., it reaches a dead end. As the human is not part of the natural, stable host-virus relationship, these infections have little if any effect on the evolution of the virus and its natural host. To a first approximation, dead-end hosts are transparent participants. However, such logic may be too simplistic; infection by a less virulent mutant or infection of a more resistant individual may be the first step in establishing a new host-virus interaction.

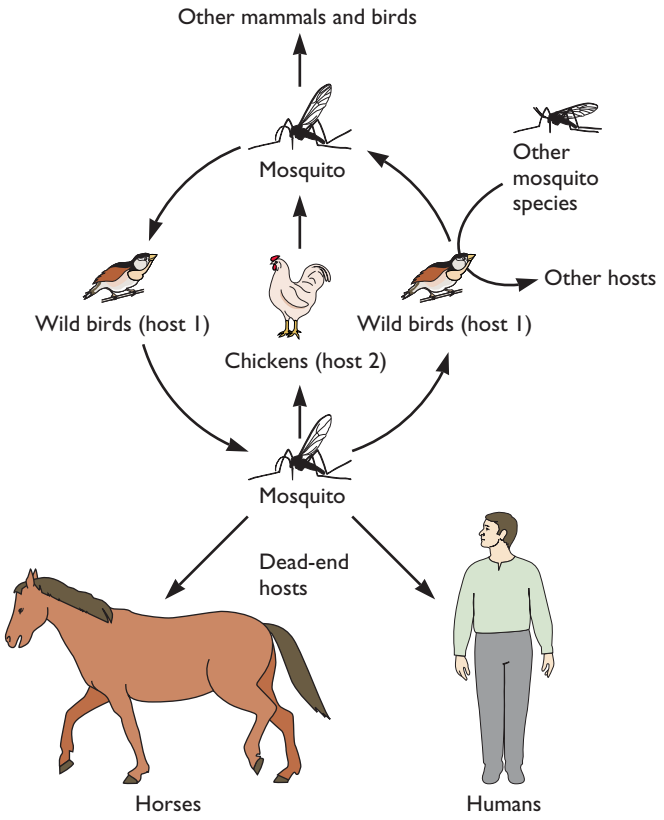


Figure 10.10 The dead-end host scenario as illustrated by a complex host-virus relationship. This arbovirus infection illustrates how multiple host species can maintain and transmit a virus in the ecosystem. In this example, the virus population is maintained in two different hosts (wild birds and domestic chickens) and is spread among individuals by a mosquito vector. The virus replicates in both species of bird and in the mosquito. Disease is likely to be nonexistent or mild in these species, as the hosts have adapted to the infection. A third host (in this example, horses or humans) occasionally is infected when bitten by a mosquito that previously fed on an infected bird. Horses and humans are dead-end hosts and contribute little to the spread of the natural infection, but they may suffer from serious, life-threatening disease. Occasionally another species of biting insect (e.g., other mosquito species) can feed on an infected individual (bird, horse, or human) and then transmit the infection to another species not targeted by the original mosquito vector.

Human diseases like yellow fever and dengue fever provide excellent examples of the dead-end interaction (Box 10.15). The viruses causing these diseases are endemic in the tropics and maintain a stable relationship with their natural host and insect vectors. When humans begin to develop a tropical area by clearing forests and building roads, dams, canals, and towns, they are at risk for being bitten by mosquitoes and other insects. The natural host-virus interaction that existed before the intrusion changes, and humans may experience new viral infections spread by insect vectors. A similar situation exists for some flaviviruses transmitted by ticks, which in turn are carried by rodents. Humans often experience European tick-borne encephalitis as dead-end hosts (Fig. 10.11).

The lethality of the Marburg and Ebola filoviruses in humans is typical of dead-end host infection. Filoviruses are single-stranded, (–) RNA viruses causing severe hemorrhagic manifestations in infected humans (Appendix A). Disease onset is sudden, with 25 to 90% fatality rates reported. Virus spreads through the blood and replicates in many organs, causing focal necrosis of the liver, kidneys, lymphatic organs, ovaries, and testes. Capillary leakage, shock, and acute respiratory disorders are observed in fatal cases. Patients usually die rapidly of intractable shock without evidence of an effective immune response. Even when recovery is under way, survivors do not have detectable neutralizing antibodies. The infection clearly overwhelms a particular individual, but apparently does not spread widely; human infections tend to cluster in local areas. Such viruses can be transmitted to other humans only by close personal contact with infected blood and tissue. Although the natural hosts for many filoviruses remain elusive, these viruses must have established a stable interaction with an animal host, and infect humans only inadvertently. Some investigators have argued that the natural host is responsible for the apparent "geographic containment" of Ebola virus in central Africa. Humans are not the only dead-end hosts for Ebola virus: gorillas are susceptible, and large numbers have died from Ebola virus infection.

Many animal models of disease might be considered examples of dead-end interactions. For example, herpes simplex virus is a human virus, but when it is introduced into mice, rabbits, or guinea pigs in the laboratory, these animals become infected and show pathogenic effects that mimic some aspects of the human disease. However, in their natural environment, these animals contribute nothing to the transmission or survival of the virus, and therefore provide experimental models of the dead-end interaction.

Rodent vectors play critical roles in the introduction of new viruses into populations in areas where these animals abound. Most hemorrhagic disease viruses, including Lassa, Junin, and Sin Nombre viruses, are endemic in rodents, their natural hosts. The viruses establish a persistent infection, and the rodents show few if any ill effects. Substantial numbers of virus particles are excreted in urine, saliva, and feces to maintain the virus in the rodent population. However, infection by such rodent viruses can cause local, often lethal, outbreaks in humans as dead-end hosts. Humans become infected only because they happen to come in contact with rodent excretions containing infectious virus particles.

BOX 10.15 DISCUSSION

Yellow fever virus: humans change the pattern and pay the price

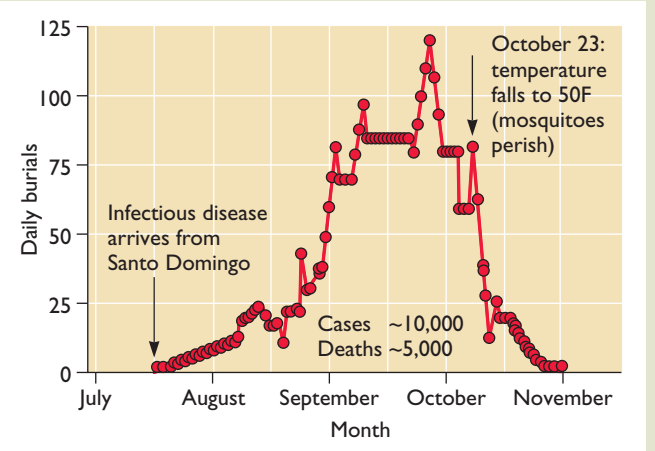
In tropical forests, yellow fever virus is maintained by a monkey-mosquito cycle. Neither the monkey nor the mosquito is the worse for wear. Various mosquito species serve as vectors, including *Aedes* species in Africa and *Haemagogus* species in the Americas. If humans blunder into mosquito-infected areas, they stand a chance of being bitten by the infected insects and contracting the disease. Yellow fever came to the New World with the colonists and the slave trade, where it wreaked havoc among the indigenous human populations.

Humans were not the only species to suffer from the invasion. New World monkeys also died when infected with yellow fever virus, indicating that they were as unequipped to handle this new infection as were the indigenous humans.

Yellow fever virus and its mosquito vector spread by ship to the burgeoning populations of the U.S. South and East Coast with ease. Cities hit hard were New Orleans; Charleston, SC; Philadelphia; New York; and Boston. In 1800 Thomas Jefferson lamented, "Yellow Fever will discourage the growth of great cities in our nation." In a striking example of an emerging disease, 15% of the population of Philadelphia died of yellow fever in 1793. Neighboring New Jersey and Maryland attempted to bar panicked Philadelphians from entering their states.

The contribution of mosquitoes to the transmission of yellow fever was first glimpsed by Carlos Finlay in Cuba in 1880 and was established firmly by Walter Reed in 1900 when the disease was a problem during construction of the Panama Canal in the Central American jungle. A vaccine was developed in 1937 to contain the disease. Even with the vaccine, more than 10,000 people die of yellow fever every year in South America.

Yellow fever deaths in Philadelphia in the summer and early fall of 1793. From N. Nathanson, *ASM News* **63:**83–88, 1997, with permission.



Bats are vectors of several dead-end, zoonotic infections (Fig. 10.9 and 10.12). Hendra virus, Nipah virus, rabies virus, and the progenitor of the SARS coronavirus are known to enter the human population via bats. The Old World fruit bats (genus *Pteropus*), commonly called flying foxes, are widely distributed in southeast Asia, Australia, and the Indian subcontinent. These fruit bats naturally harbor Hendra and Nipah viruses. Despite having high antibody titers, the animals exhibit no obvious disease. High virulence in humans and our complete lack of therapeutic interventions require that these viruses be studied only under the highest biological and physical containment (biosafety level 4 [BSL4]). Accordingly, we know very little about their biology and pathogenesis. In addition, we know next to nothing about fruit bat ecology and how humans and livestock become infected. Considerably more effort is required in the field before we can begin to understand the epidemiology of many zoonotic infections that produce the dead-end host scenario.

The Resistant Host

All living things are exposed continuously to viruses of all types, yet the vast majority of these interactions are uneventful. This may be because the host cells are not susceptible, not permissive, or both. A more likely possibility is that the primary physical, intrinsic, and innate defenses are so strong that potential invaders are diverted or destroyed upon contact. In other cases, organisms may become infected and produce some virions, but the infection is cleared rapidly without activation of the host's acquired immune system (Chapter 5). This outcome is in contrast to an inapparent infection, in which an immune response is mounted but the individual exhibits no signs of disease.

We include the resistant host in our analysis because the transition between the inability and ability of a host to support viral replication need not be insurmountable. Indeed, **xenotransplantation** (the use of animal organs in humans) is thought provoking. Not only does transplantation bypass physical and innate defenses by surgery,

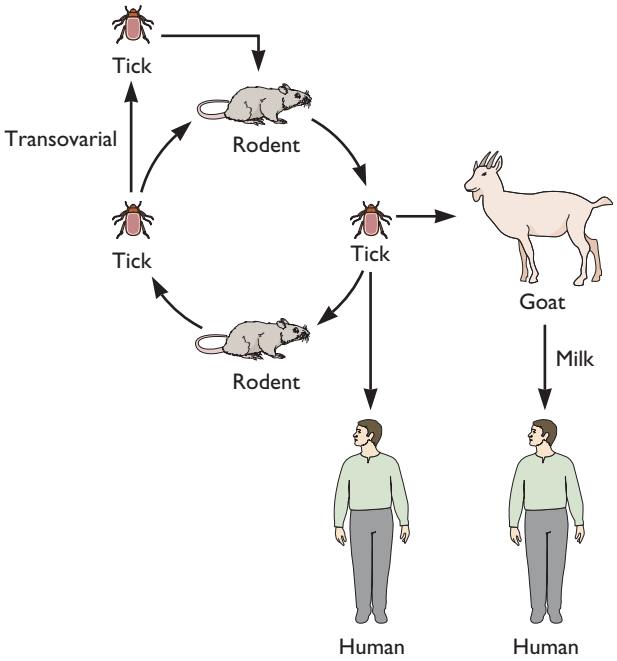


Figure 10.11 Replicative cycle of the central European tick-borne flavivirus may involve zoonotic infections. European tick-borne flavivirus infection is maintained and spread by multiple host infections. Congenital transmission in the tick maintains the virus in the tick population as they feed upon rodents. The infected newborn ticks have adapted to the infection and thrive. The virus is transmitted from the tick to a variety of animals, including cows, goats, and humans. Humans can also be infected by drinking milk from an infected goat, sheep, or cow. This zoonotic infection is another example of the dead-end host interaction.

but also drugs suppress the immune response. As a result, virus particles or genomes in xenografts have direct access to the once-resistant host in the absence of crucial antiviral defenses. As many of these viruses can infect human cells or have close, human-adapted relatives, the xenotransplantation patient represents a source of new viral diversity. The outcome of such an experiment in viral evolution is not predictable, and the experiment should not be attempted.

Encountering New Hosts: Fundamental Problems in Ecology

All virus-host interactions are governed by the concentrations of the participants and the probability of productive encounters. Rare chance encounters of viruses with new hosts may give rise to infections that are never seen, or at least never appreciated. These rare single-host infections may not be transmitted among humans for any number of reasons including insufficient quantity of progeny virus shed, limited duration of shedding, and small numbers of

Fruit bats (flying foxes): a source of zoonotic infections

- Four previously unknown paramyxoviruses isolated from flying foxes since 1995, including Nipah and Hendra viruses
- Three cause severe disease in domestic animals (horses and pigs) and are known to infect humans

Figure 10.12 Emergence of Nipah and Hendra viruses from natural infections of fruit bats. All of the *Pteropus* species are considered flying foxes. *Pteropus vampyrus* is the Malayan flying fox (found in peninsular Malaysia), and *Pteropus conspicillatus* is the spectacled flying fox (found in far northern Queensland, Australia, and Papua New Guinea). *P. vampyrus* is one of the species that carries Nipah virus, and *P. conspicillatus* carries Hendra virus. Bat photograph courtesy of Juliet Pulliam, Princeton University. See B. Eaton et al., *Nat. Rev. Microbiol.* **4:**23–35, 2006.

new human hosts exposed to the infected individual. In addition, the progeny virus produced in the new host may not have the genetic repertoire to facilitate high levels of replication and transmission.

Several ecological and social parameters facilitate the transmission of infection to new hosts in natural populations (Tables 10.4 and 10.5). Living together and sharing resources facilitates inter- and intraspecies transmission. Droughts concentrate many species at water holes; destruction of habitat forces new interactions. Predators eat their prey and become unwitting "test tubes" for cross-species infection by viruses found in tissues of the prey, no matter

Table 10.4 Human actions can promote large-scale changes in virus ecology

Air travel
Dams and water impoundments
Irrigation
Rerouting of wildlife migration patterns
Wildlife parks
Hot tubs
Air conditioning
Blood transfusion
Xenotransplants
Long-distance transport of livestock and birds
Moral and societal changes with regard to drug abuse and sex
Massive deforestation
Millions of used tires
Uncontrolled urbanization
Day care centers

Table 10.5 Ecological and social parameters facilitate transmission of infection to new hosts

Transmission parameter	Action or example
Contact with bodily fluids of infected hosts	Predation, hunting, and consumption of wild game; intimate contact with infected animals in the wild, at zoos, or in the home
Sharing of a resource with different species	Infected fruit bats, pigs, and humans share the same space
Sharing of insect or rodent vectors	Japanese encephalitis virus infection is spread by mosquitoes that feed on herons, people, and pigs
Encroachment by one species into the habitat of another	Humans enter the jungle and are bitten by mosquitoes that are part of a virus-bird infectious cycle

if aerosols, excretion, or close mucosal contact normally transmits the infection.

Successful Encounters Requires Access to Susceptible and Permissive Cells

Potential new hosts must have cells with accessible receptors that can engage virion ligands. The influenza virus hemagglutinin protein has a high affinity for sialosaccharides found on the cell surfaces of many different host species. The linkage of the terminal sialic acid/galactose residues plays a crucial role in tropism. Avian influenza virions bind sialic acid α(2,3)-galactose-terminated oligosaccharides, whereas the human influenza virus hemagglutinin proteins bind tightly to oligosaccharides carrying a terminal α(2,6)-linked galactose. Cells of the human respiratory tract do display the α(2,3)-galactose-terminated oligosaccharides, but they lie deep in respiratory tissues. Conversely, sialic acid with terminal α(2,6) linkages abounds in the more accessible regions of the upper respiratory tract. This anatomical fact appears to be a prime reason why humans cannot be infected easily with avian influenza viruses.

Nipah virus was first identified during an outbreak in swine and humans in Malaysia in 1998 and 1999 (Fig. 10.12). Nipah virus infection of bats apparently is nonpathogenic, but copious quantities of virions are secreted in urine and feces. Two salient facts are that pig farmers often plant mangoes and durian trees next to pig pens, and fruit bats are messy eaters. When pigs come in contact with partially eaten contaminated fruit, they suffer a respiratory disease, and efficiently spread virions in the environment by sneezing and via mucous secretions.

For reasons that only now are becoming clear, bats and pigs established a one-way conduit for a zoonotic infection of humans. In rural Indonesian communities, humans often share accommodation with domestic swine, facilitating zoonotic infection. In addition, slaughterhouse workers are exposed to infected animals. Remarkably, when Nipah virus infects humans, it causes encephalitis, not respiratory infection. While often lethal for the infected human (Nipah virus killed 105 of 265 infected people in the Malaysian outbreak mentioned above), the infection is contained in infected brain tissue and does not spread. This state of affairs is apparently changing: Nipah virus isolated in India can infect the human upper respiratory tract, and these strains spread efficiently among humans in close contact.

Population Density and Health Are Important Factors

Two predominant parameters influencing the spread of infection are the population density and the health of individuals in that population. Close personal contact, either by direct methods (e.g., aerosols or sexual contact) or by indirect exposure (e.g., water or sewage), is also required. Variables such as duration of immunity and the quantity of virions produced and shed from each individual have marked effects on spread of infection. As discussed in Chapter 5, at least half a million people in a more or less confined urban setting are required to ensure a large enough annual supply of susceptible hosts to maintain measles virus in a human population. When this large population of interacting hosts is not available, measles virus dies out. One can reduce the effective population by splitting the half million hosts into small groups physically separated from social discourse, as exemplified by quarantine or the use of sanitariums to isolate infected patients. Another way is by immunizing the group such that the large majority are immune and cannot propagate the virus. If these or similar actions are not taken, our ever-expanding and increasingly interactive human population will ensure the maintenance and continued evolution of measles virus.

The age distribution of any potential host population is also important. For example, babies and the elderly are commonly more susceptible to a given virus than is the general population and, consequently, serve as sources of transmission. Predictably, prevention of infection in these groups tends to reduce the overall infection rate in the population at large. The distribution of poor and wealthy individuals in a population can influence infection rates. Malnourished individuals are more susceptible to disease than those who are well fed.

The age, health, and genetic variation in any given outbred host population contribute to the unpredictable nature of viral infection. Perhaps not so obvious are the

other variables that modulate infection, notably, seasonal variations. Respiratory infections caused by adenoviruses and rhinoviruses often occur in the spring, but respiratory infections caused by coronaviruses and respiratory syncytial viruses tend to occur in the winter. Most arbovirus infections are experienced in the summer. While it is clear that insect-mediated infections are not likely to occur when temperatures drop below freezing for months at a time, the seasonal variations for other viral diseases are not easy to explain.

The Need for Experimental Analysis of Host-Virus Interactions

The definitions of different host-virus interactions listed above highlight some of the problems of coming to grips with virus evolution in natural populations. How do we do experiments and test hypotheses? Unfortunately, such experiments are complicated by a variety of issues, including safety, cost, and social and political issues, as well as by the uncertainty of the relevance of laboratory models to natural disease and virus spread. Natural infections occur in outbred populations in complicated settings with unknown ecological parameters, features that are difficult to model in the laboratory. To understand the mechanics of viral evolution, we must be able to quantify the relationship between viral virulence (ability to cause disease) and the rate of transmission. We need facts and figures to determine the importance of interspecies transmission in the establishment of new viruses. More quantitative and qualitative data are required to understand the variables that maintain quasispecies. Certainly, if we are to understand viral evolution and emerging viral infections, we must be knowledgeable about basic viral ecology.

We understand in principle that most virus populations survive in nature only because of **serial infections** among individuals (a chain of transmission). Quantitative measures of these interactions, determination of the molecular mechanisms responsible for them, and development of model systems that can predict them are sorely needed. Mathematical models are being developed to describe patterns of disease transmission in complex groups. Such models should help to determine the critical population size necessary to support the continual transmission of viruses with differing incubation periods, and the dynamics of persistent viral infections. The value of such models remains to be seen, simply because it is difficult to perform a controlled experiment in nature.

Learning from Accidental Natural Infections

Our understanding of the dynamics of a viral infection in a large outbred human population is rudimentary, at best. What we do know is based predominantly on a limited number of accidental "experiments." Two classic examples are provided by hepatitis B virus and poliovirus infections. During World War II, large doses of infectious hepatitis B virus were accidentally introduced into approximately 45,000 soldiers when they were injected with a contaminated yellow fever vaccine. Surprisingly, only 900 (2%) came down with clinical hepatitis, and fewer than 36 developed severe disease. Similarly, in 1955, 120,000 school-aged children were vaccinated with an improperly inactivated poliovirus vaccine. About half had preexisting antibodies to poliovirus thanks to inapparent infections by wild virus. Of the remainder, about 10 to 25% were infected by the vaccine, as determined by the appearance of antibodies. More than 60 cases of paralytic poliomyelitis were documented among these infected children, and the remainder escaped disease. These two experiments tell us that even when a large number of individuals are infected with a virulent virus, the outcome cannot be predicted; we have only a rudimentary understanding of why this is so. One of the more classic cases of deliberate release of a virus and the resulting effects on a wild host population is the attempt to use viral infection to rid Australia of rabbits (Box 10.16).

Expanding Viral Niches: Snapshots of Selected Emerging Viruses

Poliomyelitis: a Disease of Modern Sanitation

Host populations change with time, and each change can have unpredictable effects on virus evolution. Analysis of poliomyelitis, a diseased caused by poliovirus infection, provides an instructive example. The disease is ancient, postulated by some to be present over 4,000 years ago (see Volume I, Chapter 1). For centuries, the host-virus relationship was stable, and infection was endemic in the human population. Poliomyelitis epidemics were unheard of (or, at least, not written about), but we imagine that occasional bouts of disease were obvious in scattered areas. This state of affairs changed radically in the first half of the 20th century, when large annual outbreaks of poliomyelitis appeared in Europe, North America, and Australia. Retrospective analysis established that the viral genome did not change or evolve substantially (Fig. 10.13A). How can the emergence of epidemic poliomyelitis be explained?

The answer is that humans changed their lifestyle on an unprecedented scale. Poliomyelitis is caused by an enteric virus spread by oral-fecal contact. As a consequence, endemic disease was characteristic of life in rural communities, which generally had poor sanitation and small populations. Because virions circulated freely, most children were infected at an early age and developed antibodies to at least one of the serotypes. Maternal

BOX 10.16 EXPERIMENTS

A classic experiment in virus evolution: deliberate release of rabbitpox virus in Australia

In 1859, 24 European rabbits were introduced into Australia for hunting, and, lacking natural predators, the friendly rabbits reproduced to plague proportions. In 1907, the longest unbroken fence in the world (1,139 miles long) was built to protect portions of the country from invading rabbits. Such heroic actions were to no avail. As a last resort, the rabbitpox virus, myxoma virus, was released in Australia in the 1950s in an attempt to rid the continent of rabbits. The natural hosts of myxoma virus are the cottontail rabbit, the brush rabbit of California, and the tropical forest rabbit of Central and South America. The infection is spread by mosquitoes, and infected rabbits develop superficial warts on their ears. However, European rabbits are a different species and are killed rapidly by myxoma virus. In fact, the infection is 90 to 99% fatal!

In the first year, the infection was amazingly efficient in killing rabbits, with a 99.8% mortality rate. However, by the second year the mortality dropped dramatically to 25%. In subsequent years, the rate of killing was lower than the reproductive rate of the rabbits, and hopes for 100% eradication were dashed. Careful epidemiological analysis of this artificial epidemic provided important information about the evolution of viruses and hosts.

The infection spread rapidly during spring and summer, when mosquitoes are abundant, but slowly in winter, as expected. Given the large numbers of rabbits and virus particles, and the almost 100% lethal nature of the infection, advantageous mutations were quickly selected. Within 3 years, less-virulent viruses appeared, as did rabbits that survived the infection. The host-virus interaction observed was that predicted for an evolving host coming to an equilibrium with the pathogen. A balance is struck: some infected rabbits die, but they die more slowly, and many rabbits survive.

What was learned? To paraphrase the Rolling Stones: you always get what you select, but you don't often get what you want. Probably the most obvious lesson was that the original idea to eliminate rabbits with a lethal viral infection was flawed. Powerful selective forces that could not be controlled or anticipated were at work. In this experiment, the viral genomes acquired mutations resulting in an attenuated infection: fewer rabbits were killed, infected rabbits were able to survive over the winter, and in the spring mosquitoes spread the infection. Moreover, rabbits that were more resistant to, or tolerant of, the infection were selected.

Surprisingly, more experiments in the biological control of rabbits are under way in Australia. One line of experimentation uses a lethal rabbit calicivirus, while another employs a genetically engineered myxoma virus designed to sterilize, but not kill, rabbits. The latter viruses encode a rabbit zona pellucida protein, and infected rabbits synthesize antibodies against their own eggs (so-called immunocontraception).

antibodies, which protect newborns, were also prevalent, as most mothers had experienced a poliovirus infection at least once. An important consideration is that most infected children do not develop paralysis, the most visible symptom of poliomyelitis. Paralysis is a more frequent result when older individuals are infected. Even the most virulent strains of poliovirus cause 100 to 200 subclinical infections (inapparent infections) for every case of poliomyelitis. These inapparent infections in children provided a form of natural vaccination. As childhood disease and congenital malformations were not uncommon in rural populations, the few individuals who developed poliomyelitis were not seen as out of the ordinary. No one noticed endemic poliovirus.

However, during the 19th and 20th centuries, industrialization and urbanization changed the pattern of poliovirus transmission. Improved sanitation broke the normal pattern and effectively stopped natural vaccination. In addition, dense populations and increased travel provided new opportunities for rapid spread of infection. As a result, children tended to encounter the virus for the first time at a later age, without the protection of maternal antibodies, and were at far greater risk for developing paralytic disease. Consequently, epidemic poliovirus

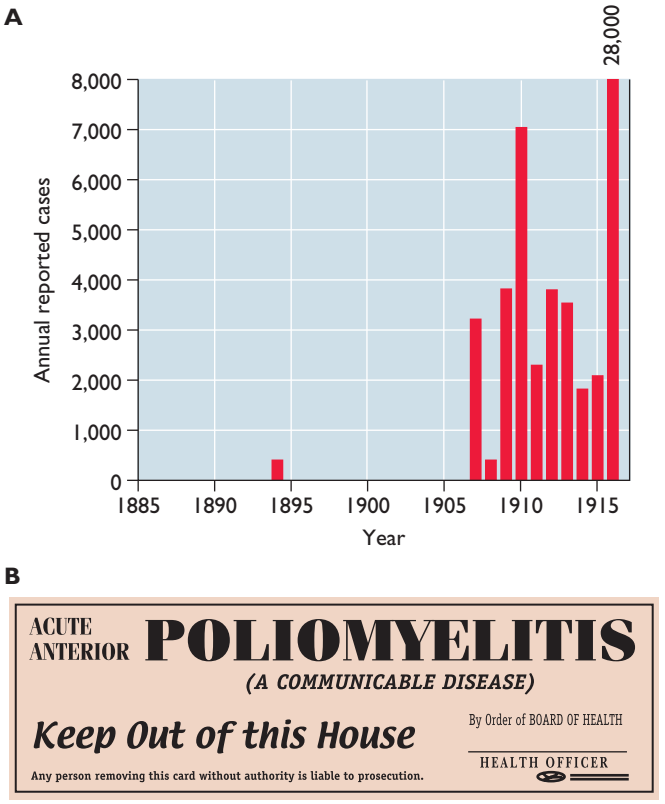


Figure 10.13 Poliovirus in the early 20th century. (A) The emergence of paralytic poliomyelitis in the United States, 1885 to 1915. From N. Nathanson, *ASM News* **63:**83–88, 1997, with permission. **(B)** Board of Health quarantine notice, San Francisco, CA, circa 1910.

infections emerged time and time again in communities across Europe, North America, and Australia. Until vaccines became available, quarantine was the only public health defense (Fig. 10.13B).

Widespread use of inexpensive, effective poliovirus vaccines has since controlled the epidemic. As the virus has no host other than humans, it should be possible to eradicate it by vaccinating sufficient people to end the spread of the virus. Accordingly, the World Health Organization targeted the eradication of poliovirus by 2005 with a massive worldwide vaccination program. The goal was not achieved because of social, religious, and political variables that are difficult if not impossible to control. Poverty, social problems, and economic conditions often conspire to prevent vaccination of children in inner cities and in poorer countries. There is hope that poliomyelitis will soon be a disease of the past, as a consequence of the worldwide poliovirus eradication program. However, given that it may be impossible to eliminate all sources of the virus, vaccination may be part of public health programs indefinitely.

Smallpox and Measles: Diseases of Exploration and Colonization

Explosive epidemic spread may occur when a virus enters a naive population (**the evolving host-virus interaction**) (Boxes 10.15 and 10.16). The resulting infections can be frightening, often devastating, and appear to "come out of the blue." Charles Darwin was aware of this phenomenon, as he wrote in *The Voyage of the Beagle*: "Wherever the European has trod, death seems to pursue the aboriginal."

Consider the classic lethal epidemics of measles and smallpox caused by two diverse but well-known viral scourges of human populations throughout history. History records that smallpox reached Europe from the Far East in 710 A.D. and attained epidemic proportions in the 18th century as populations grew and became concentrated. The effects on society are hard to imagine today, but as an example, at least five reigning monarchs died of smallpox.

Smallpox virus continued its spread around the world when European colonists and slave traders moved to the Americas and Australia. This viral infection changed the balance of human populations in the New World. Some say that viral infections were responsible for the elimination of a myriad of native languages and the nearly exclusive use of Spanish and Portuguese in South America. This suggestion may not be hyperbole. The first recorded outbreak of smallpox in the Americas occurred among African slaves on the island of Hispaniola in 1518, and the virus rapidly spread through the Caribbean islands. Within 2 years, this toehold of smallpox in the New World enabled the conquest of the Aztecs by European colonists. When Hernán Cortez first visited the Yucatan Peninsula in 1518 and began his conquest, his soldiers infected no one with smallpox virus. However, in 1520, smallpox reached the mainland from Cuba. Within 2 years, 3.5 million Aztecs were dead, more than could be accounted for by the bullets and swords of the small band of conquistadors. Smallpox spread like wildfire in the native population (which unfortunately was highly interactive and of sufficient density for efficient virus transmission). It reached as far as the Incas in Peru before Francisco Pizarro made his initial invasion in 1533. As is true in most smallpox epidemics, some Aztecs and Incas survived, but those who did were then devastated by measles virus, probably brought in by Cortez's and Pizarro's men. Conquest occurred by a one-two virological punch rather than by military prowess. Slave traders (who were most likely immune to infection) were populating Brazil with their infected human cargo at approximately the same time, with the same horrible result. The devastation of indigenous peoples by these viruses was also recapitulated in the colonization of North America and continued

into the 20th century as contaminated explorers infected isolated groups of Alaskan Inuit and native populations in New Guinea, Africa, South America, and Australia.

Hantavirus Pulmonary Syndrome: Human Disease Resulting from Changing Climate and Animal Populations

In 1993, a small but alarming epidemic of a highly lethal infectious disease occurred in the Four Corners area of New Mexico. Individuals who were in excellent health developed flu-like symptoms that were followed quickly by a variety of pulmonary disorders, including massive accumulation of fluid in the lungs, and death. Rapid action by local health officials and a prompt response by the Centers for Disease Control and Prevention were instrumental in discovering that these patients had low-level, cross-reacting antibodies to hantaviruses. These members of the family *Bunyaviridae* were previously associated with renal diseases in Europe and Asia and were well known to scientists, who associated them with viral hemorrhagic fever during the Korean War. Hantaviruses commonly infect rodents and are endemic in these populations around the world. Using PCR technology, scientists from the Centers for Disease Control and Prevention found that the patients were infected with a new hantavirus. Field biologists discovered that this virus was found in a rodent called the deer mouse (*Peromyscus maniculatus*), which is common in New Mexico. Hantavirus pulmonary syndrome has invariably been associated with the presence of this virus in New Mexico, as well as a few other isolated incidents around North America. The virus, which was given the name Sin Nombre virus (no-name virus), is an example of an emerging virus, endemic in rodents, that causes severe problems when it crosses the species barrier and infects humans.

The reason why humans became infected with Sin Nombre virus is still being debated, but one popular idea is that a dramatic increase in the deer mouse population was an important factor. In 1992 and 1993, higher than normal rainfall resulted in a bumper crop of piñon nuts, a favorite food for deer mice and local humans. Mouse populations increased in response, and contacts with humans inevitably increased as well. Hantavirus infection is asymptomatic in mice, but virions are excreted in large amounts in urine and droppings, where they are quite stable. Human contact with contaminated blankets or dust from floors or food storage areas provided ample opportunities for infection. Hantavirus syndrome is rare because humans are not the natural host, and apparently are not efficient vehicles for virus spread. However, the hantavirus zoonotic infection serves as a warning that potential human diseases lurk in the wild if we inadvertently intrude upon another host-parasite relationship.

Caliciviruses: Underappreciated but Highly Effective Zoonotic Agents

Caliciviruses are (+) strand RNA viruses and are a common causes of virus-induced vomiting and diarrhea in humans. The Centers for Disease Control and Prevention estimates that 23 million cases of acute gastroenteritis in the United States are due to members of the *Norovirus* genus in the family *Caliciviridae*. Remarkably, 50% of all food-borne outbreaks of gastroenteritis are caused by norovirus infection. Other common calicivirus-promoted symptoms include skin blistering, pneumonia, abortion, organ inflammation, and coagulation or hemorrhage. The epidemiology of calicivirus infections is poorly understood. Until recently, a common way to identify these viruses was by determining their unusual structure, visible only by electron microscopy, hardly a facile diagnostic tool. Today, diagnosis of calicivirus infection is accomplished by accurate and rapid PCR technology. Humans often become infected from water and food sources, and we now understand that the ocean and its marine animals are a major reservoir of these interesting viruses. Four genera are recognized in the family *Caliciviridae: Norovirus* (formerly known as Norwalk agent or Norwalk-like virus), *Sapovirus*, *Lagovirus,* and *Vesivirus.* Only noroviruses and sapoviruses cause human disease, and to date, all have resisted attempts at cultivation. Only the marine caliciviruses can be propagated *in vitro* and are known to infect terrestrial hosts, including humans.

The host range of these marine viruses is astounding. One virus serotype can infect five genera of seals, cattle, three genera of whales, donkeys, foxes, opaleye fish, horses, domestic swine, primates, and humans. The degree of exposure of land-based hosts to marine caliciviruses may be substantial, especially in confined areas such as shallow bays where aquatic mammals breed and calf. Infected whales excrete more than 10^{13} calicivirus particles daily, and the viruses remain viable for more than 2 weeks in cold seawater. Other prime sites for calicivirus encounters are aquatic theme parks and cruise ships. Human contact with contaminated water and sewage guarantees more outbreaks of calicivirus disease.

SARS: the Rise and Fall of a Highly Transmissible Zoonotic Infection

A new human coronavirus evolved in China at the end of the 20th century. As we now understand it, the disease called severe acute respiratory syndrome (SARS) first appeared in Guangdong Province in China in the fall of 2002. A Chinese doctor who treated these patients traveled to Hong Kong on 21 February 2003 and stayed on the ninth floor of the Hotel Metropole. He became ill and died in the hospital on February 22. The infection spread to 10 people

staying in the hotel, who then flew to Singapore, Vietnam, Canada, and the United States before symptoms were evident. Little did they know that they were making history—the first major viral epidemic to be spread by air travel. This small number of infected people efficiently spread the new coronavirus around the world, such that about 8,000 people in 29 different countries became infected in less than a year. The fatality rate was almost 1 in 10, a chilling statistic that activated health organizations worldwide. The scientific enterprise mobilized with unprecedented speed and cooperation, such that the causative agent was identified and sequenced within a few months.

We now know that this coronavirus originated in bats and was previously known to infect occasionally only palm civets and ferret badgers (Fig. 10.9). Chance transmission to civets subsequently consumed by humans in China apparently selected for mutations that expanded the virus host range and facilitated human transmission. Crucial mutations were in the viral ligand that enable virions to bind a human receptor. Amazingly enough, the epidemic never reached pandemic proportions, despite billions of susceptible hosts and widespread seeding of infected people around the world. While public health officials did a remarkable job in quarantine and diagnosis, it appears that the virus quickly evolved to be less virulent and less transmissible. After a frightening few months, SARS all but disappeared from the human population. However, field workers continue to isolate the virus from bats, its natural host, as well as from animals that share territory with the infected bats.

Human Immunodeficiency Virus: a Pandemic from a Zoonotic Infection

The origin of human immunodeficiency virus was an enigma until two lines of research converged. The first was a remarkable study published in 1998. This study provided a time point reference for when the virus entered the human population (see Chapter 6). The second line of research required isolation and analysis of simian lentiviruses. Based on sequence homology between chimpanzee and human lentiviruses, the progenitor of human immunodeficiency virus type 1 can now be traced to a few transmissions from chimpanzees to a human in West Central Africa. This precursor of contemporary human immunodeficiency virus acquired new mutations, giving it increased tropism and propensity to spread among humans. How did the chimpanzee virus infect a human? Viral ecologists and epidemiologists now have strong evidence that humans are exposed to many zoonotic infections by the bushmeat trade in West Africa, which involves killing and consumption of wild animals, including chimpanzees, gorillas, other primates, and rodents. It is now clear that chimpanzee retroviruses infected humans, giving rise to the M and N clades of human immunodeficiency virus type 1. Curiously, the O clade viruses first identified in Cameroon in 1994 appear to have arisen independently and may have come from gorillas, which harbor a very closely related retrovirus. Remarkably, a chance encounter of primate retroviruses with human bushmeat hunters established human immunodeficiency virus type 1 on the planet (Fig. 10.9).

The progenitor of human immunodeficiency virus type 1 was on the path to extinction because the population of wild chimpanzees had dropped to about 150,000 animals living in isolated troops. The new human host exceeds 6 billion individuals, and this niche is rapidly filling: more than 1 in 100 humans are now infected.

Shortly after the recognition of human immunodeficiency virus type 1 in the 1980s, a second related subtype, called type 2, was identified in Portugal and subsequently has been found in many countries (Chapter 6). Type 2 is not as widely distributed as type 1, being more commonly found in Africa. Interestingly, the membrane proteins of the type 2 human virus have homology to those of the sooty mangabey and strains of simian immunodeficiency virus. In fact, humans infected with human immunodeficiency virus type 2 have antibodies that cross-react with the simian virus proteins. These observations have led some scientists to speculate that the human type 2 virus was derived from transmission from the sooty mangabey into humans, most likely due to the bushmeat trade.

Host Range Can Be Expanded by Mutation, Recombination, or Reassortment

Given the large numbers of viruses and ample opportunities for infection, new host-virus interactions will certainly occur. How will they be recognized? Because the new host population will have had no experience with the virus, the infection will have unpredictable consequences (e.g., the dead-end or the evolving host-virus scenario). Such an invasion may be marked only by an immune response (i.e., production of antibodies and seroconversion) and no obvious disease. Infection could also result in mild disease, the symptoms of which may or may not be noteworthy; occasionally, astute health care workers may notice unusual symptoms in local population centers. However, sometimes the invasion can be dramatic, as in the global epidemics caused by canine parvovirus, influenza virus, and human immunodeficiency virus.

Canine Parvovirus: Cat-to-Dog Host Range Change by Two Mutations

In 1978 canine parvovirus, a member of the family *Parvoviridae,* was first identified simultaneously in several countries around the world as the cause of a new

enteric and myocardial disease in dogs. Canine parvovirus apparently evolved from the feline panleukopenia virus after two or three mutations made the latter pathogenic and highly transmissible in the dog population. Feline panleukopenia virus infects cats, mink, and raccoons, but not dogs. When the new canine virus emerged, it was able to infect feline cells in culture, but did not replicate in cats. Because canine parvovirus appeared less than 30 years ago, it has been possible to analyze dog and cat tissue collected in Europe in the early 1970s to search for the progenitor canine parvovirus. Some fascinating studies have tracked and identified the virus mutants causing the epidemic. The ancestor of canine parvovirus began infecting dogs in Europe during the early 1970s, as deduced by the appearance of antibodies in dogs in Greece, The Netherlands, and Belgium. In 1978, evidence of virus infection was obvious in Japan, Australia, New Zealand, and the United States, suggesting that the new virus had spread around the world in less than 6 months. The stability of the new virus, its efficient fecal-oral transmission, and the universal susceptibility and behavior of the world's dog population were important factors in the emergence of this new virus.

We now understand the genetic changes that created a new pathogen from a well-known virus. Only two amino acid substitutions in the VP2 capsid protein were necessary to change the tropism from cats to dogs. These substitutions are both necessary and sufficient to alter host range, because changing only the feline virus VP2 sequence to the canine virus sequence enabled the feline virus to replicate in cultured canine cells and in dogs. These critical amino acids are located on a raised region of the capsid that surrounds the threefold axis of icosahedral symmetry in the $T = 1$ capsid (Fig. 10.14). These differences in the capsid structure control the tropism for cells by affecting virion binding to host transferrin receptor, the protein used to establish infection. Feline panleukopenia virions bind only to the feline transferrin receptor. In contrast, canine parvovirus virions bind to both the feline and canine transferrin receptors.

The emergence of canine parvovirus was the result of an hitherto unappreciated high rate of mutation and positive selection for mutations in the major capsid gene. The viral genome continues to evolve rapidly. Indeed, the original antigenic variant, called type 2, was only a transient player in virus evolution. It was completely replaced within 2 years by new canine parvovirus strains called type 2a and 2b, which contain additional amino acid substitutions in the VP2 protein.

The principle to be learned from canine parvovirus emergence is that host range switches by well-adapted viruses are rare, but when a switch occurs, the outcome can be severe for the new host population, New variants evolve and spread widely through the nonimmune and nonadapted population (the evolving host-virus scenario). The emergence of the canine parvovirus group provided an extraordinary opportunity to study virus-host adaptation and host-range shifts in the field.

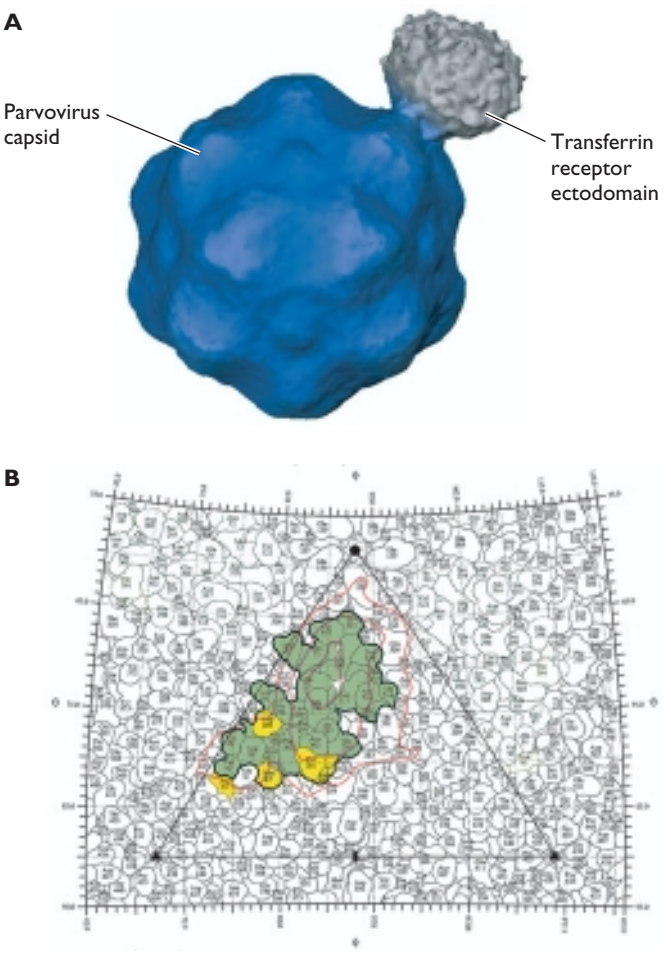


Figure 10.14 The transferrin receptor mediates canine and feline parvovirus host range. The transferrin receptors for feline and canine parvoviruses have a large extracellular domain (ectodomain) that is a homodimer of a single protein. The binding of the canine parvovirus virion to the ectodomain of canine transferrin receptor is determined by combinations of amino acid residues on the surface of the capsid. To examine the interactions in more detail, cryo-electron microscopy was used to determine the structure of the purified ectodomain of the feline transferin receptor bound to canine parvovirus capsids. Only a small number of transferin receptors bind to each capsid, and in the model, one such complex is shown **(A)**. The binding site (footprint) of the transferrin receptor on the surface of the canine parvovirus capsid is shown in green on a representation of the surface-exposed amino acids of the capsid, with one of the 60 asymmetric units of the icosahedron indicated **(B)**. Residues that are known to affect binding of the canine transferrin receptor or host range are indicated in yellow. Figures prepared by Susan Hafenstein and Colin Parrish, Cornell University. See S. Hafenstein et al., *Proc. Natl. Acad. Sci. USA* **104:**6585–6589, 2007.

Influenza Epidemics and Pandemics: Escaping the Immune Response by Genetic Reassortment

Influenza, a disease with symptoms that have remained unchanged for centuries, is caused by a virus with a constantly changing genome (Fig. 10.15). This disease is the paradigm for the situation in which continued evolution of the virus in several host species is essential for its maintenance. We now know that all mammalian influenza viruses are of avian origin, but mammal-to-mammal transmission can occur. Considerable attention has been focused on the catastrophic influenza pandemic in 1918 that claimed over 25 million lives (Table 10.6). Sequencing data indicate that this H1N1 virus is likely to be the ancestor of all current human influenza viruses, as well as the H1N1 and H3N2 viruses circulating in the world's swine population. The 1918 pandemic was devastating, with an estimated 28% of the population of the United States being infected. Worldwide spread was facilitated by massive worldwide troop movements as a consequence of World War I. Mortality rates were over 2.5%, compared with the normal mortality of less than 0.1%. The unprecedented virulence of the 1918 influenza virus strain raised concerns that it may emerge again, as the genes from this virus still circulate in the avian influenza virus population. Reconstruction of the 1918 virus from viral nucleic acid amplified from archived tissues established that no single gene was responsible for its virulence. Rather, it appears that almost all the viral genes contributed and that the virulence was due to an overreaction of the intrinsic and innate immune response (a so-called cytokine storm, or systemic cytokine toxicity). It is likely that this virus entered the human population directly from avian sources without much adaptation.

The influenza virus serotypes in circulation are virulent, but not to the extent of the 1918 virus. Epidemiologists estimated that influenza virus kills an average of 36,000 people per year in the United States, and the number may

Figure 10.15 Phylogenetic trees for 41 influenza A virus NP genes rooted to influenza B virus NP (B/Lee/40). The nucleotide tree is shown on the left. The horizontal distance is proportional to the minimum number of nucleotide differences to join nodes and NP sequences. Vertical lines are for spacing branches and labels and have no other meaning. Blue animal symbols denote the five host-specific lineages. Pink animal symbols denote viruses in the lineage that have been transmitted to other hosts. The amino acid tree is shown on the right. The blue and pink animal symbols are the same as for the nucleic acid tree. Adapted from R. G. Webster and Y. Kawaoka, *Semin. Virol.* **5**:103–111, 1994, with permission.

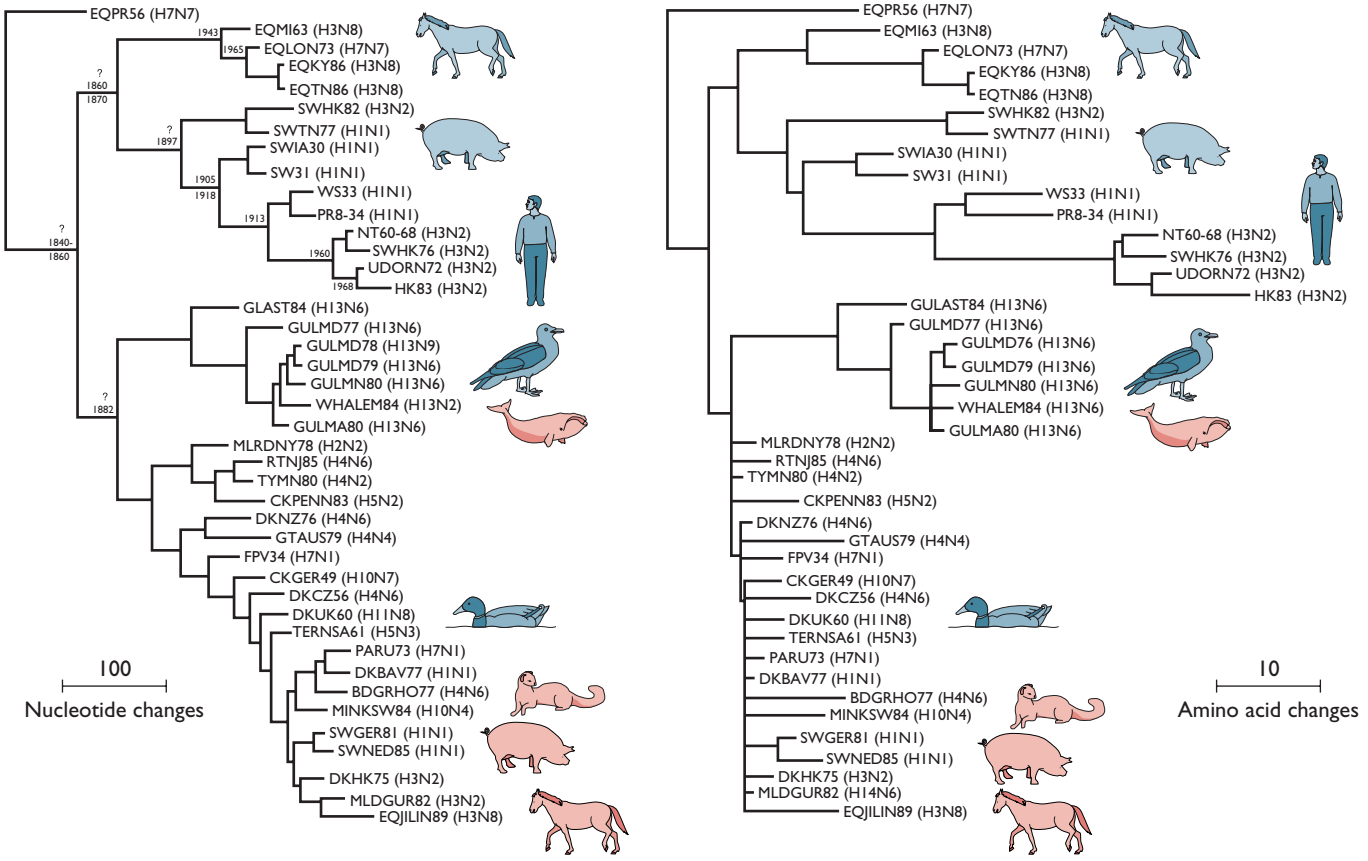


Table 10.6 The 1918–1919 influenza pandemic: one of history's most deadly events[a]

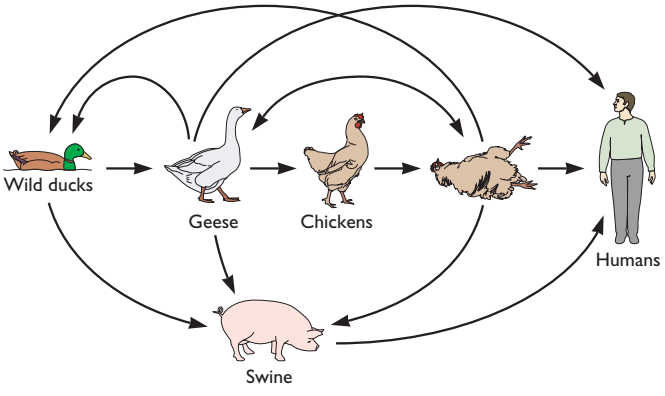
Event	No. of deaths (millions)
Influenza pandemics (1918–1919)	20–40
Black Death (1348–1350)	20–25
AIDS pandemic (through 2006)	22
World War II (1937–1945)	15.9
World War I (1914–1918)	9.2

[a] Data from *The New York Times*, 21 August 1998, and from the World Health Organization.

surge to 50,000 in severe seasons. More than 90% of the victims are 65 or older. In the 1990s, three influenza A virus serotypes predominated, with the H3N2 serotype being particularly virulent.

The life cycle of influenza virus, while comparatively well understood at the molecular level, is remarkable for its complexity in nature (Box 10.5; Fig. 10.16). New influenza viruses constantly emerge from migratory populations of aquatic birds to infect humans, pigs, horses, domestic poultry, and aquatic mammals. In birds, influenza virus replicates in the gastrointestinal tract and is excreted in large quantities, a most efficient virus distribution system. The widespread dispersal of virus in water, the facile changing of hosts, and the ease of genetic reassortment form an engine for creation of new pathogenic strains.

Figure 10.16 Emergence and transmission of H5N1 influenza virus. H5N1 influenza virus has its origins in wild waterfowl, where it was relatively nonpathogenic. Infection is thought to have spread to domestic ducks and chickens, where it evolved to be highly pathogenic in chickens. It then spread back to domestic ducks and geese, where it reassorted its genome with those of other influenza viruses of aquatic birds. The resulting new reassortants spread directly to domestic chickens, humans, and swine. These infections were facilitated by mutations in their PB2, HA, NA, and NS genes that made them more pathogenic to domestic and wild waterfowl and humans. Spread to humans without an intervening "mixing host" such as a pig raises the spectre of a pandemic in the human population.



Humans are not the only hosts that suffer from infections caused by this ever-changing virus. Outbreaks of swine and avian influenza devastate operations that produce these animals for food. Despite large-scale immunization programs, virulent strains of swine influenza virus continue to emerge. When pigs are infected experimentally with an avirulent influenza virus mutant, a virulent strain can arise within a few days and cause disease. Poultry producers have similar experiences with avian influenza. A single mutation (changing a threonine to lysine in the hemagglutinin gene) in a relatively attenuated avian strain caused the 1983 epidemic that devastated commercial chicken production in Pennsylvania. The direct transfer of a virulent avian H9N1 virus to humans with lethal consequences was first documented in 1997. Similar transfer of the lethal avian H5N1 virus to humans (but with little to no human-human transmission) reminds us how quickly host-virus interactions can change (Box 10.17; Fig. 10.16).

Even in this myriad of hosts and genetic exchanges, there is some remarkable stability that is crucial to the survival of influenza virus. One surprising finding is that the avian viral genome has not changed much in more than 60 years, in contrast to the genomes of human and other nonavian viruses. Avian viral genomes exhibit mutation and reassortment rates as high as those of human and swine viruses, but only those with neutral mutations are selected and maintained in the bird population. While virulent mutants do arise occasionally, in general, birds infected with avian influenza viruses experience no overt pathogenesis. These properties indicate that influenza virus is in evolutionary stasis in birds. The avian host apparently provides the stable reservoir for influenza virus gene sequences that emerge as recombinants capable of transspecies infection.

Retrovirus Pathogenicity Change by Recombination

Retroviruses provide instructive examples of the acquisition of entirely new genes by recombination with the host genome. Such recombination can yield viruses with unexpected, and frequently lethal, pathogenic potential. A retrovirus can acquire a cellular proto-oncogene and become an acutely oncogenic virus (Chapter 7). One well-known example is feline leukemia virus, a highly conserved retrovirus that infects cats throughout the world. The virus causes lymphosarcomas and a range of degenerative diseases, including anemia and thymic atrophy. However, the sarcomas are caused not by feline leukemia virus but rather by new viruses with genomes that result from recombination of host and viral DNA sequences. These recombinant viruses are acutely oncogenic and are defective because their envelope genes have been replaced by cellular sequences. As a

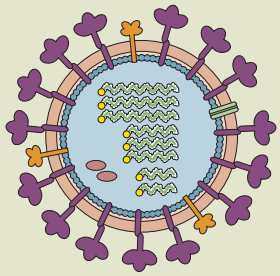
BOX 10.17 DISCUSSION

H5N1 influenza virus, a global phenomenon worth understanding

Highly pathogenic H5N1 variants of influenza virus moved from Asia to India to Europe and to Africa in the space of 10 years. At least three distinct lineages can be identified by sequence analysis in Southeast Asia, Europe, northern Africa, and the Middle East. They continue to evolve, and the new variants are moving around the world in wild birds. Wild aquatic birds, notably waterfowl, are considered the natural reservoirs of low-pathogenicity avian influenza viruses and sources of infection for other species. Highly pathogenic avian influenza viruses evolve in domestic poultry from H5 or H7 subtypes of low-pathogenicity viruses and become established in these species, despite their considerably increased virulence, possibly because domestic poultry are kept at very high densities.

Of the 250 people known to have been infected with the H5N1 serotype, more than half died. The transmission of this virus to humans required close contact with bodily fluids of infected birds. Human-to-human transmission was rare and difficult to demonstrate.

Several species of cats can be infected with and can transmit the H5N1 virus. This phenomenon was first noted at zoos in Thailand when tigers and leopards died



after being fed infected chickens. Although H5N1 infection moves poorly from poultry to humans, it spreads freely from poultry to cats, and then among the cats. Transmission of virus from cats to humans has not been demonstrated—yet.

Efforts to control the avian infections have led to the culling of more than 250 million domestic birds in Asia.

Migratory birds can carry the virus along their migration routes, as demonstrated by the remarkably rapid spread from Qinghai Lake in western China to Mongolia, Russia, and Turkey. However, humans spread the infection to Europe by transporting infected birds by car, truck, and railroad. After narcotics, live birds for the pet trade are the next most commonly smuggled items brought into the United States.

> *In over 40 years of experience with influenza, the Asian H5N1 is the most virulent virus I have encountered. If it does acquire consistent human-human transmissibility—it will likely be catastrophic.*
>
> R. G. Webster

Two Important Unanswered Questions

- Will H5N1 acquire mutations enabling efficient transmission to humans and cause a pandemic?
- How soon will H5N1 spread to the Americas?

Ito, T., H. Goto, E. Yamamoto, H. Tanaka, M. Takeuchi, M. Kuwayama, Y. Kawaoka, and K. Otsuki. 2001. Generation of a highly pathogenic avian influenza A virus from an avirulent field isolate by passaging in chickens. *J. Virol.* 75:4439–4443.

Salzberg, S. L., C. Kingsford, G. Cattoli, D. J. Spiro, D. A. Janies, et al. 2007. Genome analysis linking recent European and African influenza (H5N1) viruses. *Emerg. Infect. Dis.* **13:**713–718.

Webster, R. G., Y. Guan, M. Peiris, and H. Chen. 2006. H5N1 influenza continues to circulate and change. *Microbe* **1:**559–565.

consequence of unregulated expression of the oncogene from the integrated defective virus, tumors arise rapidly, often with lethal consequences. Several host genes, including c-*abl,* c-*kit,* and c-*sis,* have been incorporated into the genomes of different feline sarcoma viruses.

Some Emergent Viruses Are Truly Novel

Classically, viruses were identified by the diseases they caused; more recently, they have been identified by being grown in culture to characterize their components. Certainly, viruses exist that have never been described or grown in cultured cells. Many of these unculturable viruses cause serious, widespread disease. For example, rotaviruses infect essentially all children younger than 4 years of age in the United States, causing severe diarrhea. However, these viruses were not recognized until the 1970s, simply because the technology for finding them did not exist. Associating a disease or syndrome with a particular infectious agent used to be impossible if the agent could not be cultured. Fortunately, with powerful recombinant DNA, hybridization, and nucleic acid amplification technology, we are now able to detect and characterize such hitherto unknown viruses with comparative ease.

Discovery of Previously Unknown Hepatitis Viruses in the Blood Supply by Recombinant DNA Technology

One of the first examples of the contemporary quantum leap in diagnostic capacity occurred with the recognition of hepatitis C virus, a member of the family *Flaviviridae.* With the development of specific diagnostic tests for hepatitis A and B viruses in the 1970s, it became clear that most cases of hepatitis that occur after blood transfusion are caused by other agents. Even after more than 10 years of research, the identity of these so-called non-A, non-B (NANB) agents remained elusive. In the late 1980s, Chiron Corporation, a California biotechnology company, isolated a DNA copy of a fragment of the hepatitis C virus

genome from a chimpanzee with NANB hepatitis. This remarkable feat was accomplished by examining DNA from about a million DNA clones for production of proteins that were recognized by serum from a patient with chronic NANB hepatitis. The sequence analysis of these DNA clones identified a (+) strand RNA genome of about 10,000 nucleotides with high homology to known flaviviral genomes. The availability of the hepatitis C virus genome sequence made possible the development of diagnostic reagents that effectively eliminated the virus from the U.S. blood supply, reducing the incidence of transfusion-derived NANB hepatitis significantly. Viral proteins produced from the DNA clones were also instrumental in establishing mechanism-based screens for antiviral drugs (Chapter 9).

These studies provide an excellent example of how a previously unknown human pathogen was identified and then analyzed by cloning technology. Interestingly, hepatitis C virus was not the only previously hidden virus hidden in the human blood supply; the same technology permitted the discovery of several viruses, including TT virus, a ubiquitous human circovirus of no known consequence.

A Revolution in Diagnostic Virology

Recombinant DNA, PCR, sequencing, and hybridization technology are causing a revolution in diagnostic virology. New nucleic acid sequences can be associated with diseases and characterized in the absence of standard virological techniques. The etiological agent of SARS was identified using a "virochip" with characteristic oligonucleotides from all the viral genomes that had been sequenced. However, because of high sensitivity and the potential for contamination by adventitious viruses, care must be taken when using any PCR-based or hybridization method. Without proper controls, these techniques have the potential to associate a particular virus incorrectly with a disease, confounding the deduction of etiology. The spurious association of multiple sclerosis with infection by human T-lymphotropic virus type 1 is an instructive example. Similarly, a variety of studies in which PCR identified human herpesviruses in the brains of patients who had died of Alzheimer's disease, implying a causal link between the disease and the virus, have been published. These conclusions are called into question by the finding of the same viruses in the tissues of similarly aged patients who died of other causes. These examples illustrate the fact that the new technologies do not circumvent the time-honored Koch's postulates, which require that the infectious agent be isolated from a diseased patient, grown, and used to cause the same disease.

While early detection and precise identification of viruses are important in public health and must be pursued with vigor, the information is of limited value if no treatment or follow-up is available for the putative disease-causing agent. We do not have a robust armamentarium of antiviral drugs against even the most common pathogenic viruses. Therefore, developing treatments for previously unknown viruses discovered by these powerful techniques will be challenging.

Perceptions and Possibilities

Infectious Agents and Public Perceptions

While emerging virus infections are well known to virologists, in recent years they have become the subject of widespread public interest and concern. One reason is that less than 30 years ago, many people were eager to close the book on infectious diseases. The public perception was that wonder drugs and vaccines had microbes under control. Obviously, in a few short years, this optimistic view has changed dramatically. Announcements of new and destructive viruses and bacteria appear with increasing frequency. The reality of the human immunodeficiency virus pandemic and its effects at every level of society have attracted worldwide attention, while exotic viruses like Ebola virus capture front-page headlines. Movies and books bring viruses to the public consciousness more effectively than ever before. After the events of 11 September 2001, concern that terrorists might use infectious agents was widespread (Box 10.18). Consequently, it is important to separate fact from fiction and hyperbole. The widespread interest in potentially dangerous viral infections reminds us that a little knowledge can be a dangerous thing: scientists, as well as the public, can be misled about host-virus interactions simply by semantics and jargon.

Virus Names Can Be Misleading

Unfortunately, it is common to name a virus by the host from which it was isolated. By using the name **human** immunodeficiency virus for the virus that causes AIDS, we give short shrift to its nonhuman origins. Canine parvovirus indeed causes disease in puppies, but it is clearly a feline virus that recently changed hosts. Similarly, canine distemper virus is not confined to dogs but can cause disease in lions, seals, and dolphins. Well-known viruses can cause new diseases when they change hosts. Much is implied, and more is ignored, about the host-virus interaction when the virus is given a host-specific name.

The Importance of Pathogenic and Nonpathogenic Viruses

It is not unusual to think that disease-causing viruses are important whereas nonpathogens are uninteresting

BOX 10.18 DISCUSSION

Viral infections as agents of war and terror

Infectious agents have a documented capacity to cause harm, and can cause epidemics as well as pandemics. Fearsome and deadly viruses abound, ranging from universal scourges such as smallpox virus and influenza virus, to the less widely distributed, but no less deadly, hemorrhagic fever viruses. Any viral infection that can kill, maim, or debilitate humans, their crops, or their domesticated animals has the potential to be used as a biological weapon. Obviously, a biological attack need not cause mass destruction to be an instrument of terror, as was demonstrated by the far-reaching effects of the introduction of bacteria causing anthrax into the United States mail system. Society has only a limited set of responses to frightening outbreaks: vaccination, quarantine, and anti-infective drugs. For example, the unintentional 1947 outbreak of smallpox in New York City originated from a single businessman who had acquired the disease in his travels. He died after infecting 12 others; to stop the epidemic, **over 6 million people** were vaccinated within a month.

Bioterrorism, like natural outbreaks, is a serious problem with no clear solutions. That fact has not stopped the U.S. government from redirecting billions of dollars to fund programs on counterterrorism and massive research programs on so-called classs A infectious agents. Some argue that this money would be better spent on public health research, or on naturally occurring common diseases. Others are concerned that publication of biological research data could aid terrorists, and feel that measures of information control must be considered. Practically speaking, public health officials view bioterrorism as a low-probability but high-impact event, much like Hurricane Katrina. When such events occur, they are devastating. However, the hallmarks of these calamities are that they cannot be predicted with accuracy or prevented. One can only prepare for them so that appropriate actions can be taken. Preparation must be measured, practical, and functional so that damage is minimized. For biological attacks of any kind, natural or human inspired, research and free communication of results represent our greatest strengths and are the only ways to prepare.

and irrelevant. As we have seen, a virus that is stable in one host may have devastating effects when it enters a different species. Another misconception arises from human-centered thinking. How often do we think that viruses causing human diseases are more important than those that infect mammals, birds, fish, or other hosts? As noted above, naming viruses as "human" not only is inaccurate but also gives them inflated importance. Similarly, by thinking only of human needs, we are blind to the multiple networks of interactions that constitute host-virus relationships. Indeed, viral particles have access to a broad smorgasbord of hosts, and humans often represent but one stop in the evolution of a virus.

Human Economic Interests Have Significant Effects

In a material society, it is routine to direct attention to viruses that have economic consequences as opposed to those that do not. Moreover, companies and governments generally concentrate resources on viruses that affect their economies, simply because they will be rewarded monetarily for their efforts. Consequently, research is focused on antiviral drugs and therapies that offer greater economic rewards. Such a bottom-line approach is shortsighted and does not address world health problems. Furthermore, because of the mobility of society and our expanding populations, economic impulses must be balanced more prudently by a broader worldview.

What Next?

Can We Predict the Next Viral Pandemic?

The world is currently is in the midst of the AIDS pandemic, one of the most horrible in history. At the current rate of infection, more than 50 million people around the world will die within a year or two. This global devastation continues despite the availability of effective antiviral drug therapies. It is difficult to imagine an infection of greater consequence. However, it is instructive to consider the possibility that other viruses with similar potential to wreak havoc upon humans exist.

Upon reflection, the sobering reality is that some of the most serious threats do not come from the popularized, highly lethal filoviruses (e.g., Ebola virus), the hemorrhagic disease viruses (e.g., Lassa virus), or an as yet undiscovered killer virus lurking in the wild. Rather, the most dangerous viruses are likely to be the well-adapted, multihost, evolving viruses already in the human population. Influenza virus is one that fits this description perfectly. Its yearly visits show no signs of diminishing; genes promoting pandemic spread and virulence are already circulating in the virus population, and the world is ever more prone to its dissemination. Indeed, a pandemic of influenza on the scale of the 1918 to 1919 outbreak is thought by many to be the next emerging disease most likely to affect humans on an enormous scale (Table 10.6). We were reminded of this possibility in the late 1990s, with reports from China and Southeast Asia that an avian strain of influenza virus

(H5N1) had spread directly to humans. Prior to these studies, antibodies to H5 had never been isolated from humans. Obviously, once such a virus infects the naive human population, pandemic spread is possible. Scientists from around the world are now following the trajectory of the H5N1 virus as it reassorts among the myriad influenza virus populations and interacts with the many avian hosts worldwide (Box 10.17).

Many Emerging Viral Infections Illuminate Immediate Problems and Issues

Obviously, it would be a mistake to concentrate on influenza virus to the exclusion of others, because many viral infectious diseases pose immediate and urgent problems for the world. The AIDS pandemic and our experience with SARS illustrate the ease with which a new virus can enter the human population. The secondary infections that accompany AIDS have exposed the fragility of the world's health care systems and the infrastructure of developing countries. The known outbreaks of new, exotic viruses, or well-known viruses that have invaded new geographic niches, as well as the possible cross-species interactions, challenge the standard methods of diagnosis, epidemiology, treatment, and control.

Humans Constantly Provide New Venues for Meeting

To understand and control emerging virus infections, humans must recognize the scale of the problem (Fig. 10.7). The examples of poliovirus, measles virus, and smallpox virus demonstrate that viruses can suddenly cause illness and death on a catastrophic scale following a change in human behavior. Current technological advances and changing social behaviors continue to influence the spread of viruses. Some human-introduced environmental changes that are significant to virology are listed in Table 10.4. Most did not exist 50 years ago, and each one brings humans and viruses into new situations. Probably the most critical fact is that the human population is as large as it ever has been, and is still growing. As a consequence, humans are interacting among themselves and with the environment on a scale unprecedented in history.

Many changes listed in Table 10.4 have major effects upon the transmission of viruses by vectors, such as insects and rodents. When humans interfere in a natural host-virus interaction, cross-species infection is possible. Population movements, the transport of livestock and birds, the construction and use of irrigation systems, and deforestation provide not only new contacts with mosquito and tick vectors, but also mechanisms for transport of infected hosts to new geographic areas.

Humans can provide novel habitats for viruses, as demonstrated by used tires, an unexpected vehicle for movement of viruses and their hosts. Several species of tropical mosquitoes (e.g., *Aedes* species) prefer to breed in small pockets of water that accumulate in tree trunks and flowers in the tropics. The used tire has provided a perfect mimic of this breeding ground, and, as a consequence, the millions of used tires (almost all carrying a little puddle of water inside) accumulating around the world provide a mobile habitat for mosquitoes and their viruses. In the United States, such used tires are shipped all around the country for recycling, transmitting mosquito larvae to new environments literally overnight. As a result, the mosquito hosts for dengue and yellow fever viruses may be given the chance to establish a new range in New Jersey, thanks to the shipment of used tires for recycling from the South. It remains to be seen how the new insect vector will impinge on human health in the northeastern United States, but mosquito-borne viral diseases such as dengue fever clearly are of major concern.

Puerto Rico had five dengue fever epidemics in the first 75 years of the 20th century but had six recent epidemics in 10 years, with major economic costs. Simultaneously, Brazil, Nicaragua, and Cuba experienced their first dengue fever epidemics in 50 years. Brazil reported 180,000 cases in 1996. It is suspected that this viral infection is emerging because the mosquito host has new avenues for spread and many governments have poor, or no, vector control programs.

Another contemporary example of humans moving viruses to new hosts comes from transport of livestock. African swine fever virus, a member of the family *Iridoviridae,* causes a serious viral disease that is threatening the swine industries of developing and industrialized countries. Unlike any other DNA virus, it is spread by soft ticks of the genus *Ornithodoros*. African swine fever virus was spread from Africa to Portugal in 1957, to Spain in 1960, and to the Caribbean and South America in the 1960s and 1970s by long-distance transport of livestock and their resident infected arthropods. Similarly, the rapid spread of H5N1 avian influenza virus from China to Europe was probably mediated by transport of infected birds across national borders.

The construction of dams and irrigation systems can change host-virus interactions on a large scale. The creation of vast areas of standing water has introduced new sources of viruses, hosts, and vectors. The 1987 outbreak of Rift Valley fever in Mauritania along the Senegal River was associated with the new Diama Dam, which created conditions ideal for mosquito propagation. Not only do water impoundments affect insects, but also they alter the population and migration patterns of waterfowl and other animals and the viruses they carry. Previously separated hosts and viruses are brought together as a consequence.

In industrialized countries, the increasing need for day care centers has led to new opportunities for viral transmission and other types of day care-associated illness. In the United States, more than 12 million children are in day care centers for several hours a day, and the vast majority are under 3 years of age. As most parents can testify, respiratory and enteric infections are common, and infections spread easily to other children, day care workers, and the family at home.

Many of the examples discussed above involve human interactions on a large scale and reflect an aphorism called **the law of unintended consequences**: human actions often can produce unforeseen effects. Unfortunately, these unforeseen effects often have substantial negative or momentous consequences (Table 10.4). Sociologists, including the late Robert Merton, provided some insight into the concept, which has been used in developing policy and predicting the potential outcome of large-scale human activities (e.g., environmental impact statements). He stated five reasons that lead to unintended consequences: **ignorance** (we can't anticipate everything); **error** (things that worked in other situations may not apply to the current situation); **immediate interest** (long-term interests are ignored); **basic values, which may require or prohibit certain actions, even if the long-term result is unfavorable** (the long-term consequences may even change the basic values); and **self-defeating prophecy** (fear of some consequence drives people to find solutions before the problem occurs; thus nonoccurrence of the problem is unanticipated).

Preventing Emerging Virus Infections

The modernization of society and the expanding human population have led to spread of infection and selection of new virus variants. We cannot turn back the clock; however, experience and common sense can guide us. The requirements for control of new viral infections range from the obvious to the ideal. Implementation of these ideas will take money and integration of the public and private sectors at a level almost unprecedented today. Two ideas are at the forefront: safe water and better nutrition. If ways and means could be found to provide pure water and sewage disposal for the world, infection rates would drop dramatically. Better nutrition will ensure that natural human defenses are up to the task. Rodent and insect control in population centers would go far to reduce infections. Recognizing when human activities are likely to disrupt endemic disease patterns would be a major step forward. Comparative medicine, the study of disease processes across species including humans, should be fostered. Teams of medical doctors and veterinarians should work together to study zoonotic infections in the field, which are the root cause of many emerging viral infections. It will be beneficial if government agencies that deal with animal and human health share resources and communication.

Because of the potential for rapid spread of viruses via air travel and urban development, a system of global surveillance and early warning is required for primary-care physicians and health care workers. With modern sequencing technology, it is conceivable that we could monitor all viral pathogens circulating in humans. When a new (or old) viral disease is suspected, the agent could be identified and characterized and the information could be shared widely. Methods should be developed for rapid diagnosis in the field, and primary health care workers must have access to databases. Baseline data on endemic disease and vector prevalence need to be compiled and included in this early-warning database. Active or passive vaccines could be made available, although the infrastructure for accomplishing anything more than local action is almost nonexistent and solutions are expensive to implement. Finally, responsible public and professional education is essential at all levels, as is more scientific research on host-parasite interactions. Inherent in this last proposal is the need for the scientific community to develop better mechanisms for sharing and developing the fruits of scientific research and making their work known, appreciated, and applied by the general public.

Perspectives

The relationships of viruses and their hosts are in constant flux. The perspectives of evolutionary biologists, ecologists, and epidemiologists are required to understand why this is so. At present, the interplay of environment and genes, as well as the interactions of virus and host populations, barely can be articulated, let alone studied in the laboratory. For viral infections, rapid production of huge numbers of progeny, the tolerance to amazing population fluctuations, and the capacity to produce enormous genetic diversity provide the adaptive palette that ensures survival. As Joshua Lederberg said, "In the battle by attrition, humans have a real problem competing with microorganisms. Here we are, here are the bugs, they're looking for food, we're their meal in one sense or another, how do we compete?"

One obvious reason for our success in the competition is that our intrinsic and immune defense systems are capable of recognizing and destroying invading viruses. A less obvious, but equally important, reason is that the current viral hosts represent progeny of survivors of past interactions with viruses. This experience is recorded in the vast gene pool of survivors. Indeed, heterozygosity at the many major histocompatibility complex alleles is known to confer a strong selective advantage on human populations in the battle against microbes. Viral infections have far-reaching

effects, ranging from shaping of the host immune system in survivors to eliminating entire populations. Clearly, survival of host and virus is a delicate balance: once the balance is disturbed, the host, the virus, or both will be changed or even eliminated. Given the ever-changing viral populations and the drastic modifications of the ecosystem that have accompanied the current human population explosion, we are hard pressed to predict the future. Perhaps the most sobering fact for humans is that despite our intellect and ability to adapt, virus particles continue to infect and even kill us. In some senses, virus populations are, and always will be, at the top of the evolutionary ladder. The challenge is to keep them at bay and to strive to stay at least one step ahead.

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APPENDIX A
Diseases, Epidemiology, and Disease Mechanisms of Selected Animal Viruses Discussed in This Book

This appendix presents key facts about the pathogenesis of selected animal viruses that cause human disease. Information about each virus or virus group is presented in three sections. In the first section, the viruses and associated diseases are listed. The second section, "Epidemiology," is outlined in four key areas: transmission, distribution of the virus, those at risk or risk factors, and vaccines or antiviral drugs. The third section, "Disease mechanisms," provides simple images that will enable the reader to visualize infection and the resulting pathogenesis. Each of the three sections can be made into a slide for lectures or teaching, providing a "snapshot" of the pathogenesis of a specific virus.

Adenoviruses

Virus	Disease
51 adenovirus serotypes that infect humans, classified into six subgroups	Respiratory diseases • Febrile upper tract infection • Pharyngoconjunctival fever • Acute disease • Pertussis-like disease • Pneumonia Other diseases • Acute hemorrhagic cystitis • Epidemic keratoconjunctivitis • Gastroenteritis

Epidemiology

Transmission
- Respiratory droplets, fecal matter, fomites
- Close contact
- Poorly sanitized swimming pools
- Ophthalmologic instruments (eye infections)

Distribution of virus
- Ubiquitous
- No seasonal incidence

At risk or risk factors
- Children aged <14 years
- Day care centers, military camps, swimming clubs

Vaccines or antiviral drugs
- Live, attenuated vaccine; serotypes 4 and 7 have been produced for the military

Disease mechanisms

Virus infects mucoepithelial cells of respiratory and gastrointestinal tract, conjunctiva, and cornea

Virus persists in lymphoid tissue (tonsils, adenoids, and Peyer's patches)

Antibody is essential for recovery from infection

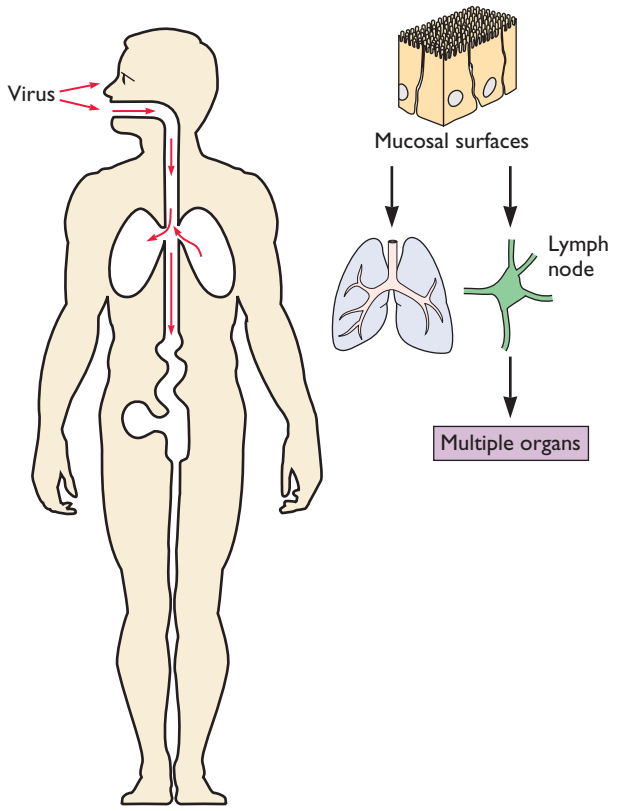


Figure 1

Arenaviruses

Virus	Disease
Lymphocytic choriomeningitis virus	Febrile, flu-like myalgia; meningitis
Lassa virus	Lassa fever: severe systemic illness, increased vascular permeability, shock
Junin virus	Argentine hemorrhagic fever: similar to Lassa fever but more extensive bleeding
Machupo virus	Bolivian hemorrhagic fever

Epidemiology

Transmission
- Contact with infected rodents or their secretions or body fluids

Distribution of virus
- Lymphocytic choriomeningitis virus: hamsters and house mice in Europe, Americas, Australia, possibly Asia
- Other arenaviruses: Africa, South America, United States
- No seasonal incidence

At risk or risk factors
- Lymphocytic choriomeningitis virus: contact with pet hamsters, areas with rodent infestation
- Other arenaviruses: habitat of rodents

Vaccines or antiviral drugs
- No vaccines
- Antiviral drug: ribavirin

Disease mechanisms

Persistent infection of rodents caused by neonatal infection and induction of immune tolerance

Viruses infect macrophages and release mediators of cell and vascular damage

Tissue destruction caused by T-cell immunopathology

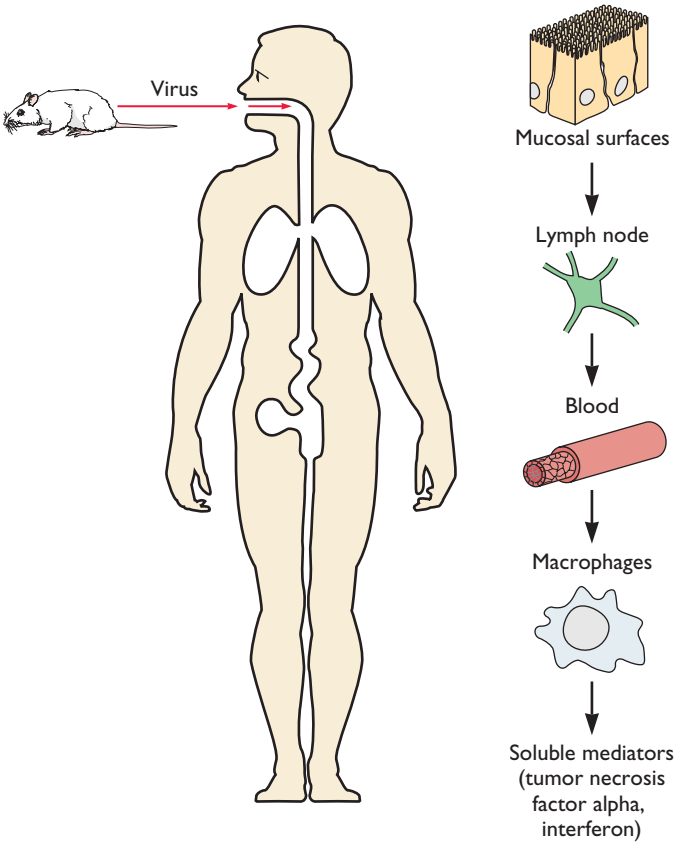


Figure 2

Bunyaviruses

Virus	Vector	Disease
Bunyavirus (49 species) • Bunyamwera virus • California encephalitis virus • La Crosse virus • Oropouche virus	Mosquito	Febrile illness, encephalitis, febrile rash
Hantavirus (22 species) • Hantaan virus	None	Hemorrhagic fever with renal syndrome, adult respiratory distress syndrome
• Sin Nombre virus	None	Hantavirus pulmonary syndrome, shock, pulmonary edema
Nairovirus (6 species) • Crimean-Congo hemorrhagic fever virus	Tick	Sandfly fever, hemorrhagic fever, encephalitis, conjunctivitis, myositis
Phlebovirus (9 species) • Rift Valley fever virus • Sandfly fever virus	Fly	Hemorrhagic fever

Epidemiology

Transmission
- Arthropod bite
- Rodent excreta

Distribution of virus
- Depends on distribution of vector or rodents
- Disease more common in summer

At risk or risk factors
- People in area of vector, e.g., campers, forest rangers, woodspeople

Vaccines or antiviral drugs
- None

Disease mechanisms

Primary viremia, then secondary viremia leads to virus spread to target tissues, including central nervous system, various organs, and vascular endothelium

Antibody essential for controlling viremia

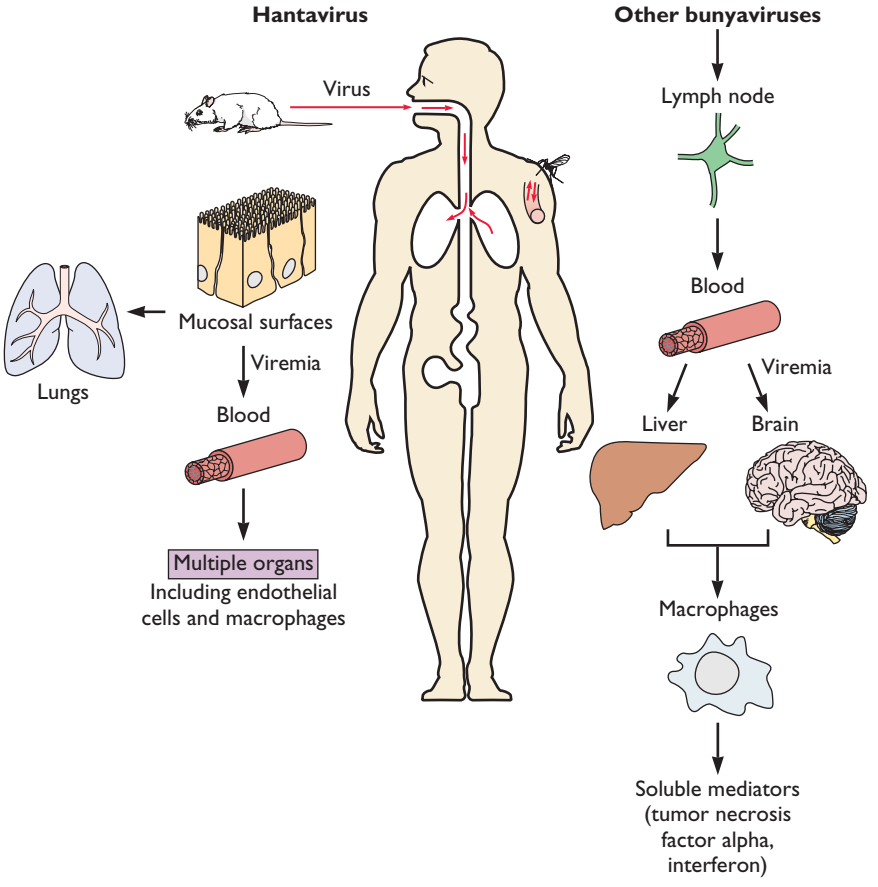


Figure 3

Caliciviruses

Virus	Disease
Logovirus *Norovirus* Norwalk virus	Gastroenteritis
Sapovirus Sapporo virus	Gastroenteritis

Epidemiology

Transmission
- Fecal-oral route from contaminated water and food
- Virions are resistant to detergents, drying, and acid

Distribution of virus
- Ubiquitous
- No seasonal incidence

At risk or risk factors
- Children in day care centers
- Schools, resorts, hospitals, nursing homes, restaurants, cruise ships (due to infected food handlers)

Vaccines or antiviral drugs
- None

Disease mechanisms

Viruses infect intestinal brush border, preventing proper absorption of water and nutrients

Viruses cause diarrhea, vomiting, abdominal cramps, nausea, headache, malaise, and fever

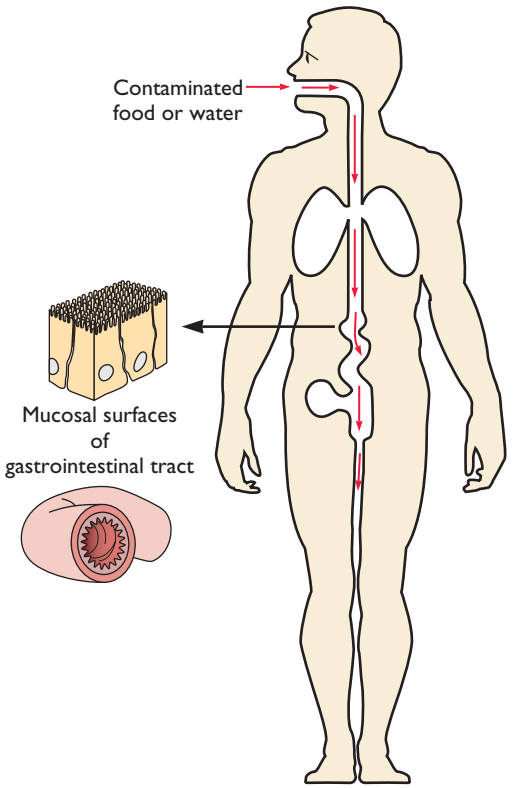


Figure 4

Filoviruses

Virus	Disease
Marburg virus Lake Victoria marburgvirus	Hemorrhagic fever
Ebola virus Reston ebolavirus	Hemorrhagic fever

Epidemiology

Transmission
- Fruit bats are reservoirs
- Contact with infected fruit bats, monkeys, or their tissues, secretions, or body fluids
- Contact with infected humans
- Accidental injection, contaminated syringes

Distribution of virus
- Endemic in monkeys in Africa
- No seasonal incidence

At risk or risk factors
- Monkey handlers
- Health care workers attending sick persons

Vaccines or antiviral drugs
- None

Disease mechanisms

Virus replication causes necrosis in liver, spleen, lymph nodes, and lungs

Hemorrhage causes edema and shock

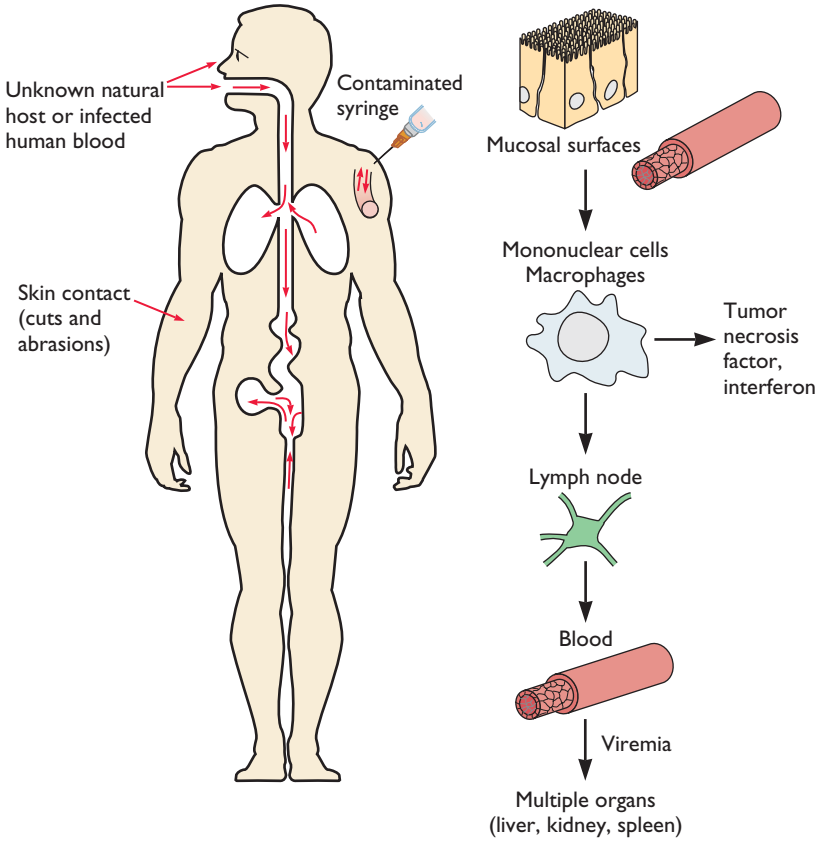


Figure 5

Flaviviruses

Virus	Vector	Disease
Flavivirus		
• Yellow fever virus	*Aedes* mosquitoes	Hepatitis, hemorrhagic fever
• Powassan virus	*Ixodes* ticks	Encephalitis
• Dengue virus	*Aedes* mosquitoes	Mild systemic; breakbone fever, dengue hemorrhagic fever, dengue shock syndrome
• Japanese encephalitis virus	*Culex* mosquitoes	Encephalitis
• St. Louis encephalitis virus	*Culex* mosquitoes	Encephalitis
• West Nile virus	*Culex* mosquitoes	Fever, encephalitis, hepatitis
Hepacivirus		
• Hepatitis C virus	None	Hepatitis (see "Viruses that cause hepatitis," next page)

Epidemiology

Transmission
- Mosquito or tick vectors

Distribution of virus
- Determined by habitat of vector
  - *Aedes* mosquito (urban areas)
  - *Culex* mosquito (forest, urban areas)
- More common in summer

At risk or risk factors
- People in niche of vector

Vaccines or antiviral drugs
- Live, attenuated vaccines for yellow fever and Japanese encephalitis
- No antiviral drugs

Disease mechanisms

Viruses are cytolytic

Viruses cause viremia and systemic infection

Nonneutralizing antibodies can facilitate infection of monocytes/macrophages via Fc receptors

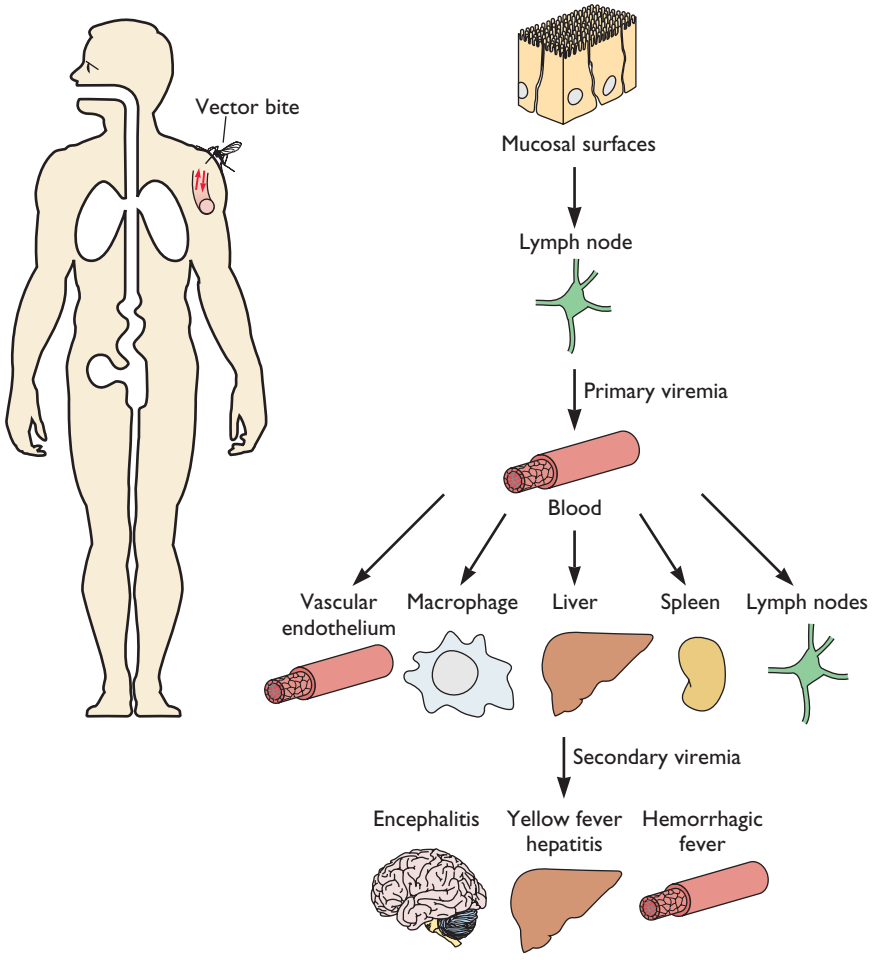


Figure 6

Viruses that cause hepatitis

Virus	Transmission	Incubation period	Mortality rate	Persistent infections	Other diseases
Hepatitis A virus (picornavirus)	Fecal-oral	15–50 days	<0.5%	No	None
Hepatitis B virus (hepadnavirus)	Parenteral, sexual	45–160 days	1–2%	Yes	Primary hepatocellular carcinoma, cirrhosis
Hepatitis C virus (flavivirus)	Parenteral, sexual	14–180 days	~4%	Yes	Primary hepatocellular carcinoma, cirrhosis
Hepatitis D virus (viroid-like; contribution to hepatitis is controversial)	Parenteral, sexual	15–64 days			
Hepatitis E virus (calicivirus)	Fecal-oral	15–50 days	1–2%; pregnant women, 20%	No	None

Epidemiology

Transmission
- Hepatitis A and E viruses
  - Fecal-oral route (contaminated food, water)
  - Transmitted by food handlers, day care workers, children
- Hepatitis B, C, and D viruses
  - Blood, semen, vaginal secretions
  - Transfusions, needle injury, drug paraphernalia, sex, breast-feeding
- Hepatitis B virus
  - Saliva, mother's milk

Distribution of virus
- Ubiquitous
- No seasonal incidence

At risk or risk factors
- Hepatitis A and E viruses
  - Children (mild disease)
  - Adults (abrupt-onset hepatitis)
  - Pregnant women (high mortality with hepatitis E virus)
- Hepatitis B, C, and D viruses
  - Children (mild, chronic infection)
  - Adults (insidious hepatitis)
  - Adults with chronic hepatitis
- Hepatitis B or C virus (hepatocellular carcinoma)

Vaccines or antiviral drugs
- Hepatitis A virus: inactivated vaccine
- Hepatitis B virus: subunit vaccine
- Hepatitis C virus: ribavirin + IFN-α

Disease mechanisms

Primary replication in hepatocytes followed by viremia

Generally not cytolytic; tissue damage caused by cell-mediated immune response

All cause acute infections

Hepatitis B and C virus may cause chronic infections that can lead to hepatocellular carcinoma

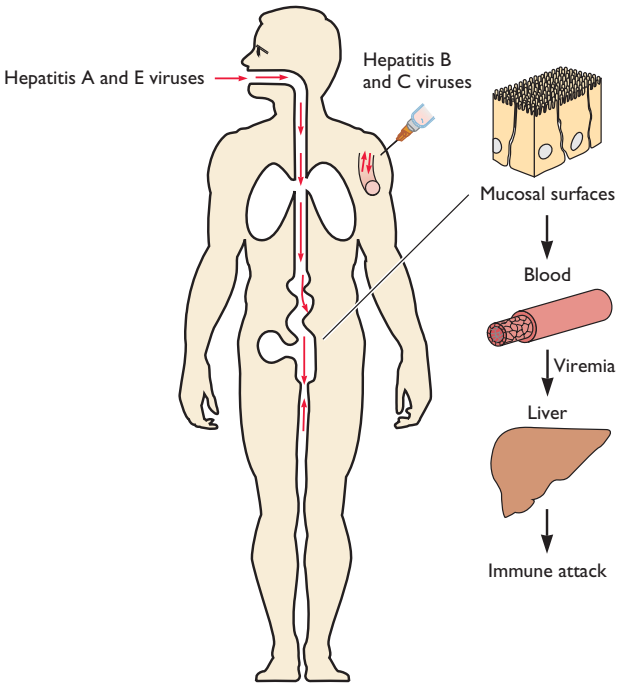


Figure 7

Herpesviruses

Herpes simplex virus

Virus	Disease
Alphaherpesviruses	
• **Herpes simplex virus types 1 and 2**	Mucosal lesions, encephalitis
• Varicella-zoster virus	Chickenpox, shingles

Epidemiology

Transmission
- Saliva, vaginal secretions, lesion fluid
- Into eyes and breaks in skin
- Herpes simplex virus type 1: mainly oral, herpes simplex virus type 2: mainly sexual

Distribution of virus
- Ubiquitous
- No seasonal incidence

At risk or risk factors
- Children (type 1) and sexually active people (type 2)
- Physicians, nurses, dentists, and those in contact with oral and genital secretions (herpetic whitlow, infection of finger)
- Immunocompromised and neonates (disseminated, life-threatening disease)

Vaccines or antiviral drugs
- No vaccine
- Antiviral drugs: acyclovir, penciclovir, valacyclovir, famciclovir, adenosine arabinoside, iododeoxyuridine, trifluridine

Disease mechanisms

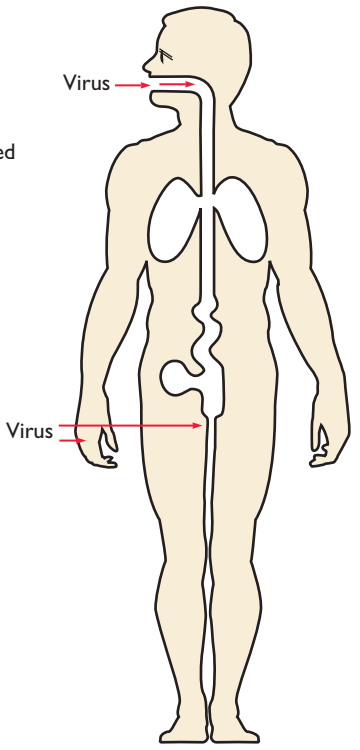
Virus spreads cell to cell, not neutralized by antibody

Cell-mediated immunopathology contributes to symptoms

Cell-mediated immunity is required for resolution of infection

Virus establishes latency in neurons

Virus is reactivated from latency by stress or immune suppression



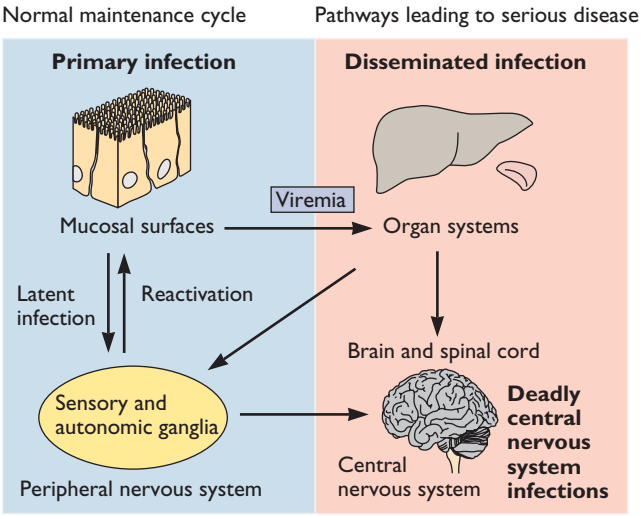


Figure 8

Herpesviruses

Cytomegalovirus

Virus	Disease
Betaherpesviruses	
• **Cytomegalovirus**	Congenital defects; opportunistic pathogen in immunocompromised patients
• Human herpesvirus 6	Roseola
• Human herpesvirus 7	Orphan virus

Epidemiology

Transmission
- Blood, tissue, and body secretions (urine, saliva, semen, cervical secretions, breast milk, tears)

Distribution of virus
- Ubiquitous
- No seasonal incidence

At risk or risk factors
- Babies whose mothers become infected during pregnancy (congenital defects)
- Sexual activity
- Blood and transplant recipients
- Burn victims
- Immunocompromised (recurrent disease)

Vaccines or antiviral drugs
- No vaccines
- Antiviral drugs: acyclovir, ganciclovir, valganciclovir, foscarnet, cidofovir, fomivirsen

Disease mechanisms

Infects epithelial and other cells

Mainly causes subclinical infections

Cell-mediated immunity required for resolution of infection

Latent infection in $CD34^+$ bone marrow progenitor cells, macrophages, other cells

Suppression of cell-mediated immunity leads to recurrence and severe disease

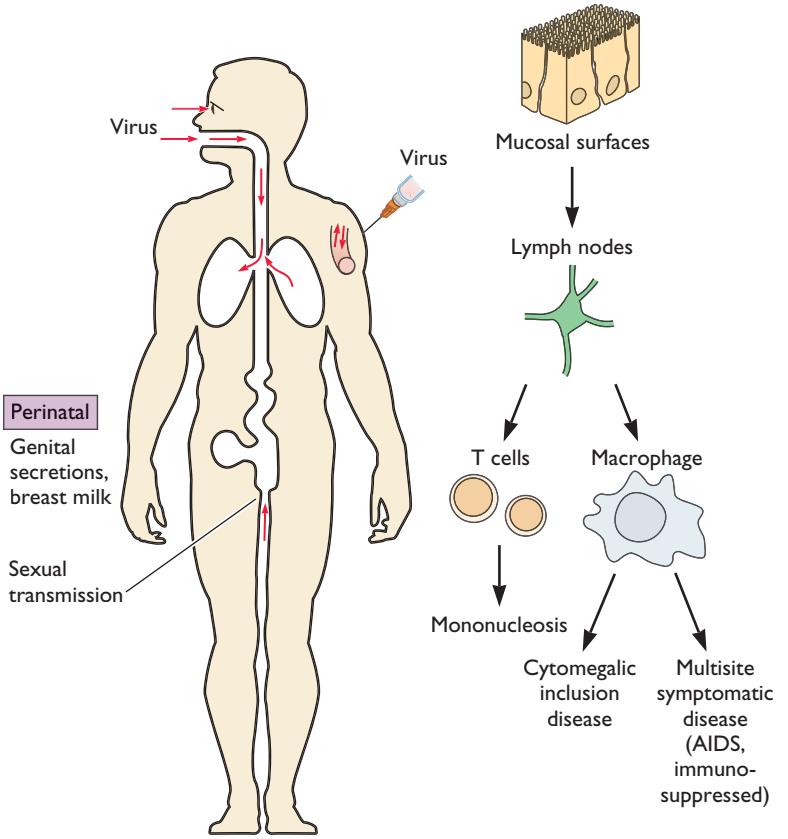


Figure 9

Herpesviruses

Epstein-Barr virus

Virus	Disease
Gammaherpesviruses	
• **Epstein-Barr virus**	Infectious mononucleosis; associated with a variety of lymphomas
• Human herpesvirus 8 (Kaposi's sarcoma-related virus)	Kaposi's sarcoma, rare B-cell lymphoma

Epidemiology

Transmission
- Saliva, close oral contact, or shared items (cup or toothbrush)

Distribution of virus
- Ubiquitous
- No seasonal incidence

At risk or risk factors
- Children (asymptomatic or mild symptoms)
- Teenagers (infectious mononucleosis)
- Immunocompromised (fatal neoplastic disease)
- Malaria (Burkitt's lymphoma)

Vaccines or antiviral drugs
- None

Disease mechanisms

Infects oral epithelial cells, B cells

Immortalizes B cells

T cells are required to control infection

T cells contribute to symptoms of **infectious mononucleosis**

Associated with lymphoma in immunosuppressed patients, Burkitt's lymphoma, and nasopharyngeal carcinoma

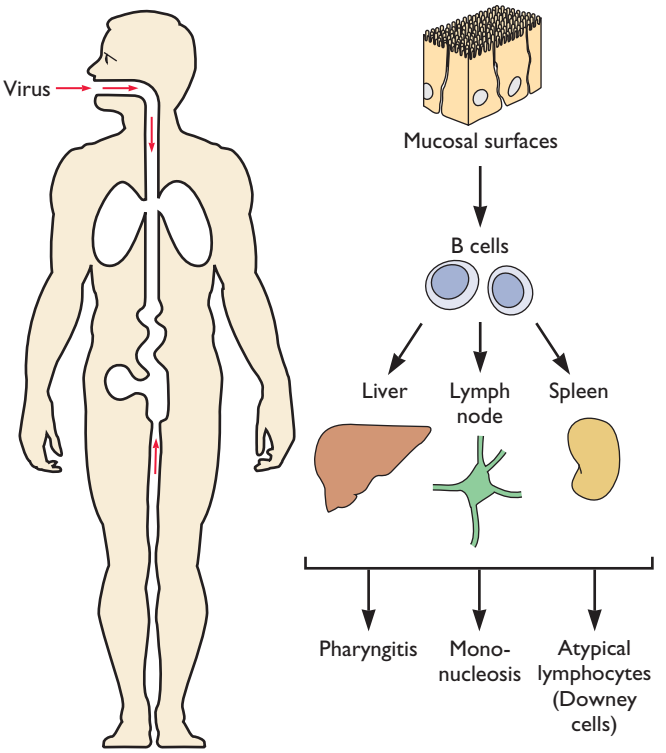


Figure 10

Herpesviruses

Varicella-zoster virus

Virus	Disease
Alphaherpesviruses	
• Herpes simplex virus types 1 and 2	Mucosal lesions, encephalitis
• **Varicella-zoster virus**	Chickenpox, shingles

Epidemiology

Transmission
- Virus is transmitted by respiratory droplets or contact

Distribution of virus
- Ubiquitous
- No seasonal incidence

At risk or risk factors
- Children (ages 5–9 years) (mild disease)
- Teenagers and adults (more severe disease, possibly pneumonia)
- Immunocompromised or neonates (fatal pneumonia, encephalitis, disseminated varicella)
- Elderly, immunocompromised (recurrent zoster)

Vaccines or antiviral drugs
- Live vaccine (Oka strain)
- Antiviral drugs: acyclovir, foscarnet

Disease mechanisms

Infects epithelial cells and fibroblasts, spread by viremia to skin, causes lesions of chicken pox

Cell-mediated immunopathology contributes to symptoms

Cell-mediated immunity is required for resolution of infection

Latent infection in neurons

Reactivation by immune suppression

Reactivation leads to zoster or shingles, formation of lesions over entire dermatome

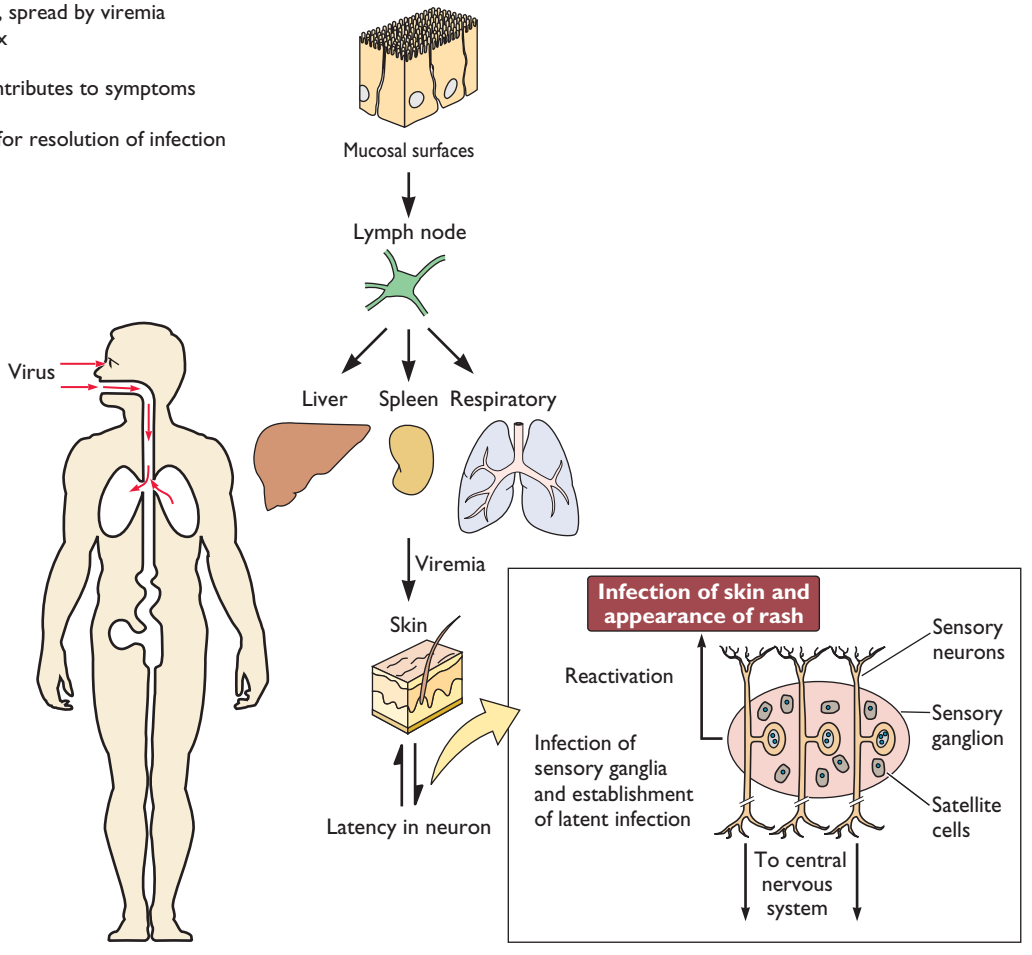


Figure 11

Orthomyxoviruses

Virus	Disease
Influenza A, B, and C viruses	Influenza • Acute febrile respiratory tract infection • Rapid onset of fever, malaise, sore throat, cough • Children may also have abdominal pain, vomiting, otitis media, myositis, croup Complications • Primary viral pneumonia • Myositis and cardiac involvement • Guillain-Barré syndrome • Encephalopathy • Encephalitis • Reye's syndrome

Epidemiology

Transmission
- Inhalation of small aerosol droplets
- Widely spread by schoolchildren

Distribution of virus
- Ubiquitous; epidemics, pandemics
- More common in winter

At risk or risk factors
- Adults (typical "flu" syndrome)
- Children (asymptomatic to severe infections)
- Elderly, immunocompromised, and those with cardiac or respiratory problems (high risk)

Vaccines or antiviral drugs
- Killed vaccine against annual strains of influenza A and B viruses
- Live, attenuated influenza A and B vaccine (nasal spray)
- Antiviral drugs: amantadine, rimantadine, zanamivir, oseltamivir

Disease mechanisms

Infects upper and lower respiratory tract

Pronounced systemic symptoms caused by cytokine response to infection

Antibodies against hemagglutinin (HA) and neuraminidase (NA) are important for protection against infection

Recovery depends upon interferon and cell-mediated immune response

Susceptibility to bacterial superinfection due to loss of natural epithelial barriers

HA and NA of influenza A virus undergo major and minor antigenic changes

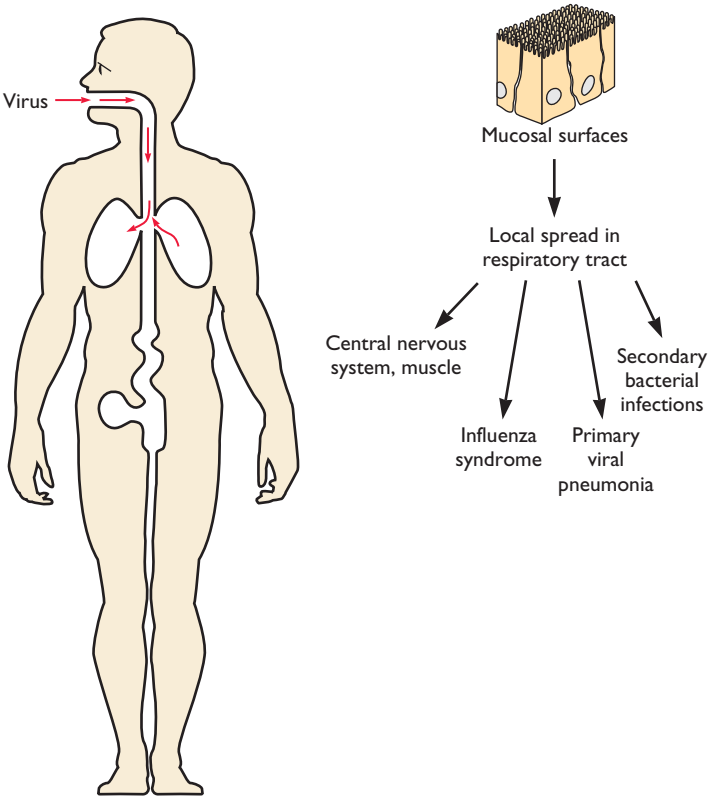


Figure 12

Papillomaviruses

Virus	Disease
Papillomavirus (90 genotypes)	Skin warts: plantar, common, and flat warts, epidermodysplasia verruciformis Benign head and neck tumors: laryngeal, oral, and conjunctival papillomas Anogenital warts: condyloma acuminatum, cervical intra-epithelial neoplasia, cancer

Epidemiology

Transmission
- Direct contact, sexual contact
- During birth, from infected birth canal

Distribution of virus
- Ubiquitous
- No seasonal incidence

At risk or risk factors
- Sexual activity

Vaccines or antiviral drugs
- Vaccine against types 6, 11, 16, and 18 (Gardasil)

Disease mechanisms

Infect epithelial cells of skin, mucous membranes

Replication depends on stage of epithelial cell differentiation

Cause benign outgrowth of cells into warts

Some types are associated with dysplasia that may become cancerous

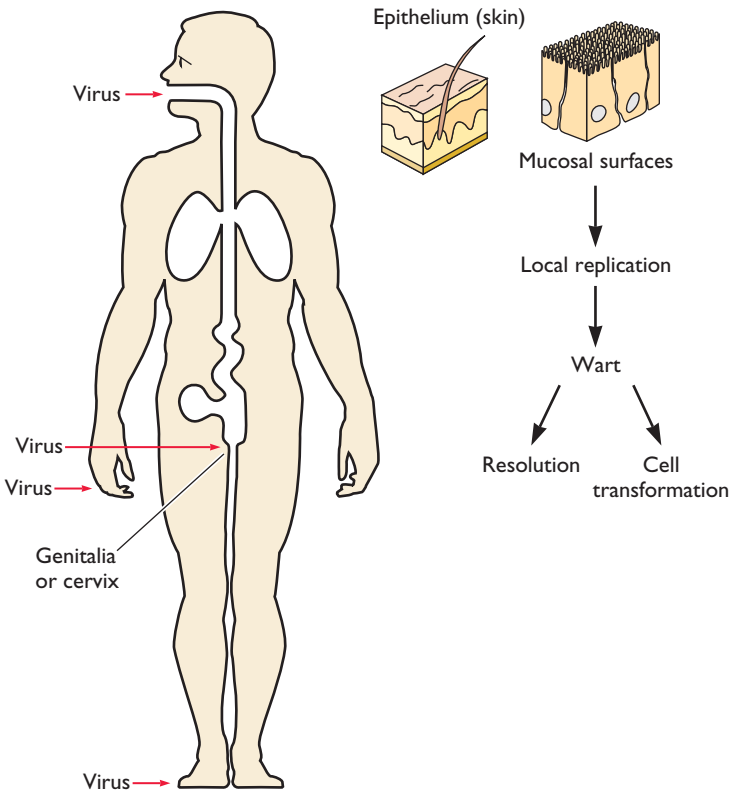


Figure 13

Paramyxoviruses

Measles virus

Virus	Disease
Morbilliviruses	
• **Measles virus**	Measles Complications: otitis media, croup, bronchopneumonia, encephalitis Subacute sclerosing panencephalitis

Epidemiology

Transmission
- Inhalation of aerosols
- Highly contagious

Distribution of virus
- Ubiquitous
- Endemic from autumn to spring

At risk or risk factors
- Adults (serious disease) and children
- Immunocompromised persons (more serious outcomes)

Vaccines or antiviral drugs
- Live, attenuated vaccine
- No antiviral drugs

Disease mechanisms

Infects epithelial cells of respiratory tract, spreads in lymphocytes and by viremia

Replicates in conjunctivae, respiratory tract, urinary tract, lymphatic system, blood vessels, and central nervous system

T-cell response to virus-infected capillary endothelial cells causes rash

Cell-mediated immunity is required to control infection

Complications are due to immunopathogenesis (postinfectious measles encephalitis) or viral mutants (subacute sclerosing panencephalitis)

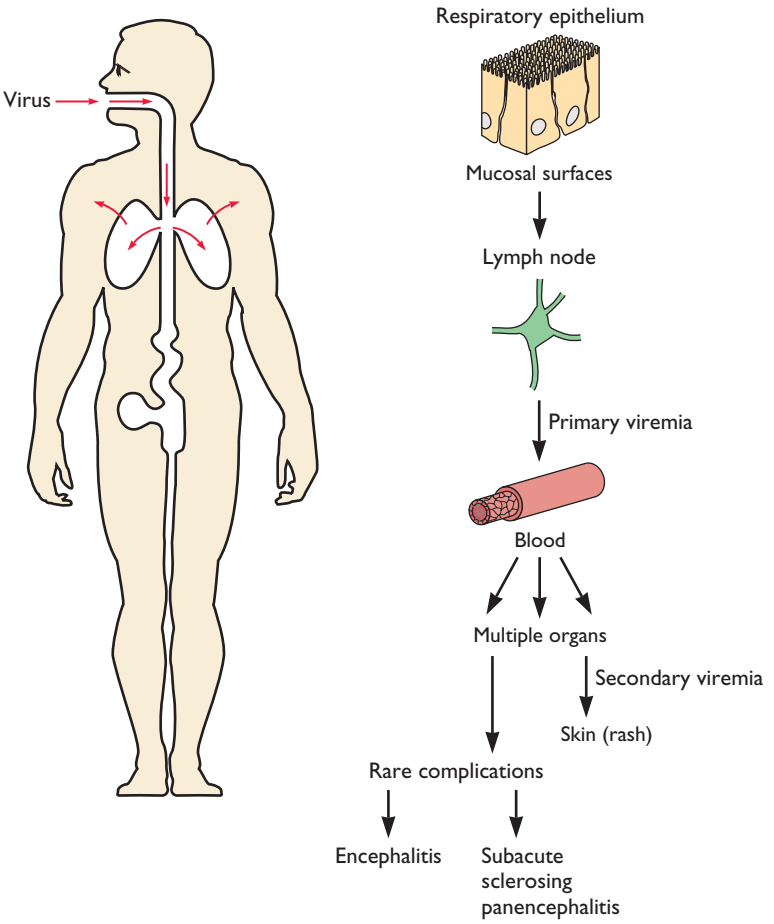


Figure 14

Paramyxoviruses

Mumps virus

Virus	Disease
Paramyxoviruses	
• Parainfluenza virus types 1–4	Cold-like symptoms, bronchitis, croup
• **Mumps virus**	Mumps

Epidemiology

Transmission
- Inhalation of aerosols
- Highly contagious

Distribution of virus
- Ubiquitous
- Endemic in late winter, early spring

At risk or risk factors
- Adults (serious disease) and children
- Immunocompromised persons (more serious outcomes)

Vaccines or antiviral drugs
- Live, attenuated vaccine
- No antiviral drugs

Disease mechanisms

Infects epithelial cells of respiratory tract, spreads by viremia

Replicates in salivary glands, testes, respiratory tract, and central nervous system

Cell-mediated immunity is required to control infection

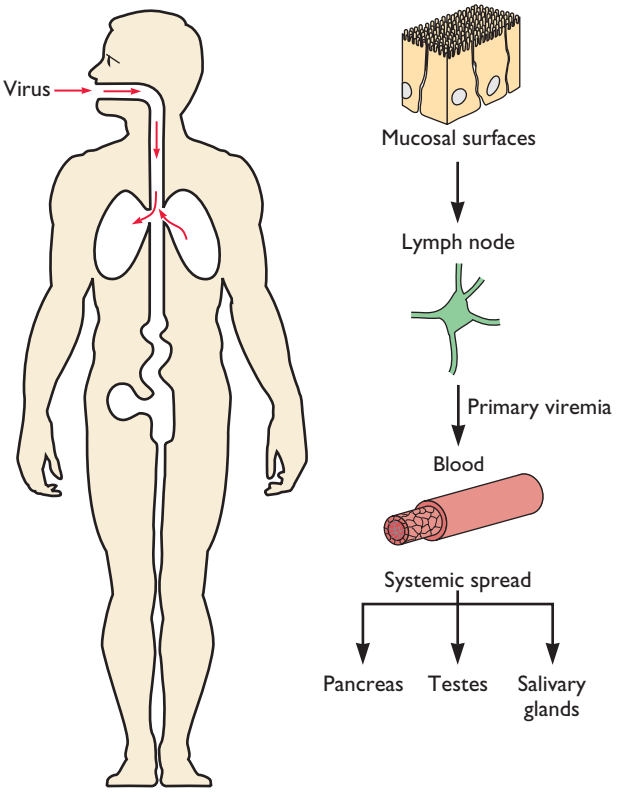


Figure 15

Paramyxoviruses

Respiratory syncytial virus

Virus	Disease
Pneumoviruses	
• **Respiratory syncytial virus**	Bronchiolitis, pneumonia, febrile rhinitis, pharyngitis, common cold

Epidemiology

Transmission
- Inhalation of aerosols

Distribution of virus
- Ubiquitous
- Incidence is seasonal

At risk or risk factors
- Infants (bronchiolitis, pneumonia)
- Children (mild disease to pneumonia)
- Adults (mild symptoms)

Vaccines or antiviral drugs
- No vaccine
- Antiviral drug: ribavirin for infants

Disease mechanisms

Infects the respiratory tract, does not spread systemically

May cause bronchitis, febrile rhinitis, pharyngitis, common cold, or pneumonia

Bronchiolitis probably caused by the host immune response

In newborns, the infection may be fatal because narrow airways are blocked by virus-induced pathology

Infants are not protected from infection by maternal antibody

Reinfection may occur after a natural infection

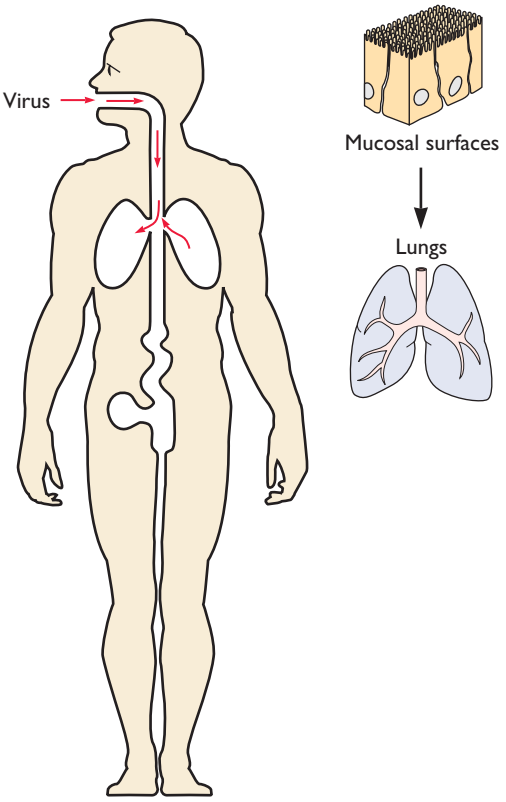


Figure 16

Parvoviruses

Virus	Disease
B19 parvovirus	Erythema infectiosum (fifth disease) Aplastic crisis in patients with chronic hemolytic anemia Acute polyarthritis Abortion
Adeno-associated virus	Commonly infects humans, not associated with illness

Epidemiology

Transmission
- Respiratory and oral droplets

Distribution of virus
- Ubiquitous
- Fifth disease most common in late winter and spring

At risk or risk factors
- Children in elementary school (fifth disease)
- Parents of infected children
- Pregnant women (fetal infection and disease)
- Patients with chronic anemia (aplastic crisis)

Vaccines or antiviral drugs
- None

Disease mechanisms

In utero infection

Virus infects mitotically active erythroid precursor cells in bone marrow

Biphasic disease
Flu-like phase, viral shedding during viremia
Later phase: erythematous maculopapular rash, arthralgia, and arthritis caused by circulating virus-antibody immune complexes

Aplastic crisis in patients with chronic hemolytic anemia is caused by depletion of erythroid precursors and destabilization of erythrocytes

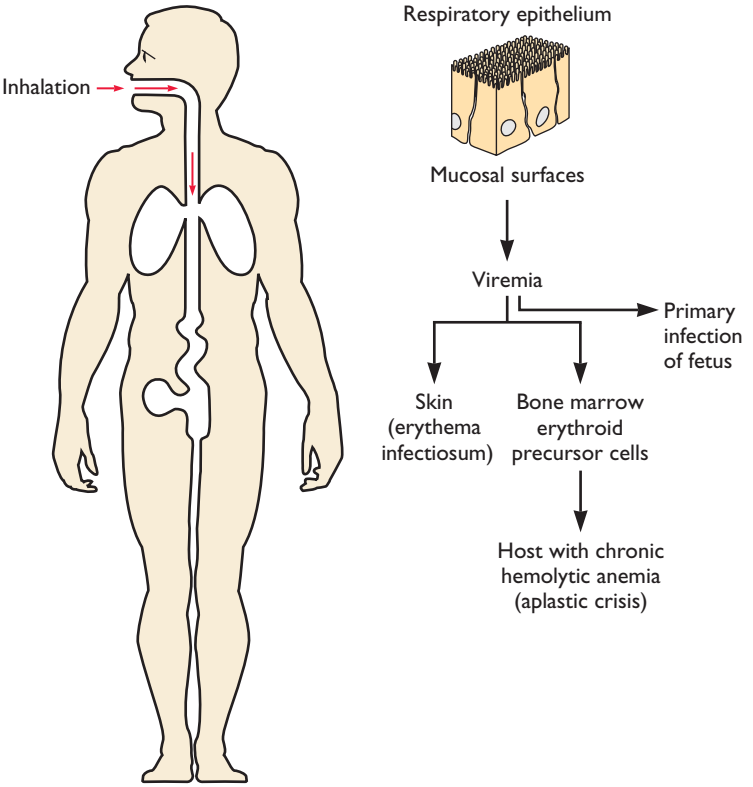


Figure 17

Picornaviruses

Virus	Paralytic disease	Encephalitis, meningitis	Carditis	Neonatal disease	Pleurodynia	Herpangina	Hand-foot-and-mouth disease	Rash disease	Acute hemorrhagic conjunctivitis
Poliovirus types 1–3	+	+							
Coxsackie A viruses 1–24	+	+	+			+	+	+	+
Coxsackie B viruses 1–6	+	+	+	+	+			+	
Echoviruses 1–33	+	+	+	+				+	
Enterovirus 70	+								+
Enterovirus 71	+	+					+		
Parechoviruses 1–3	+	+							
Rhinoviruses 1–100									

Virus	Respiratory tract infections	Undifferentiated fever	Diarrhea, gastrointestinal disease	Diabetes, pancreatitis	Orchitis	Disease in immunodeficient patients	Congenital anomalies
Poliovirus types 1–3	+	+				+	
Coxsackie A viruses 1–24	+	+				+	+
Coxsackie B viruses 1–6		+		+	+		+
Echoviruses 1–33	+	+	+			+	
Enterovirus 70							
Enterovirus 71							
Parechoviruses 1–3	+	+	+				
Rhinoviruses 1–100	+						

Epidemiology

Transmission
- Enteroviruses: fecal-oral
- Rhinoviruses: inhalation of droplets, contact with contaminated hands

At risk or risk factors
- Poliovirus
  - Young children (asymptomatic or mild disease)
  - Older children, adults (asymptomatic to paralytic disease)
- Coxsackievirus and enterovirus (newborns and neonates at highest risk for serious disease)
- Rhinovirus (all ages)

Distribution of virus
- Ubiquitous; poliovirus is nearly eradicated
- Enteroviruses: disease most common in summer
- Rhinovirus: disease most common in early autumn, late spring

Vaccines or antiviral drugs
- Poliovirus: live oral or inactivated polio vaccines
- No vaccines for other enteroviruses or rhinoviruses
- No licensed antiviral drugs

Figure 18

Disease mechanisms

Virus
Enteroviruses

Disease
Enter oropharyngeal or intestinal mucosa

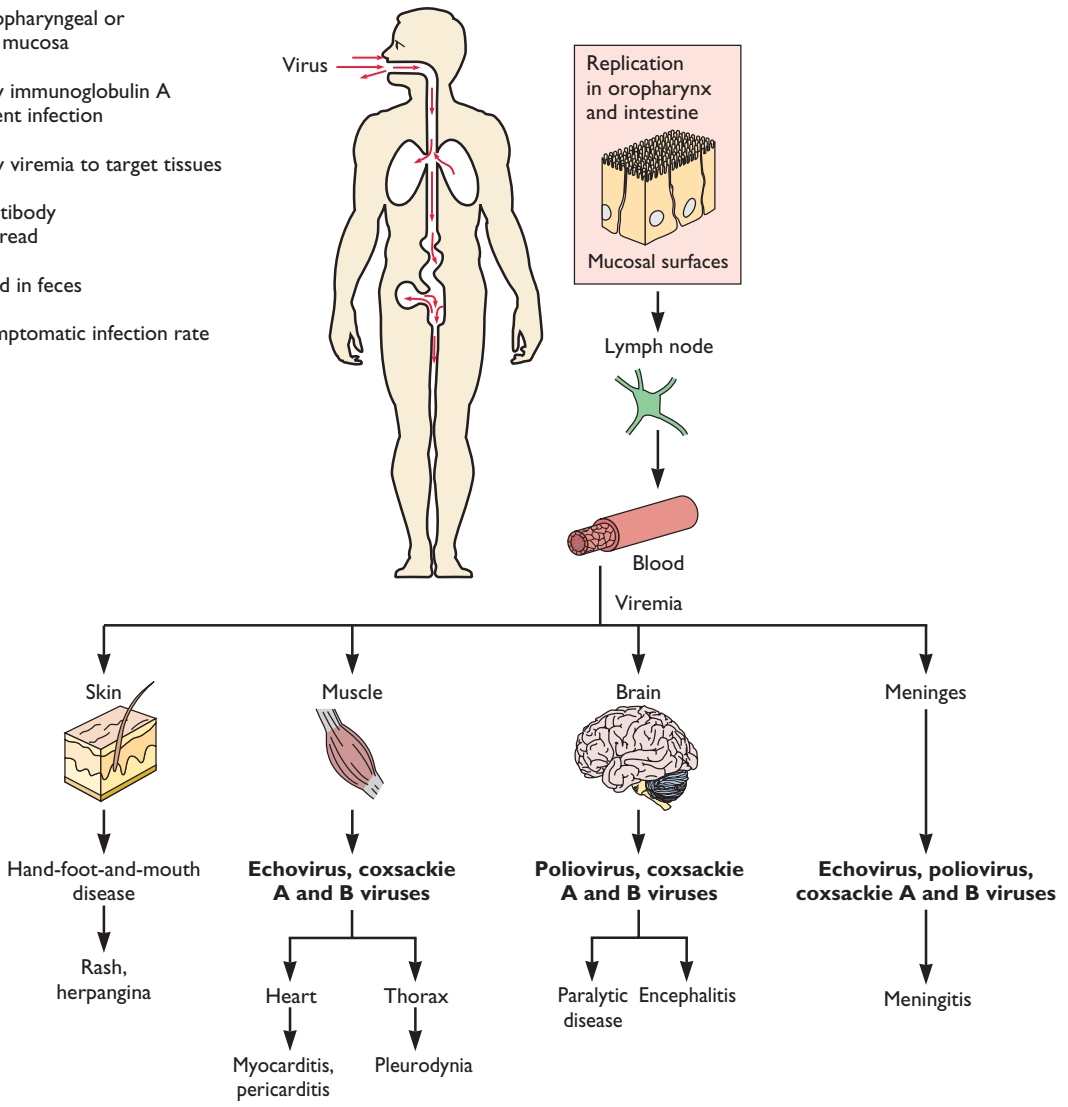
Secretory immunoglobulin A can prevent infection

Spread by viremia to target tissues

Serum antibody blocks spread

Virus shed in feces

High asymptomatic infection rate



Virus
Rhinoviruses

Disease
Enter upper respiratory tract, where infection is usually limited

Major factor in asthma exacerbations

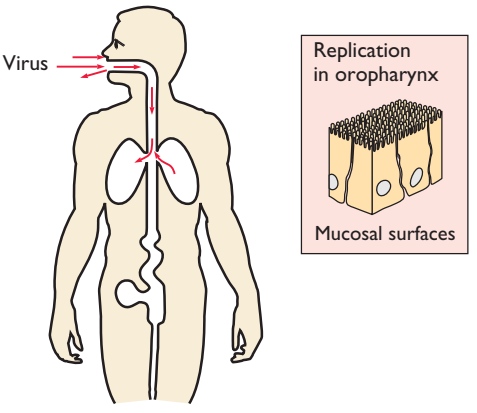


Figure 19

Polyomaviruses

Virus	Disease
Polyomavirus • BK virus	Renal disease in immunosuppressed patients
• JC virus	Progressive multifocal leukoencephalopathy in immunosuppressed patients

Epidemiology

Transmission
• Inhalation of aerosols

Distribution of virus
• Ubiquitous
• No seasonal incidence

At risk
• Immunocompromised persons

Vaccines or antiviral drugs
• None

Disease mechanisms

Acquired through the respiratory route, spread by viremia to kidneys early in life

Infections are usually asymptomatic

Virus establishes persistent and latent infection in organs such as the kidneys and lungs

In immunocompromised people, JC virus is activated, spreads to the brain, and causes progressive multifocal leukoencephalopathy; oligodendrocytes are killed, causing demyelination

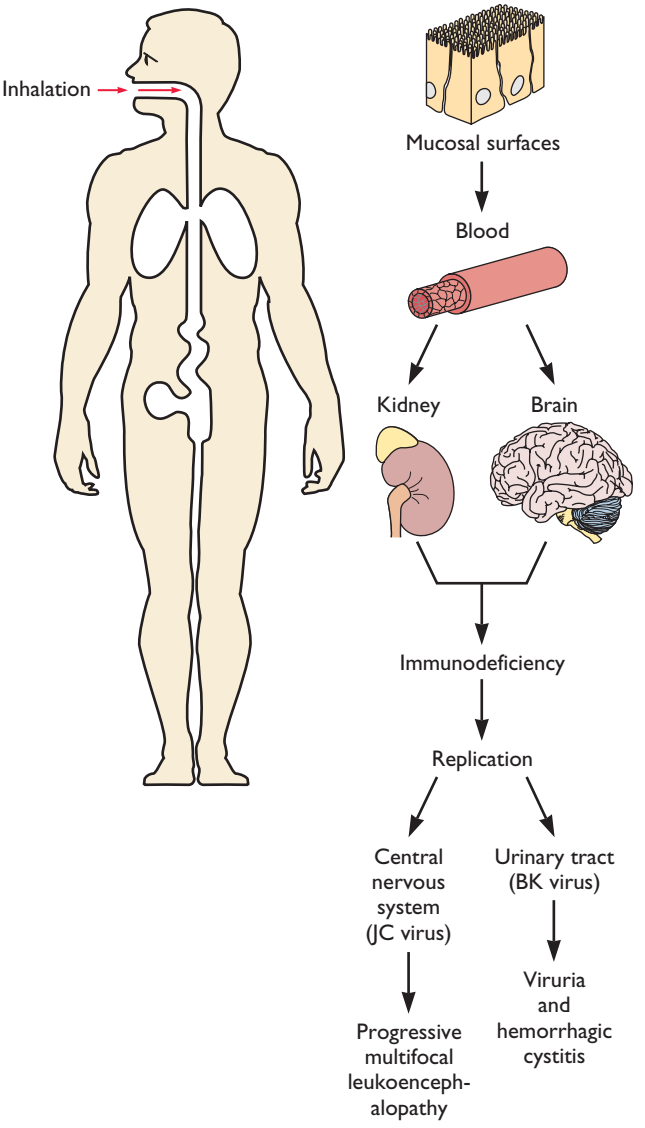


Figure 20

Poxviruses

Virus	Disease
Variola virus	Smallpox
Vaccinia virus (smallpox vaccine)	Encephalitis and vaccinia necrosum (complications of vaccination)
Orf virus	Localized lesion
Cowpox virus	Localized lesion
Pseudocowpox virus	Milker's nodule
Monkeypox virus	Generalized disease
Bovine papular stomatitis virus	Localized lesion
Tanapox virus	Localized lesion
Yaba monkey tumor virus	Localized lesion
Molluscum contagiosum virus	Disseminated skin lesions

Epidemiology

Transmission
- Smallpox: respiratory droplets, contact with virus on fomites
- Other poxviruses: direct contact or fomites

At risk or risk factors
- Molluscum contagiosum: sexual contact, wrestling
- Zoonoses: animal handlers (contact with lesion)

Distribution of virus
- Ubiquitous
- No seasonal incidence
- Natural smallpox has been eradicated

Vaccines or antiviral drugs
- Live vaccine against smallpox (vaccinia virus)
- No antiviral drugs

Disease mechanisms

Infects respiratory tract, spreads through lymphatics and blood

Molluscum contagiosum and zoonoses transmitted by contact

Sequential infection of multiple organs

Cell-mediated and humoral immunity important to resolve infection

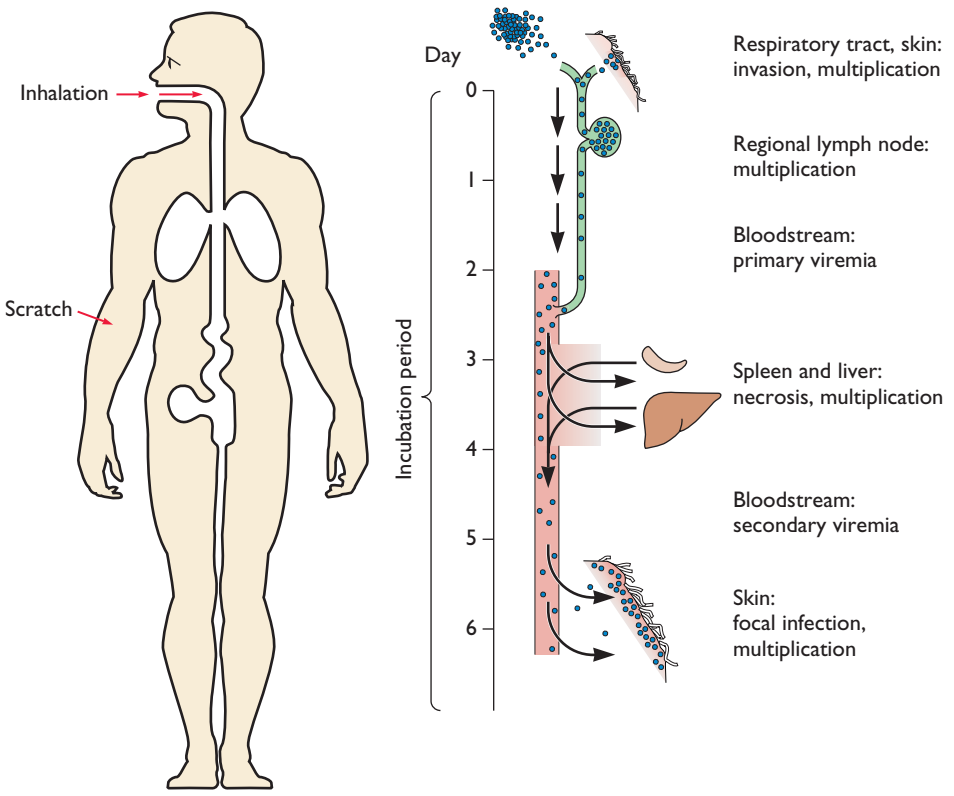


Figure 21

Reoviruses

Rotavirus

Virus	Disease
Orthoreovirus	Mild upper respiratory tract disease, gastroenteritis, biliary atresia
Coltivirus	Colorado tick fever: febrile disease, headache, myalgia (zoonosis)
Rotavirus	Gastroenteritis

Epidemiology

Transmission
- Fecal-oral route

Distribution of virus
- Ubiquitous (type A)
- Less common in summer

At risk
- Rotavirus type A
  - Infants <24 months of age (gastroenteritis, dehydration)
  - Older children (mild diarrhea)
  - Undernourished persons in underdeveloped countries (diarrhea, dehydration, death)
- Rotavirus type B
  - Infants, older children, adults in China (severe gastroenteritis)

Vaccines or antiviral drugs
- Live, attenuated, oral vaccines available

Disease mechanisms

nsP4 is a viral enterotoxin that causes diarrhea

Disease is serious in infants <24 months old, asymptomatic in adults

Large quantities of virions released in diarrhea

Immunity to infection depends on immunoglobulin A in gut lumen

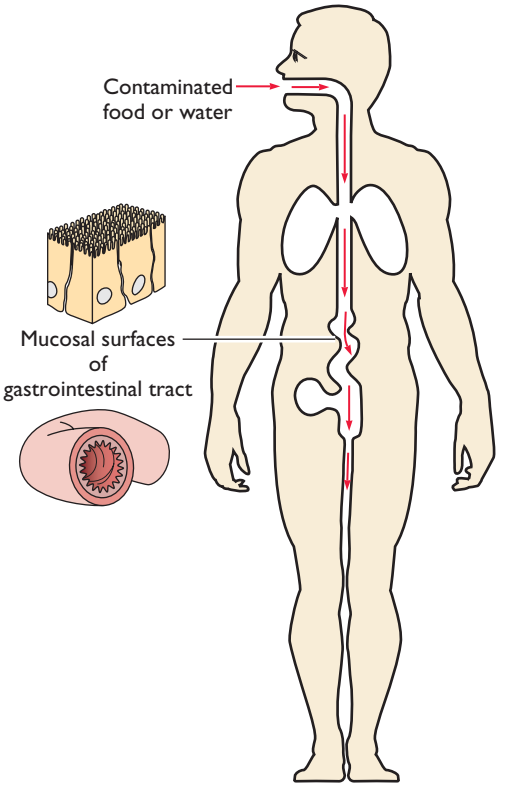


Figure 22

Retroviruses

Human T-lymphotropic virus type 1

Virus	Disease
Deltaretrovirus	
• **Human T-lymphotropic virus type 1**	Adult T-cell leukemia Tropical spastic paraparesis
• Human T-lymphotropic virus type 2	Hairy-cell leukemia
• Human T-lymphotropic virus type 5	Malignant cutaneous lymphoma

Epidemiology

Transmission
- Virus in blood
  Transfusions, needle sharing among drug users; infected lymphocytes must be present
- Virus in semen
  Anal and vaginal intercourse
- Perinatal transmission
  Transplacental passage of infected maternal lymphocytes, infected lymphocytes in breast milk

Distribution of virus
- Ubiquitous
- No seasonal incidence

At risk
- Intravenous drug users
- Homosexuals and heterosexuals with many partners
- Prostitutes
- Newborns of virus-positive mothers

Vaccines or antiviral drugs
- No vaccines
- No antiviral drugs

Disease mechanisms

Infects T lymphocytes

Remains latent or replicates slowly, induces clonal outgrowth of T-cell clones

Long latency period (30 years) before onset of leukemia

Infection leads to immunosuppression

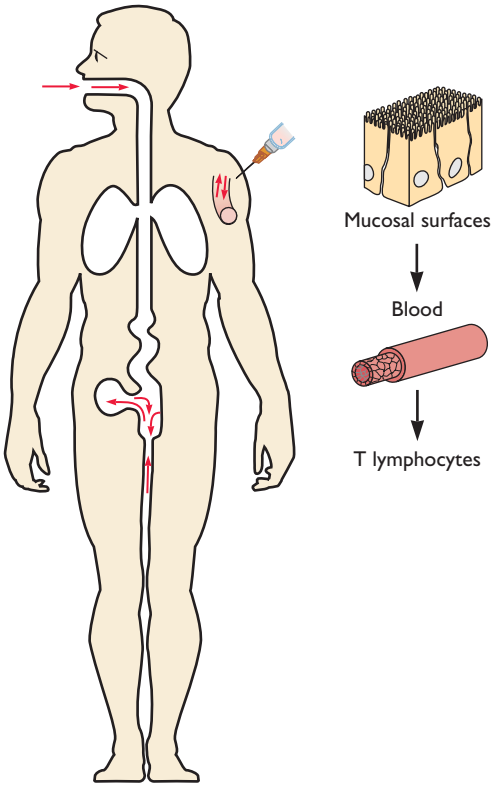


Figure 23

Retroviruses

Human immunodeficiency virus types 1 and 2

Virus	Disease
Lentivirus • **Human immunodeficiency virus types 1 and 2**	Acquired immune deficiency syndrome (AIDS)

Epidemiology

Transmission
- Virus in blood
  - Transfusions, needle sharing among drug users, needle sticks in health care workers, tattoo needles
- Virus in semen and vaginal secretions
  - Anal and vaginal intercourse
- Perinatal transmission
  - Intrauterine and peripartum transmission; breast milk

Distribution of virus
- Ubiquitous
- No seasonal incidence

At risk
- Intravenous drug users
- Homosexuals and heterosexuals with many partners
- Prostitutes
- Newborns of virus-positive mothers

Vaccines or antiviral drugs
- No vaccines
- Antiviral drugs
  - Nucleoside analog reverse transcriptase inhibitors (e.g., azidothymidine, dideoxycytidine)
  - Nonnucleoside reverse transcriptase inhibitors (e.g., nevirapine, delavirdine)
  - Protease inhibitors (e.g., saquinavir, ritonavir)
  - Integrase inhibitors (e.g., raltegravir, elvitegravir)
  - Fusion inhibitors (e.g., enfuvirtide, maraviroc)

Disease mechanisms

Infects mainly $CD4^+$ T cells and macrophages

Lyses $CD4^+$ T cells, persistently infects macrophages

Infection alters T-cell and macrophage function; immunosuppression leads to secondary infection and death

Infects long-lived cells, establishing reservoir for persistent infection

Infected monocytes spread to brain, causing dementia

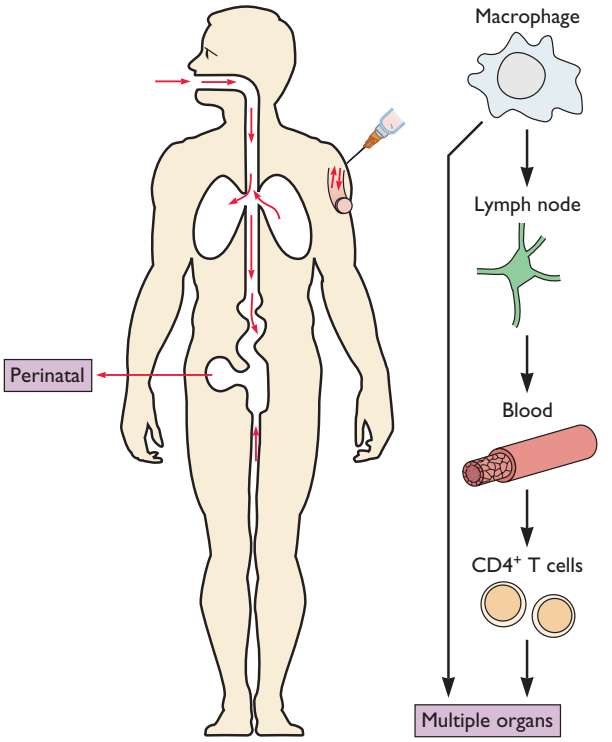


Figure 24

Rhabdoviruses

Rabies virus

Virus	Disease
Lyssavirus	
• **Rabies virus**	Rabies
• Related viruses of rodents and bats	Rarely cause rabies-like encephalitis
Vesiculovirus	
• Vesicular stomatitis virus	Flu-like illness

Epidemiology

Transmission
- Reservoir: wild animals
- Vectors: wild animals, unvaccinated dogs and cats
- Bite of rabid animal (virus in saliva), aerosols (in caves harboring rabid bats)

Distribution of virus
- Ubiquitous, except certain islands
- No seasonal incidence

At risk or risk factors
- Animal handlers, veterinarians
- Those in countries with no pet vaccinations or quarantine

Vaccines or antiviral drugs
- Vaccines for pets and wild animals
- Inactivated virus vaccine for at-risk personnel, postexposure prophylaxis
- No antiviral drugs

Disease mechanisms

Replicates in muscle at bite site

Incubation period of weeks to months, depending on inoculum and distance of bite from central nervous system

Infects peripheral nerves and travels to brain

Replication in brain causes hydrophobia, seizures, hallucinations, paralysis, coma, and death

Spreads to salivary glands, from which it is transmitted

Postexposure immunization can prevent disease due to long incubation period

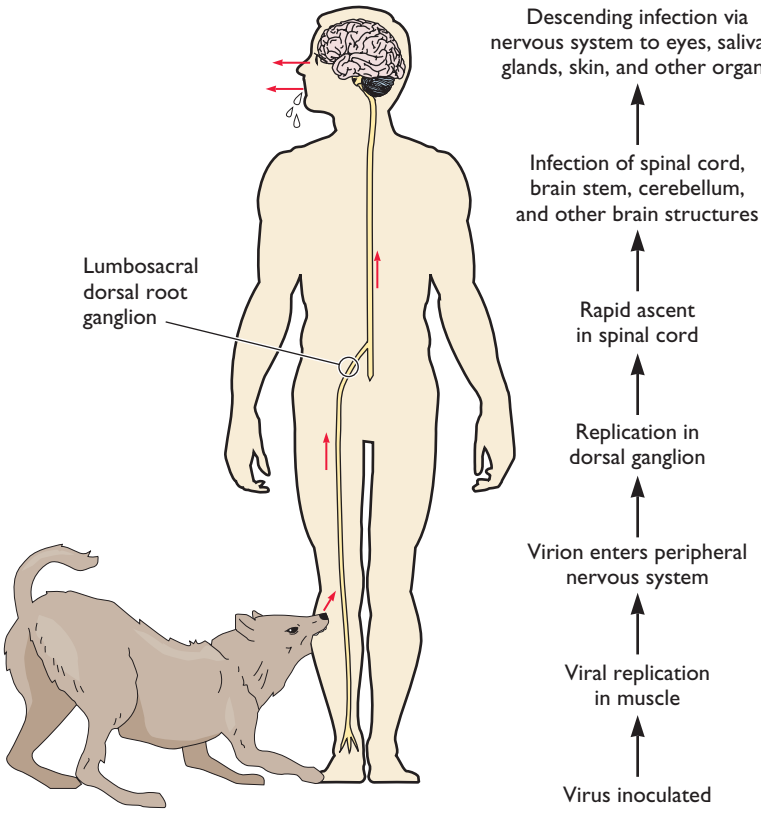


Figure 25

Togaviruses

Virus	Vector	Disease
Alphaviruses		
• Sindbis virus	*Aedes* mosquitoes	Subclinical
• Semliki Forest virus	*Aedes* mosquitoes	Subclinical
• Venezuelan equine encephalitis virus	*Aedes, Culex* mosquitoes	Mild systemic; severe encephalitis
• Eastern equine encephalitis virus	*Aedes, Culiseta* mosquitoes	Mild systemic; encephalitis
• Western equine encephalitis virus	*Culex, Culiseta* mosquitoes	Mild systemic; encephalitis
• Chikungunya virus	*Aedes* mosquitoes	Fever, arthralgia, arthritis
Rubella virus	None	Rubella

Epidemiology

Transmission
- Alphavirus: mosquito vectors
- Rubella virus: respiratory route

Distribution of virus
- Arthropod-borne viruses: determined by habitat of vector
  Aedes mosquito: urban areas
  Culex mosquito: forest, urban areas
  Most common in summer
- Rubella virus: ubiquitous

At risk
- Arthropod-borne viruses: people in niche of vector
- Rubella virus: neonates <20 weeks old (congenital defects), children (mild rash), adults (more severe disease, arthritis, arthralgia)

Vaccines or antiviral drugs
- Live, attenuated vaccine for rubella virus
- No antiviral drugs

Disease mechanisms

Viruses are cytolytic (except rubella virus)

Cause viremia, systemic infection

Antibodies limit virus spread by viremia (e.g., to fetus in pregnant host)

Cell-mediated immunity important to resolve infection

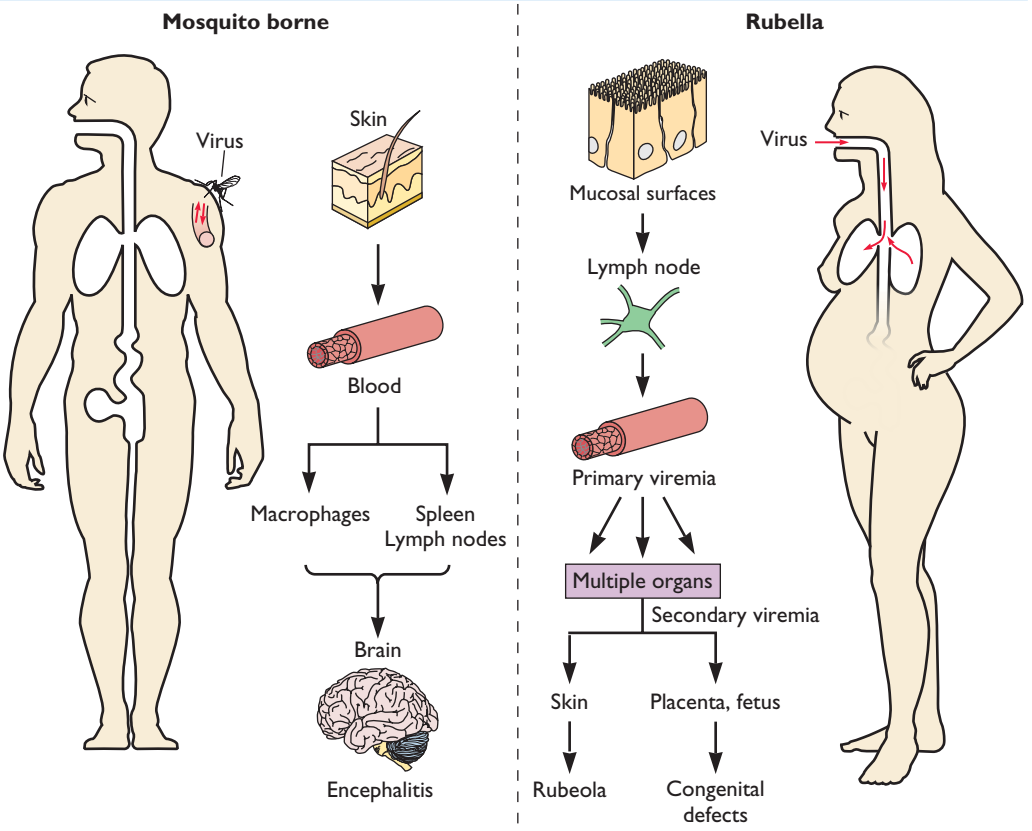


Figure 26

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APPENDIX B
Unusual Infectious Agents

Introduction

In Volume I, Chapter 3, we discuss the functions specified by small and large viral genomes. A fundamental question about size is, What is the minimal genome necessary to sustain an infectious agent? Taken to the extreme, the question becomes, Could an infectious agent exist without any genome at all? The subviral agents called viroids, satellites, and prions provide answers to these questions. The adjective "subviral" was coined, in part, because these agents did not fit into the standard taxonomy schemes for viruses. The Subviral RNA Database (http://subviral.med.uottawa.ca/cgi-bin/home.cgi) boasts about 2,000 nucleotide sequences for viroids and related RNA molecules. These infectious agents are widespread, and, perhaps more important, infection by some is deadly.

Viroids

Potato spindle tuber viroid, which was discovered in 1971, is the prototype for the smallest known nucleic acid-based agents of infectious disease, the **viroids** (Fig. 1). Viroids are unencapsidated, small, circular, single-stranded RNA molecules that replicate autonomously when introduced mechanically into host plants. Infection by some viroids causes economically important diseases of crop plants, while others appear to be benign, despite their widespread presence in the plant world. Two examples of economically important viroids are coconut cadang-cadang viroid (which causes a lethal infection of coconut palms) and apple scar skin viroid (which causes an infection that results in visually unappealing apples that cannot be sold). Viroids have been classified in the families *Avsunviroidae* (group A viroids) and *Pospiviroidae* (group B viroids).

The circular single-stranded RNA molecules of all viroids range from 246 to 399 nucleotides in length and are extensively base paired, so that the RNA appears as a 50-nm rod in the electron microscope. There is no evidence that viroids encode proteins or mRNA. Unlike viruses, which are parasites of host translation machinery, **viroids are parasites of cellular transcription**

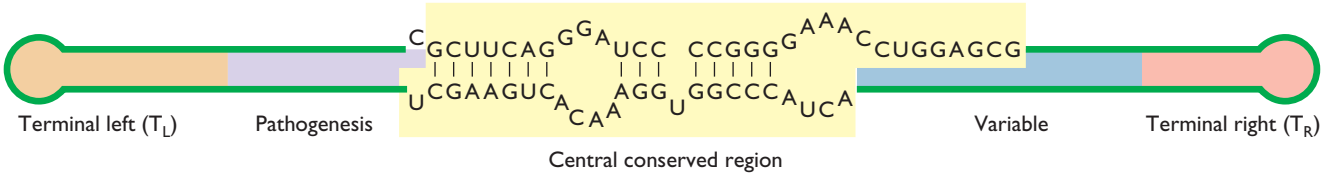
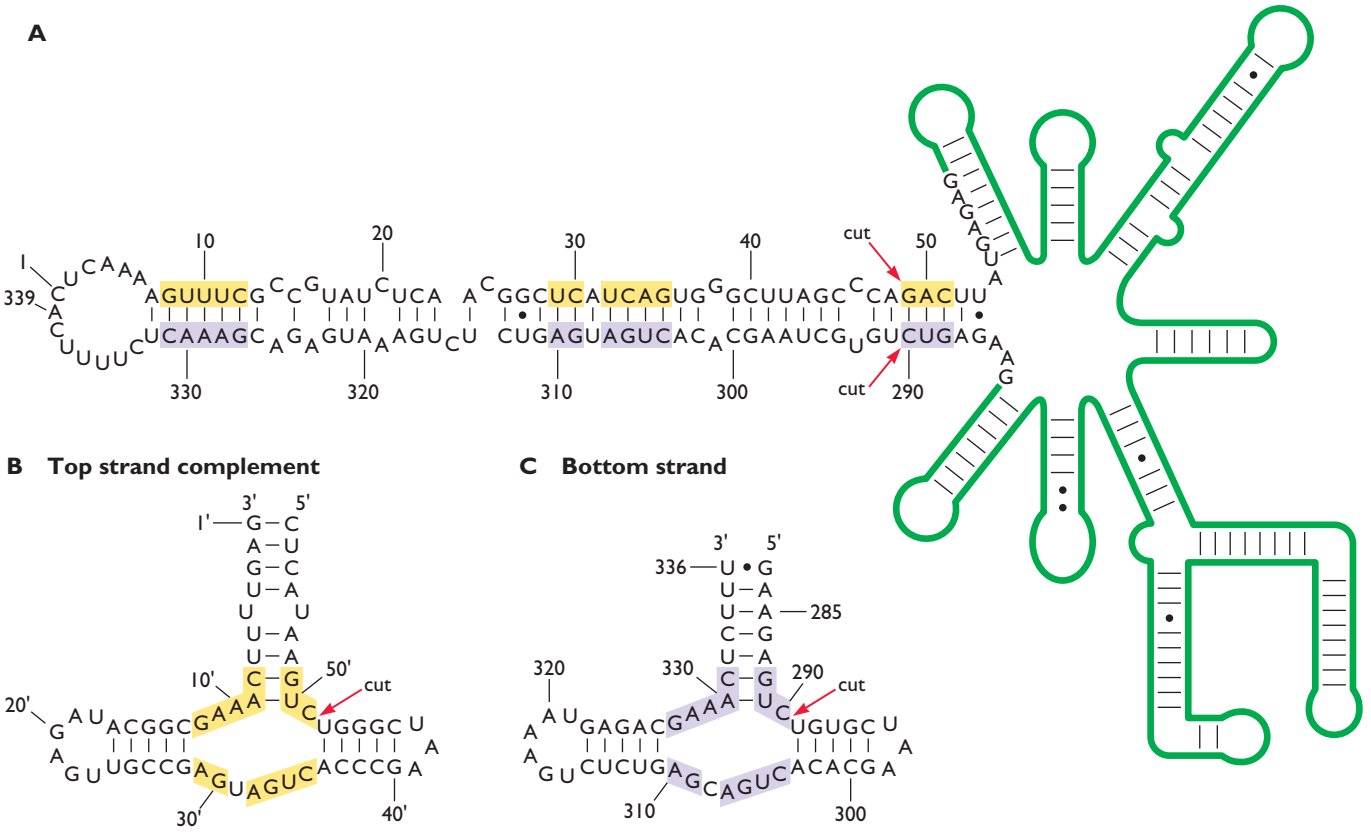


Figure 1 Model of the potato spindle tuber viroid (PSTVd), a viroid that does not form a ribozyme. The RNA strand is shown as a green closed loop. Four functional domains are indicated by different colors. The nucleotides in the upper strand of the central conserved region can form a stable stem-loop. See R. Flores, F. Di Serio, and C. Hernandez, *Semin. Virol.* **8:**65–73, 1997, for more information.

proteins: they depend on RNA polymerase II for replication. Several well-studied group A viroid RNA molecules are functional ribozymes, and this activity is essential for replication (Fig. 2). Group B viroid RNA molecules have no detectable ribozyme activity and instead require a cellular RNase for replication. In general, replication occurs in the nucleus, and most viroids can also be found in the nucleoli of infected cells.

Our current understanding is that the disease-causing viroids were transferred from wild plants used for breeding modern crops. The modern widespread prevalence of viroids can be traced to the use of genetically identical plants (monoculture), worldwide distribution of breeding lines, and mechanical transmission by contaminated farm machinery. As a consequence, these unusual pathogens now occupy niches around the planet that never before

Figure 2 The predicted secondary structure of peach latent mosaic viroid, a ribozyme-forming RNA. **(A)** The circular RNA backbone is indicated by the thick green line. Hydrogen bonds are indicated by dashes, and G · U pairs are indicated by black dots. The nucleotides involved in forming the ribozyme structures are extended to the left and are numbered arbitrarily with respect to the ribozyme sequence. When copied, the top strand forms a hammerhead ribozyme. The bottom strand also forms a hammerhead ribozyme. Conserved nucleotides in the *Avsunviroidae* are boxed and shaded, and the cleavage sites are indicated by red arrows. **(B)** The top strand complement ribozyme (the prime symbol indicates a complement of the sequence shown in panel A). **(C)** The bottom strand hammerhead ribozyme. See R. Flores, F. Di Serio and C. Hernandez, *Semin. Virol.* **8:**65–73, 1997, and M. Pelchat, D. Levesque, J. Ouellet, et al., *Virology* **271:**37–45, 2000, for more information.



were available to them. As might be expected for an RNA replicon, mutations accumulate at high frequency. In addition, the viroid genome encodes no gene products and infection requires no host receptors, allowing viroids to evolve with amazing speed.

Satellites

Satellites are small, single-stranded RNA molecules that lack genes required for their replication but do replicate in the presence of another virus (the **helper virus**). Unlike viroids, satellite genomes encode one or two proteins. Typical satellite genomes are 500 to 2,000 nucleotides of single-stranded RNA. We recognize two classes of satellites: viruses and nucleic acids. **Satellite viruses** are distinct particles that were discovered in preparations of their helper viruses. These particles contain nucleic acid genomes that encode a structural protein that encapsidates the satellite genome. Satellite viruses are **not** defective viruses derived from the helper virus: their genomes have no homology to the helper. **Satellite nucleic acids** (sometimes called virusoids) are distinguished from satellite viruses by virtue of their packaging by a capsid protein encoded in the helper virus genome.

Most satellites are associated with plant viruses, but a well-known example of a human satellite virus is hepatitis delta satellite virus with its helper, hepatitis B virus. In plants, satellites and satellite viruses cause disease symptoms not seen with the helper virus alone. However, although hepatitis delta satellite virus is associated with human hepatitis, its contribution to disease remains controversial. Hepatitis delta satellite virus appears to be a hybrid between a viroid and a satellite. The genome is 1.7 kb of circular single-stranded RNA that folds upon itself in a tight rodlike structure (70% base paired). The RNA molecule is also a ribozyme, and this activity is required for replication. These properties resemble those of viroid genomes. On the other hand, the genome encodes a protein (delta) that encapsidates the genome, a property shared with satellite nucleic acids. Two functionally distinct forms of the delta protein are made as a result of RNA editing (see Volume I, Chapter 10). The hepatitis delta satellite virion comprises the satellite nucleocapsid packaged within an envelope that contains the surface protein of the helper, hepatitis B virus.

The world abounds with RNA replicons that share structural and functional characteristics of satellites and viroids (Table 1). Similarities to introns, transposons, and RNAs found in the signal recognition particle have been noted, but the origin of satellites and viroids remains an enigma. Moreover, we have only a modest understanding of the mechanisms by which these minimal pathogens cause disease. The speed of their divergence in the absence of obvious selection and the ease with which they can be manipulated make them useful models to study the evolution of infection and pathogenesis.

Table 1 Viroids and satellites

Property	Viroids		Satellites
	Group A	Group B	
Requires coinfection with helper virus	No	No	Yes
Encodes protein	No	No	Yes
Replication	By host RNA polymerase and viroid ribozyme	By host RNA polymerase and host RNase	By helper virus replication proteins

Prions and Transmissible Spongiform Encephalopathies

Can infectious agents exist without genomes? This question challenges our definitions of what constitutes an infectious agent, but the answer seems to be yes. The question arose with the discovery and characterization of infectious agents associated with one group of slow diseases, now called **transmissible spongiform encephalopathies (TSEs)**. These diseases are rare, but always fatal, neurodegenerative disorders that afflict humans and other mammals (Table 2). Each year, thousands of individuals worldwide are diagnosed with spongiform encephalopathies, and about 1% of these cases arise by infection. There is concern that this low rate of infection may increase, because some TSEs spread when animals ingest infected tissues. At the end of 2002, 120 individuals had succumbed to variant Creutzfeldt-Jakob disease, an affliction ascribed to consumption of meat from

Table 2 Transmissible spongiform encephalopathies[a]

TSE diseases of animals
Bovine spongiform encephalopathy (BSE) (mad cow disease)
Chronic wasting disease (CWD) (deer, elk)
Exotic ungulate encephalopathy (EUE) (nyala and greater kudu)
Feline spongiform encephalopathy (FSE) (domestic and wild cats)
Scrapie in sheep and goats
Transmissible mink encephalopathy (TME)
TSE diseases of humans
Creutzfeldt-Jakob disease (CJD)
Fatal familial insomnia (FFI)
Gerstmann-Sträussler syndrome (GSS)
Kuru
Variant CJD (vCJD)

[a]From S. B. Prusiner (ed.), *Semin. Virol.* **7**:157–223, 1996.

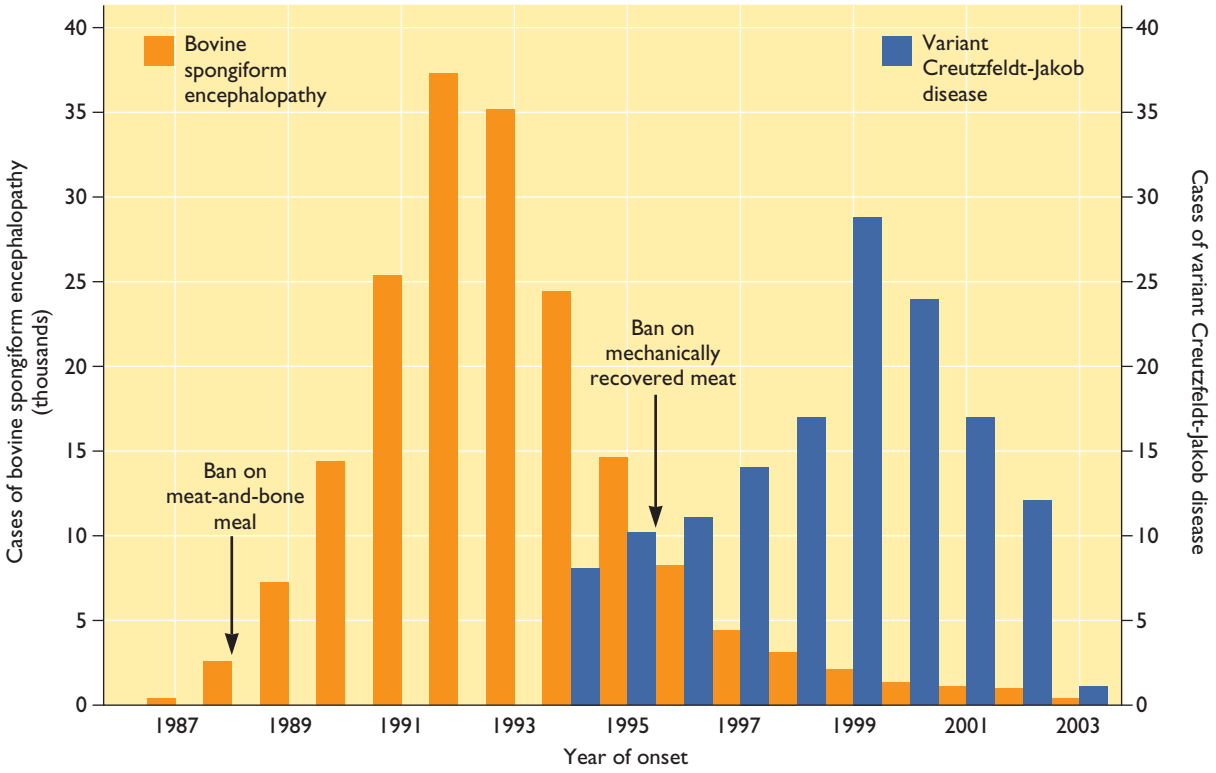


Figure 3 Time course of the reporting of bovine spongiform encephalopathy in cattle and variant Creutzfeldt-Jakob disease in humans in the United Kingdom over a period of 9 years. The peak of the bovine epidemic was in 1992, and the peak of the human disease was in 1999. The incidence of both is now rare. Data obtained from two websites (http://www.defra.gov.uk/animalh/bse/statistics/incidence.html and The 2004 Institute of Food Science and Technology Information Statement on BSE and Variant Creutzfeldt-Jakob Disease [http://www.ifst.org/hottop5.htm]).

animals with bovine spongiform encephalopathy (Fig. 3). While some investigators still contend that viruses or virus-like particles cause TSEs, most now are convinced that these diseases result from infectious proteins called **prions**.

Human TSEs

Several lines of evidence indicated that human spongiform encephalopathies might be caused by an infectious agent. Carleton Gajdusek and colleagues studied the disease **kuru**, found in the Fore people of New Guinea. Kuru spread among women and children as a result of ritual cannibalism of the brains of deceased relatives. When cannibalism stopped, so did kuru. William Hadlow made the seminal observation that lesions in the brains of humans with spongiform encephalopathy were similar to lesions in the brains of animals with scrapie. It was known that scrapie was transmissible to other animals, and experiments by others soon demonstrated that human spongiform encephalopathy can be transmitted from humans to chimpanzees and other primates. Since 1957, the spongiform encephalopathies of animals and humans have been considered to have common features. Because of this unifying idea, experimental systems that enabled a more detailed understanding of this complex diseases were established. In humans, the spongiform encephalopathies fall into three classes, **infectious, familial,** and **sporadic,** distinguished by how the disease is acquired initially (Box 1).

Hallmarks of TSE Pathogenesis

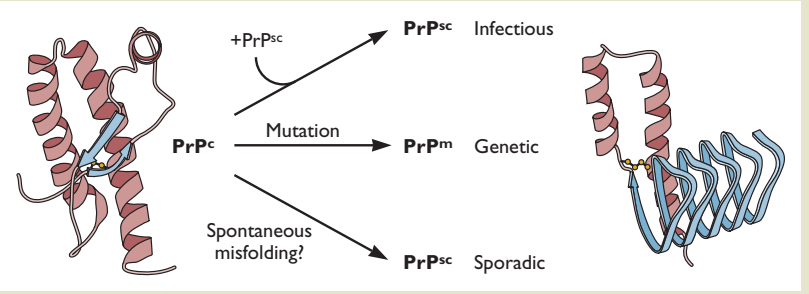
For most TSEs, the presence of an infectious agent can be detected definitively only by injection of organ homogenates into susceptible recipient species. Clinical signs of infection commonly include cerebellar ataxia (defective motion or gait) and dementia, with death occurring after months or years. The infectious agent first accumulates in the lymphoreticular and secretory organs and then spreads to the nervous system. In model systems, spread of the disease from the site of inoculation to other organs and

BOX I TERMINOLOGY

Characteristics of the human spongiform encephalopathies

An **infectious** (or **transmissible**) **spongiform encephalopathy** is exemplified by kuru and iatrogenic spread of disease to healthy individuals by transplantation of infected corneas, the use of purified hormones, or transfusion with blood from patients with **Creutzfeldt-Jakob disease (CJD).** The recent epidemic spread of bovine spongiform encephalopathy (mad cow disease) among cattle in Britain can be ascribed to the practice of feeding processed animal by-products to cattle as a protein supplement. Similarly, the new human disease, variant CJD, arose after consumption of beef from diseased cattle.

Familial spongiform encephalopathy is associated with an autosomal dominant mutation in the *prnp* gene. Familial CJD, for example, in contrast to sporadic CJD, is an inherited disease.



Sporadic CJD is a disease affecting fewer than a million individuals worldwide, usually late in life (from age 50 to 70). About 65% of all spongiform encephalopathies are due to sporadic CJD. As the name indicates, the disease appears with no warning or epidemiological indications. Kuru may have been originally established in the small population of Fore people in New Guinea when the brain of an individual with sporadic CJD was eaten.

The important principle is that diseases of **all three classes** can usually be transmitted experimentally or naturally to primates by inoculation or ingestion of diseased tissue.

the brain requires dendritic and B cells. The disease agent then invades the peripheral nervous system and spreads from there to the spinal cord and brain. Once the infectious agent is in the central nervous system, the characteristic pathology includes severe astrocytosis, vacuolization (hence the term "spongiform"), and loss of neurons. Occasionally, dense fibrils or aggregates (sometimes called plaques) can be detected in brain tissue at autopsy. There is little indication of inflammatory, antibody, or cellular immune responses. The time course, degree, and site of cytopathology within the central nervous system are dependent upon the particular TSE agent and the genetic makeup of the host.

Identification of the First Agent Causing TSE

One of the best-studied TSE diseases is scrapie, so called because infected sheep tend to scrape their bodies on fences so much that they rub themselves raw. A second characteristic symptom, skin tremors over the flanks, led to the French name for the disease, *tremblant du mouton.* Motor disturbances then manifest as a wavering gait, staring eyes, and paralysis of the hindquarters. There is no fever, but infected sheep lose weight and die, usually within 4 to 6 weeks of the first appearance of symptoms. Scrapie has been recognized as a disease of European sheep for more than 200 years. It is endemic in some countries, for example, the United Kingdom, where it affects 0.5 to 1% of the sheep population per year.

Sheep farmers discovered that animals from diseased herds could pass the affliction to a scrapie-free herd, implicating an infectious agent. In 1939, infectivity from extracts of scrapie-affected sheep brains was shown to pass through filters with pores small enough to retain everything but viruses. In the 1970s, ultracentrifugation studies indicated that the agent was heterodisperse in size and density. Even to this day, purification of the infectious agent to homogeneity has not been achieved.

Physical Nature of the Scrapie Agent

The physical nature of the infectious agent has been a major point of contention. As early as 1966, scientists found that scrapie infectivity was considerably more resistant than that of most viruses to ultraviolet (UV) and ionizing radiation. For example, the scrapie agent is 200-fold more resistant to UV irradiation than polyomavirus and 40-fold more resistant than a mouse retrovirus. Other TSE agents exhibit similar UV resistance. On the basis of this relative resistance to UV irradiation, some investigators argued that TSE agents are viruses well shielded from irradiation whereas others claimed that TSE agents have little or no nucleic acid at all.

The infectivity of scrapie agent is also more resistant to chemicals, such as the combination of 3.7% formaldehyde and autoclaving routinely used to inactivate viruses. While it is possible to reduce infectivity by 90 to 95% after several hours of such treatment, complete elimination is

exceedingly difficult. This fact has led to unfortunate infections by seemingly sterile instruments used in neurosurgery. Suffice it to say that TSE agents are not typical infectious agents.

Prions and the *prnp* Gene

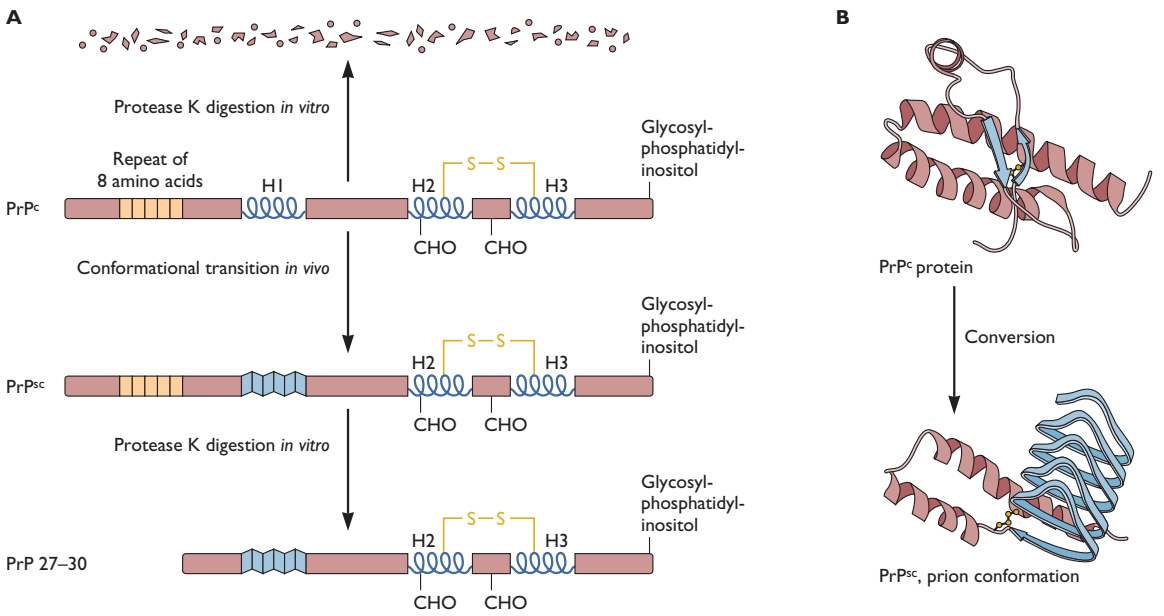
The unconventional physical attributes and slow infection pattern originally prompted many to argue that TSE agents are not viruses at all. For example, in 1967 the mathematician J. S. Griffith made three suggestions as to how scrapie may be mediated by a host protein, not by a nucleic acid-carrying virus. His thoughts were among the first of the "protein-only" hypotheses to explain TSE.

An important breakthrough occurred in 1981, when characteristic fibrillar protein aggregates were visualized in infected brains. These aggregates could be concentrated by centrifugation and remained infectious. Stanley Prusiner and colleagues developed an improved bioassay, as well as a fractionation procedure that allowed the isolation of a protein with unusual properties from scrapie-infected tissue. This protein is insoluble and relatively resistant to proteases. Sequence analysis of this protein led to the cloning of the *prnp* gene, which is highly conserved in the genomes of many animals, including humans. Expression of the *prnp* gene is now known to be essential for the pathogenesis of common TSEs.

The *prnp* gene encodes a 35-kDa membrane-associated glycoprotein (PrP) found in many neurons. Mice lacking the *prnp* gene develop normally and have few obvious defects. In another study, the PrP protein was shown to be synthesized in hematopoietic stem cells and was important for their renewal. It is strongly linked to human TSE diseases. At least 18 mutations in the human *prnp* gene are associated with familial TSE diseases. Furthermore, specific *prnp* mutations appear to be associated with susceptibility to different strains of TSE (see below).

Prusiner named the scrapie infectious agent a **prion** (from the words "protein" and "infectious"). His unconventional proposal was that an altered form of the PrP protein causes the fatal encephalopathy characteristic of scrapie. Prusiner's controversial protein-only hypothesis caused a firestorm among those who study infectious disease. The hypothesis is that the essential pathogenic component **is** the host-encoded PrP protein with an altered conformation, called PrP^{sc} ("PrP-scrapie"; also called PrP^{res} for "protease-resistant form"). Furthermore, in the simplest case, PrP^{sc} is proposed to have the property of converting normal PrP protein into more copies of the pathogenic form (Fig. 4). An important finding in this regard is that mice lacking both copies of their *prnp* gene are resistant to infection. In recognition of his work on this problem, Prusiner

Figure 4 The conversion of nonpathogenic, α-helix-rich PrP^{c} protein to the β-sheet-rich conformation of Prp^{sc}, the pathogenic prion. (A) PrP^{c} is the mature normal cellular protein. The precursor is 254 amino acids long with a signal sequence that is removed. Twenty-three amino acids of the carboxy terminus also are removed as the glycosylphosphatidylinostitol (GPI) linker is added. PrP^{sc} is the β-sheet-rich, pathogenic prion. This conformation is relatively resistant to protease K digestion, in contrast to PrP^{c}, as indicated. This protease K-resistant PrP fragment of PrP^{sc} is diagnostic of the prion protein. H1, H2, and H3 are helical regions of PrP^{c}. The yellow boxes are repeats of 8 amino acids [P(Q/H)GGGWGQ]. CHO indicates two N-linked carbohydrate chains. S–S indicates disulfide bonds. (B). Ribbon diagram of the PrP^{c} and PrP^{sc} protein backbones with α-helices in red and β-sheets in blue. From P. Chien, J. Weissman, and A. DePace, *Annu. Rev. Biochem.* **73:**617–656, 2004, with permission.



was awarded the Nobel Prize in physiology or medicine in 1997.

The conformational conversion of PrP to PrP^{sc} can be described by two models: the **refolding model** and the **seeding model.** In the former, spontaneous conversion to PrP^{sc} is virtually undetectable. However, interaction of PrP with **preformed** PrP^{sc} facilitates refolding of PrP to PrP^{sc}. PrP^{sc} can be introduced from exogenous sources or by rare mutations that lower the activation energy of conversion. In the seeding model, PrP and PrP^{sc} are in equilibrium, with the formation of PrP being highly favored. PrP^{sc} can be stabilized only when it binds to an aggregate (or seed) of PrP^{sc}. Aggregates are rare, but once formed, PrP^{sc} binds avidly and the aggregate grows rapidly by fragmentation and addition of new PrP^{sc}. Convincing evidence for the seeding model has come from studies of yeast prions.

Strains of the Scrapie Prion

As a result of many serial infections of mice and hamsters with infected sheep brain homogenates, investigators have derived distinct strains of scrapie prion. Strains are distinguished by length of incubation time before the appearance of symptoms, brain pathologies, relative abundance of various glycoforms of PrP, and electrophoretic profiles of protease-resistant PrP^{sc}. Some strains also have a different host range. For example, mouse-adapted scrapie prions cannot propagate in hamsters, but hamster-adapted scrapie prions can propagate in mice. A single amino acid substitution in the hamster protein enables it to be converted efficiently by mouse PrP^{sc} into hamster PrP^{sc}. Therefore, the barrier to interspecies transmission is in the sequence of the PrP protein. Bovine spongiform encephalopathy prions have an unusually broad host range, infecting a number of meat-eating animals, including domestic cats, wild cats, and humans. A striking finding is that different scrapie strains can be propagated in the same inbred line of mice yet maintain their original phenotypes. Stable inheritance suggests to some that a nucleic acid must be an essential component and has been used as support for a viral etiology of TSE disease. The protein-only hypothesis explains the existence of strain variation by postulating that each strain represents a unique conformation of PrP^{sc}. Each of these distinctive pathogenic conformations is then postulated to convert the normal PrP protein into a conformational image of itself. The strongest evidence supporting this hypothesis comes from studies of yeast prions.

Prions and the Food Supply

The existence of TSE in our food animals is a cause for concern, for we have only a modest understanding of the molecular nature of the infectious agent, the routes of shedding and transmission, and the processes leading to the characteristic pathogenesis (Fig. 3). Efforts are currently focused on basic research aimed at protection of the food supply and finding targets for therapeutic intervention. Remarkable progress is being made. For example, sensitive diagnostic tests for prions are now being used to screen blood and tissues. An unexpected breakthrough came when researchers discovered that the antimalarial drug quinacrine blocked accumulation of infectious prions in cultured cells. The mechanism is unknown, but this lead and others are being pursued aggressively, and limited human trials of quinacrine in patients with advanced Creutzfeldt-Jakob disease are in progress. Another promising discovery is that monoclonal antibodies specific for PrP inhibit scrapie prion replication in mice and delay the development of prion disease. The hope is that similar immunotherapy strategies will benefit humans exposed to prions.

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Glossary

Abortive infection An incomplete infectious cycle; virions infect a susceptible cell or host but do not complete replication, usually because an essential viral or cellular gene is not expressed. *(Chapter 5)*

Accessibility An attribute that describes the physical availability of cells to virions at the site of infection. *(Chapter 1)*

Active immunization The process of inducing an immune response by exposure to a vaccine; contrasts with passive immunization. *(Chapter 8)*

Active viremia The presence of newly synthesized virions in the blood. *(Chapter 1)*

Acute infection A common pattern of infection in which virions are produced rapidly, and the infection is resolved and cleared quickly by the immune system; survivors usually are immune to subsequent infection. *(Chapter 5)*

Adapter proteins Proteins that have no intrinsic enzymatic functions but act as linkers between functional proteins by binding to two or more at the same time. *(Chapter 6)*

Adaptive response The immune response consisting of antibody (humoral) and lymphocyte-mediated responses; unlike the innate response, the adaptive response is tailored individually to the particular foreign invader; the adaptive response has memory: subsequent infections by the same agent are met with a robust and highly specific response. Also known as the acquired immune response. *(Chapter 4)*

Adoptive transfer The transfer of cells, usually lymphocytes, from an immunized donor to a nonimmune recipient. *(Chapter 4)*

Alternative pathway One of three pathways in the complement system; activates the C3 and C5 convertases without going through the C1-C2-C4 complex. *(Chapter 4)*

Anchorage independence The ability of some cells to grow in the absence of a surface on which to adhere; often detected by the ability to form colonies in semisolid media. *(Chapter 7)*

Antigenic drift The appearance of virions with a slightly altered surface protein (antigen) structure caused by accumulation of point mutations following passage and immune selection in the natural host. *(Chapter 5)*

Antigenic shift A major change in the surface protein of a virion when genes encoding markedly different surface proteins are acquired during infection; this process occurs when viruses with segmented genomes exchange segments, or when nonsegmented viral genomes recombine after coinfection. *(Chapter 5)*

Antigenic variation The display by virions or infected cells of new protein sequences that are not recognized by antibodies or T cells which responded to previous infections. *(Chapter 5)*

Anti-inflammatory cytokines A series of immunoregulatory molecules that control the proinflammatory cytokine response; major anti-inflammatory cytokines include interleukin-1 (IL-1) receptor antagonist, IL-4, IL-6, IL-10, IL-11, and IL-13. *(Chapter 3)*

Apoptosis A sequence of tightly regulated reactions in response to external or internal stimuli that signal DNA

damage or other forms of stress; characterized by chromosome degradation, nuclear degeneration and cell lysis; a natural process in development and the immune system, but also an intrinsic defense of cells to viral infection. Also called programmed cell death. *(Chapter 3)*

Attenuated Having mild or inconsequential instead of an normally severe symptoms or pathology as an outcome of infection; having a state of reduced virulence. *(Chapter 2)*

Autocrine growth stimulation Stimulation of cell growth by proteins produced and sensed by the same cell. *(Chapter 7)*

Autophagy A process in which cells are induced to degrade the bulk of their cellular contents by formation of specialized membrane-bounded compartments called autophagolysosomes. *(Chapter 3)*

Auxiliary proteins Proteins encoded in lentiviral genomes in addition to the three structural polyproteins, Gag, Pol, and Env; they include Tat, Rev, Nef, Vif, Vpr, and Vpu. *(Chapter 6)*

Avirulent virus A virus that causes no, or mild, disease. *(Chapter 2)*

Blind screening Screening for antiviral compounds without regard to a specific mechanism. *(Chapter 9)*

Caspases Crucial proteases in the process called apoptosis; members of a family of cysteine proteases that specifically cleave after aspartate residues. *(Chapter 3)*

CD markers *See* Cluster-of-differentiation markers.

$CD4^+$ T cells T lymphocytes that carry the coreceptor protein CD4 on their surfaces. *(Chapter 4)*

$CD8^+$ T cells T lymphocytes that carry the coreceptor CD8 on their surfaces. *(Chapter 4)*

Cell cycle The orderly and reproducible sequence in which cells increase in size, duplicate the genome, segregate duplicated chromosomes, and divide. *(Chapters 4 and 7)*

Cell-mediated response The arm of the adaptive immune response consisting of helper and effector lymphocytes. *(Chapter 4)*

Chemokines Small proteins that attract and stimulate cells of the immune defense system; produced by many cells in response to infection. *(Chapter 3)*

Clades Subtypes of human immunodeficiency virus that are prevalent in different geographic areas. *(Chapter 6)*

Classical pathway One of three complement pathways that lead to activation of C3-C5 convertases; activation occurs by direct interaction of C1q or C3b proteins with an viral protein/antibody complex on the surface of an infected cell or a virus particle. *(Chapter 4)*

Clinical latency A state of persistent viral infection in which no clinical symptoms are manifested. *(Chapter 6)*

Cluster-of-differentiation markers Distinct surface proteins that are recognized by specific monoclonal antibodies; these antibodies bind to various cluster-of-differentiation markers and are used to distinguish different cell types (e.g., CD4 on helper T cells). Also called CD markers. *(Chapter 4)*

Complement A general term referring to all the components of the complement system. *(Chapter 4)*

Complement system A set of blood plasma proteins that act in a concerted fashion to destroy extracellular pathogens and infected cells; originally defined as a heat-labile activity that lysed bacteria in the presence of antibody (it "complemented" antibody action); the activated complement pathway also stimulates phagocytosis, chemotaxis, and inflammation. *(Chapter 4)*

c-Oncogene *See* Proto-oncogene.

Contact inhibition Cessation of cell division when cells make physical contact, as occurs at high density in a culture dish. *(Chapter 7)*

Cytokines Soluble proteins produced by cells in response to various stimuli, including virus infection; they affect the behavior of other cells both locally and at a distance, by binding to specific cytokine receptors. *(Chapter 4)*

Cytopathic virus A virus that causes characteristic visible cell damage and death upon infection of cells in culture. *(Chapter 5)*

Delayed-type hypersensitivity Cell-mediated immunity caused by CD4 T cells that recognize antigens in the skin; the reaction typically occurs hours to days after antigen is injected, hence its name; it is partially responsible for characteristic local reactions to virus infection, such as a rash. *(Chapter 4)*

Dermis The layer of skin beneath the epidermis that supports the basement membrane or vascular network; it is composed of a dense connective tissue that provides support and elasticity to the skin. *(Chapter 1)*

Diapedesis The process by which viruses cross the vascular endothelium, while being carried within monocytes or lymphocytes. *(Chapter 1)*

Disseminated infection An infection that spreads beyond the primary site of infection; often includes a viremia and infection of major organs such as the liver, lungs, and kidneys. *(Chapter 1)*

DNA synthesis phase *See* S phase.

DNA vaccine A preparation of DNA containing the genes for one or more antigenic proteins; when the pure DNA preparation is injected into a test subject, the proteins are expressed, and an immune response to those proteins is elicited. *(Chapter 8)*

Emerging virus A viral population responsible for a marked increase in disease incidence, usually as result of changed societal, environmental, or population factors. *(Chapter 10)*

Endogenous antigen presentation The cellular process in which viral proteins are degraded inside the infected cell, and the resulting peptides are loaded onto major histocompatibility complex class I molecules that move to the cell surface. *(Chapter 4)*

Enhancing antibodies Antibodies that can facilitate viral infection by allowing virions to which they bind to enter susceptible cells. *(Chapter 6)*

Enterotropic virus A virus with a predilection to infect cells of the enteric system. *(Chapter 1)*

Epidemic A pattern of disease characterized by rapid and sudden appearance of cases spreading over a wide area. *(Chapter 1)*

Epidemiology The study of the incidence, distribution, and spread of infectious disease in populations with particular regard to identification and subsequent control. *(Chapter 2)*

Epidermis The external surface of the skin, composed of a keratinized stratified squamous epithelium. *(Chapter 1)*

Error threshold A mathematical parameter that measures the complexity of the information that must be maintained to ensure survival of a population. *(Chapter 10)*

Exogenous antigen presentation The cellular process in which viral proteins are taken up from the outside of the cell, digested, and the resulting peptides loaded onto major histocompatibility complex class II molecules that move to the cell surface. *(Chapter 4)*

Fitness The replicative adaptability of an organism to its environment. *(Chapter 10)*

Foci Clusters of cells that are derived from a single progenitor and share properties, such as unregulated growth, that cause them to pile up on one another. *(Chapter 7)*

Fomites Inanimate objects that may be contaminated with microorganisms and become vehicles for transmission. *(Chapter 2)*

Gap phases (G_1 and G_2) Phases in the cell cycle between the mitosis (M) and DNA synthesis (S) phases. *(Chapter 7)*

Genetic bottleneck A descriptive term evoking the extreme selective pressure on small populations that results in loss of diversity, accumulation of nonselected mutations, or both. *(Chapter 10)*

G_0 *See* Resting state.

Hematogenous spread Spread of virus particles through the bloodstream. *(Chapter 1)*

Hepatitis Inflammation of the liver. *(Chapter 1)*

Herd immunity The immune status of a population, rather than an individual. *(Chapter 8)*

Humoral response The arm of the adaptive immune response that produces antibodies. *(Chapter 4)*

Immortality The capacity of cells to grow and divide indefinitely. *(Chapter 7)*

Immune defenses Host defenses against pathogens comprising the innate and adaptive systems. *(Chapter 3)*

Immune memory A property provided by specialized B and T lymphocytes (memory B and T cells) that respond rapidly on reexposure to the viral infection that originally induced them. *(Chapter 8)*

Immunodominant Having the property of being recognized most efficiently by cytotoxic T lymphocytes and antibodies; said of peptides and epitopes. *(Chapter 5)*

Immunopathology Pathological changes partly or completely caused by the immune response. *(Chapter 4)*

Immunotherapy A treatment that provides an infected host with exogenous antiviral cytokines, other immunoregulatory agents, antibodies, or lymphocytes in order to reduce viral pathogenesis. *(Chapter 8)*

Incidence The frequency with which a disease appears in a particular population or area (e.g., the number of newly diagnosed cases during a specific period); distinct from the prevalence (i.e., the number of cases in a population on a certain date). *(Chapter 2)*

Inflammation A general term for the complex response that gives rise to local accumulation of white blood cells and fluid; initiated by local infection or damage; many different forms of this response, characterized by the degrees of tissue damage, capillary leakage, and cellular infiltration, occur after infection with pathogens. *(Chapter 4)*

Innate response The first line of immune defense; able to function continually in the host without prior exposure to the invading pathogen. This complex system comprises, in part, cytokines, sentinel cells, complement, and natural killer cells. *(Chapter 4)*

Insertional activation The mechanism of oncogenesis by nontransducing retroviruses; integration of a proviral promoter or enhancer in the vicinity of a c-oncogene results in inappropriate transcription of that gene. *(Chapter 7)*

Insertional mutagenesis Mutation in a genome caused by the integration of viral DNA or the DNA of a transposable element. *(Chapter 3)*

Instability elements *cis*-acting repressive sequences in lentiviral RNA molecules that respond to Rev protein to increase stability, nuclear export, and translatability. *(Chapter 6)*

Interfering antibodies Antibodies that can bind to virions or infected cells and block interaction with neutralizing antibodies. *(Chapter 6)*

Intrinsic cellular defenses The conserved cellular program that responds to various stresses, such as starvation, irradiation, and infection; intrinsic defenses include apoptosis, autophagy, and RNA interference; unlike immune defenses, intrinsic defenses do not include cytokines and white blood cells. *(Chapter 3)*

Killed vaccine A vaccine made by taking an authentic disease-causing virus and treating it (e.g., with chemicals) to reduce infectivity to nondetectable levels. *(Chapter 8)*

Koch's postulates Criteria developed by the German physician Robert Koch in the late 1800s to determine whether a given agent is the cause of a specific disease. *(Chapter 1)*

Kupffer cells Macrophages of the liver that are part of the reticuloendothelial system. *(Chapter 1)*

Latency-associated transcript RNA produced specifically during a latent infection by herpes simplex virus. *(Chapter 5)*

Latent infection A class of persistent infection that lasts the life of the host; few or no virions can be detected, despite continuous presence of the viral genome. *(Chapter 5)*

Lethal mutagenesis The elevation of mutation rates by exposure to a mutagen or an error-prone polymerase to the point at which the resulting population of genomes has lost fitness and is incapable of propagating. *(Chapter 10)*

Live, attenuated vaccine A vaccine made from viral mutants that have reduced virulence but are competent for replication; they often also have reduced capacity for transmission. *(Chapter 8)*

Long-latency virus A retrovirus that causes cancer in a host many years after infection; the viral genome does not encode cellular oncogenes, nor does it cause cancer by perturbing the expression of cellular oncogenes. *(Chapter 7)*

Macules Flat, colored skin lesions caused by virus replication in the dermis. *(Chapter 1)*

Mannan-binding pathway One of three complement pathways that lead to activation of C3-C5 convertases; mannose-binding, lectin-associated proteases cleave the C2 and C4 proteins. *(Chapter 4)*

Memory cells A subset of B and T lymphocytes maintained after each encounter with a foreign antigen; they survive for years and are ready to respond and proliferate upon subsequent encounter with the same antigen. *(Chapter 4)*

Metastases Secondary tumors, often at distant sites, that arise from the cells of a malignant tumor. *(Chapter 7)*

Microbicides Creams or ointments that either inactivate or block virions before they can attach and penetrate tissues. *(Chapter 9)*

Mitogens Extracellular signaling molecules that induce cell proliferation. *(Chapter 7)*

Mitosis The phase of the cell cycle in which newly duplicated chromosomes are distributed to two new daughter cells as a result of cell division. Also called M phase. *(Chapter 7)*

Molecular mimicry Sequence similarities between viral peptides and self-peptides that result in the cross-activation of autoreactive T or B cells by virus-derived peptides. *(Chapter 4)*

Monoclonal antibody-resistant mutants Viral mutants selected to propagate in the presence of neutralizing monoclonal antibodies; often carry mutations in viral genes encoding virion protein. *(Chapter 4)*

M phase *See* Mitosis.

Muller's ratchet A statement positing that small, asexual populations decline in fitness over time if the mutation rate is high. *(Chapter 10)*

Natural killer cells An abundant lymphocyte population that comprises large, granular lymphocytes; distinguished from others by the absence of B and T cell antigen receptors; these cells are part of the innate defense system. Also called NK cells. *(Chapter 4)*

Neuroinvasive virus A virus that can enter the central nervous system (spinal cord and brain) after infection of a peripheral site. *(Chapter 1)*

Neurotropic virus A virus that can infect neurons. *(Chapter 1)*

Neurovirulent virus A virus that can cause disease in nervous tissue, manifested by neurological symptoms and often death. *(Chapter 1)*

NK cells *See* Natural killer cells.

Noncytopathic virus A virus that produces no visible signs of infection in cells. *(Chapter 5)*

Nontransducing oncogenic retroviruses Retroviruses that do not encode cell-derived oncogene sequences but can cause cancer (at low efficiency) when their DNA becomes integrated in the vicinity of a cellular oncogene, thereby perturbing its expression. *(Chapter 7)*

Oncogene A gene encoding a protein that causes cellular transformation or tumorigenesis. *(Chapter 7)*

Oncogenesis The processes leading to cancer. *(Chapter 7)*

Pandemic A worldwide epidemic. *(Chapter 1)*

Pantropic virus A virus that replicates in many tissues and cell types. *(Chapter 1)*

Papules Slightly raised skin lesions caused by virus replication in the dermis. *(Chapter 1)*

Passive immunization Direct administration of the products of the immune response (e.g., antibodies or stimulated

immune cells) obtained from an appropriate donor(s) to a patient; contrasts with active immunization. *(Chapter 8)*

Passive viremia Introduction of virus particles into the blood without viral replication at the site of entry. *(Chapter 1)*

Pathogen A disease-causing virus or other microorganism. *(Chapter 1)*

Pattern recognition receptors Unique protein receptors of the innate immune system that bind common molecular structures on the surfaces of pathogens; they reside on the cell surfaces of sentinel cells, such as immature dendritic cells and macrophages. *(Chapter 3)*

Permissive Able to support virus replication when viral nucleic acid is introduced; refers to cells. *(Chapter 1)*

Permissivity A cellular environment that provides all cellular components required for viral replication. *(Chapter 1)*

Persistent infection A viral infection that is not cleared by the combined actions of the innate and adaptive immune response. *(Chapter 5)*

Plaque-forming unit (PFU) A measure of virus infectivity when measured as plaque-forming units per milliliter. *(Chapter 1)*

Polymorphic gene A gene that has many allelic forms in outbred populations. *(Chapter 4)*

Power The probability that a meaningful difference or effect can be detected, if one were to occur. *(Chapter 2)*

Prevalence The proportion of individuals in a population having a disease; the number of cases of a disease present in a particular population at a given time. *(Chapter 2)*

Primary antibody response The initial response of B cells when first exposed to an infection. *(Chapter 4)*

Primary viremia Progeny virions released into the blood after initial replication at the site of entry. *(Chapter 1)*

Prions Infectious agents comprising an abnormal isoform of a normal cellular protein but no nucleic acid; implicated as the causative agents of transmissible spongiform encephalopathies. *(Appendix B)*

Professional antigen-presenting cells Dendritic cells, macrophages, and B cells; defined by their ability to take up antigens and present them to T lymphocytes in the groove of an major histocompatibility complex class II molecule. *(Chapter 4)*

Proinflammatory cytokines Cytokines produced predominantly by activated immune cells; responsible for amplification of inflammatory reactions. *(Chapter 3)*

Proto-oncogene A normal gene that, when altered by mutation, becomes an oncogene that can contribute to cancer. Also called c-oncogene. *(Chapter 7)*

Pustules Skin lesions derived from a vesicle in which secondary infiltration of leukocytes occurs. *(Chapter 1)*

Quasispecies Virus populations that exist as dynamic distributions of nonidentical but related replicons. *(Chapter 10)*

Recombinant vaccine A vaccine produced by recombinant DNA technology. *(Chapter 8)*

Reservoir The host population in which a viral population is maintained. *(Chapter 2)*

Resting state A state in which the cell has ceased to grow and divide and has withdrawn from the cell cycle. Also called G_0. *(Chapter 7)*

Rev-responsive element (RRE) A structural element in human immunodeficiency virus RNA that is recognized by the viral Rev protein, which mediates its export from the nucleus. *(Chapter 6)*

Satellites Small, single-stranded RNA molecules that lack genes required for their replication, but do replicate in the presence of another virus (the **helper virus**). *(Appendix B)*

Satellite virus A satellite with a genome that encodes one or two proteins. *(Appendix B)*

Secondary antibody response The antibody response produced after a subsequent infection or challenge with the same antigen or virus. *(Chapter 4)*

Secondary viremia Delayed appearance of a high concentration of infectious virus in the blood as a consequence of disseminated infections. *(Chapter 1)*

Second messengers Small molecules, such as cyclic nucleotides and lipids, that are generated by some membrane-bound proteins in a signal transduction cascade, and that act as diffusible components in a signal relay. *(Chapter 7)*

Sentinel cells Dendritic cells and macrophages; migratory cells that are found in the periphery of the body and can take up proteins and cell debris for presentation of peptides derived from them on major histocompatibility complex molecule. These cells respond to recognition of a pathogen by synthesizing cytokines such as interferons. *(Chapter 4)*

Signal transduction cascade A chain of sequential physical interactions among, and biochemical modification of, membrane-bound and cytoplasmic proteins. *(Chapter 7)*

Sinusoids Small blood vessels characterized by a discontinuous basal lamina, with no significant barrier between the blood plasma and the membranes of surrounding cells. *(Chapter 1)*

Slow infection An extreme variant of the persistent pattern of infection; has a long incubation period (years) from the time of initial infection until the appearance of recognizable symptoms. *(Chapter 5)*

Slow viruses Viruses characterized by long incubation periods, typical for the genus lentivirus in the family *Retroviridae*. *(Chapter 6)*

Smoldering infection A low rate of viral replication equal to the rate of elimination. *(Chapter 5)*

S phase The phase of the cell cycle in which the DNA genome is replicated. *(Chapter 7)*

Subunit vaccine A vaccine formulated with purified components of virus particles, rather than intact virions. *(Chapter 8)*

Susceptibility The property of a cell that enables it to be infected by a particular virus (e.g., the presence of a viral receptor[s] on the cell surface). *(Chapter 1)*

Susceptible Producing the receptor(s) required for virus entry; refers to cells. *(Chapter 1)*

Systemic infection An infection that results in spread to many organs of the body. *(Chapter 1)*

Systemic inflammatory response syndrome Overexpression or a disproportionate host response that leads to large-scale release of inflammatory cytokines and stress mediators, resulting in severe pathogenesis or death. Also known as a cytokine storm. *(Chapter 4)*

Transcytosis A mechanism of transport in which material in the intestinal lumen is endocytosed by M cells, transported to the basolateral surface, and released to the underlying tissues. *(Chapters 1 and 4)*

Transducing oncogenic retroviruses Retroviruses that include oncogenic, cell-derived sequences in their genomes and carry these sequences to each newly infected cell; such viruses are highly oncogenic. *(Chapter 7)*

Transformed Having changed growth properties and morphology as a consequence of infection with certain oncogenic viruses, introduction of oncogenes, or exposure to chemical carcinogens. *(Chapter 7)*

Transforming infection A class of persistent infection in which cells infected by certain DNA viruses or retroviruses may exhibit altered growth properties and proliferate faster than uninfected cells. *(Chapter 5)*

Tropism The predilection of a virus to invade, and replicate, in a particular cell type. *(Chapter 1)*

Tumor A mass of cells originating from abnormal growth. *(Chapter 7)*

Tumor suppressor gene A cellular gene encoding a protein that negatively regulates cell proliferation; mutational inactivation of both copies of the genes associated with tumor development. *(Chapter 7)*

Vaccination Inoculation of healthy individuals with attenuated or related microorganisms, or their antigenic products, in order to elicit an immune response that will protect against later infection by the corresponding pathogen. *(Chapter 8)*

Vesicles Focal necroses that occur when an infection spreads from the capillaries to the superficial layers of the skin and replicates in the epidermis; usually contain inflammatory fluids. *(Chapter 1)*

Viral pathogenesis The processes by which viral infections cause disease. *(Chapters 1 and 2)*

Viremia The presence of infectious virions in the blood. *(Chapter 1)*

Virion An infectious virus particle. *(Chapter 1)*

Viroceptor A viral protein that modulates cytokine signaling or cytokine production by mimicking host cytokine receptors. *(Chapters 2 and 3)*

Viroids Unencapsidated, small, circular, single-stranded RNA molecules that replicate autonomously when introduced mechanically into host plants. *(Appendix B)*

Virokine A secreted viral protein that mimics cytokines, growth factors, or similar extracellular immune regulators. *(Chapters 2 and 3)*

Virulence The relative capacity of a viral infection to cause disease. *(Chapter 2)*

Virulent virus A virus that causes disease. *(Chapter 2)*

Viruria The presence of viruses in the urine. *(Chapter 2)*

Viruses Submicroscopic, obligate parasitic pathogens comprising genetic material (DNA or RNA) surrounded by a protective protein coat. *(Chapter 1)*

Virus evolution The constant change of a viral population in the face of selection pressures. *(Chapter 10)*

v-Oncogene An oncogene that is encoded in a viral genome. *(Chapter 7)*

Zoonoses (zoonotic infections) Diseases that are transferred from animals to humans. *(Chapters 2 and 10)*

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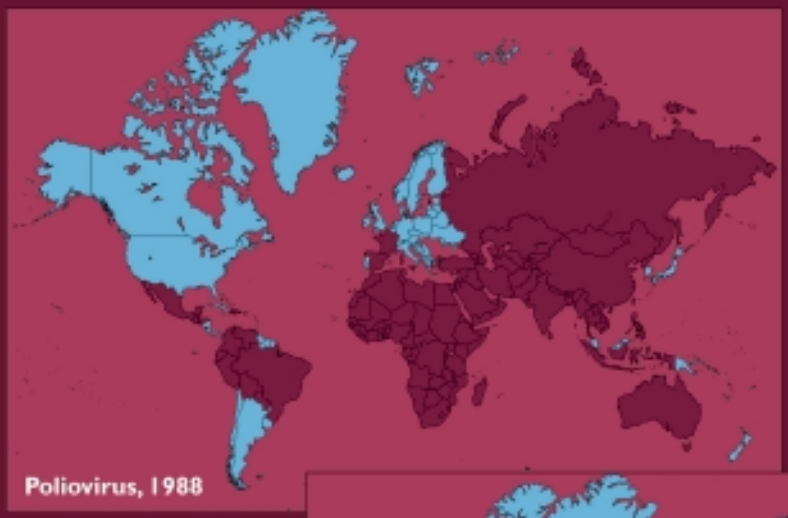

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