Current Topics in Microbiology and Immunology

163

Poxviruses

Edited by R. W. Moyer
and P. C. Turner

With 23 Figures

Springer-Verlag
Berlin Heidelberg New York London
Paris Tokyo Hong Kong Barcelona

Professor RICHARD W. MOYER, Ph.D.

PETER C. TURNER, Ph.D.

Dept. of Immunology and Medical Microbiology,
University of Florida, Gainesville, FL32610, USA

ISBN-13: 978-3-642-75607-8 e-ISBN-13: 978-3-642-75605-4
DOI: 10.1007/978-3-642-75605-4

Library of Congress Catalog Card Number 15-12910

Softcover reprint of the hardcover 1st edition 1990

Typesetting: Thomson Press (India) Ltd, New Delhi

2123/3020-543210 – Printed on acid-free paper

Preface

The purpose of this volume is to highlight some current areas of poxvirus research which are likely to be particularly fruitful in the upcoming few years. The first chapter, by Drs. Condit and Niles, discusses poxvirus genetics. Work in this area has provided mutants, produced practical procedures to simplify the manipulation of viral genes, and generated information about the molecular architecture and organization of genes characteristic of poxviruses. One of the most intensively studied regions of the viral genome is the *Hin*dIII D region of vaccinia, in which a combination of classical and molecular genetic analysis of the region has been particularly revealing. Within this region are open reading frames, some of which are expressed early and others late, organized in a fashion which is now known to be typical of these viruses. Other studies, related to temperature sensitive, drug resistant, and drug dependent mutants, are also discussed. Each of the other reviews included in this volume summarizes areas of research which have depended heavily on the genetics of the system.

The intracellular site of a poxvirus infection is mostly, if not exclusively, limited to the cytoplasm which dictates several interesting biological ramifications. For example, poxvirus transcription must occur in the cytoplasm, rather than in the nucleus. The virus copes with this situation by incorporating into the virion the enzymatic machinery necessary to initiate transcription from input virus. Such virion associated enzymes include a virus encoded, multi-subunit DNA directed RNA polymerase and the enzymes necessary to cap, methylate, and polyadenylate transcripts. A second consequence of a cytoplasmic site of replication is that virus specific enzymes and regulatory signals, including promoters, transcription termination signals, and trans-activating factors, have had the opportunity to evolve somewhat independently of those of the host cell. Therefore, the study of poxvirus transcription offers a unique system in which to examine the process of temporal transcription and regulation of gene expression. The review by

Dr. MOSS summarizes the current status of this work. A further promising area of study, which is as yet relatively untapped, is the study of the biogenesis and assembly of enzymes such as the viral RNA polymerase and of higher order multi-enzyme complexes necessary for efficient synthesis and modification of transcripts to generate functional mRNA. Finally, recent development of reliable in vitro soluble transcription systems and the results that have already been and will be obtained from their further use assures that the area of transcriptional regulation will be one of the most fruitful in the coming years.

The next two reviews, the first by Drs. DELANGE and MCFADDEN and the second by Dr. TRAKTMAN, summarize two aspects of poxvirus DNA replication: the role of structural elements in the replication process and the enzymology of DNA replication, respectively. The ends of the DNA of poxviruses are characterized by the presence of telomeres, structures which are more commonly associated with the termini of eukaryotic chromosomal DNA and are known to be intimately involved in the chromosomal replication process. The structural details of the poxvirus telomeres and the immediately adjacent region are reviewed here, as are the functions thought to be attributed to telomeres. Finally, the presence of telomeres within this relatively small DNA which can be subjected to extensive genetic manipulation has allowed incisive experiments to be performed to determine the sequences necessary for telomeric resolution during the replication process. The complementary review in this section discusses the enzymology of poxvirus DNA replication. Such studies have again been aided immeasurably by the cytoplasmic site of poxvirus development which dictates that many, if not all, of the enzymes necessary for DNA replication are virus encoded. The application of genetics to this problem has also been particularly fruitful in delineating a number of genes and, in some cases, specific enzymes involved in the process. The article discusses not only DNA replication per se but also considers both the role of DNA topology and the role of macromolecular nucleoprotein complexes in the replicative process.

In recent years, a great deal of attention has been devoted to the development of poxviruses as general live vaccine vectors; a subject which has been extensively reviewed. Requirements for a live vaccine vector system are some replication and gene expression but with a minimum of complicating side effects. The review by Drs. TURNER and MOYER discusses the virus not as a vector, but as a pathogenic entity emphasizing what role specific viral genes might have in pathogenesis and undesirable comp-

lications in the host. The identification of relevant genes and, when possible, how the proteins they encode might influence the infectious process is reviewed here. This information is essential in order to understand the process of poxvirus pathogenesis in general as well as for the intelligent design of safer yet efficacious vaccines. Many of the specific genes discussed are located within the highly variable, strain-specific, terminal regions, a fact which emphasizes both the potential of the virus to adapt to new situations and a possibility for the evolution of novel genes.

The generation of recombinant poxviruses into which antigens from a variety of sources have been cloned provides not only unique possibilities for live vaccine virus construction, but also a unique opportunity for their use as reagents or tools to gain insights into functions of the immune system. This aspect of poxvirus research is reviewed here by Drs. BENNINK and YEWDELL. Functional T cells require that antigens be expressed within the context of histocompatible cells. Fortunately, poxviruses have a broad host range and can grow within a variety of cells that can also serve as antigen presenting cells. Therefore, when cells are infected with recombinant poxviruses expressing a single foreign antigen or group of antigens from a complex organism, a rather unique opportunity is offered in which to study T cell function and interactions in response to the well-defined antigen. The article examines this aspect of poxvirus research and includes discussion of the advantages, disadvantages, and pitfalls associated with such an approach. Exploitation of the system in the future should allow one to discern the contributions of various subpopulations of immune cells to the immune process and to recovery from disease.

It is now recognized that the poxviruses, typical of eukaryotic systems in general, have the capacity to posttranslationally modify their proteins. Such modifications include: proteolytic processing, glycosylation, acylation, phophorylation, and ADP-ribosylation. These poxviruses allow one to study the posttranslational modifications within a context which is easier to manipulate both biochemically and genetically than a corresponding system derived from the uninfected cell. The current catalog of poxvirus directed posttranslational modifications is reviewed and discussed here by Drs. VANSLYKE and HRUBY, as are possible functional roles these processes might play in the regulation of gene function within the overall infectious process.

In conclusion, let me offer my thanks and appreciation to the contributors to this volume for their efforts and their

collective insight and enthusiasm in producing an integrated collection of reviews of timely topics traversing many facets of poxvirus research. A special measure of thanks is due to Dr. P.C. TURNER, my collaborator in the pathogenesis review and this volume.

Fall, 1989 R. W. MOYER

List of Contributors

(Their addresses can be found at the beginning of their respective chapters)

List of Contents

Orthopoxvirus Genetics

R. C. CONDIT and E. G. NILES

Department of Biochemistry, 140 Farber Hall, SUNY/Buffalo, Buffalo, NY 14214, USA

Current Topics in Microbiology and Immunology, Vol. 163

1 Introduction

The classical approach to virus genetics is limited primarily to the isolation and characterization of conditionally lethal, deletion, and drug resistant or dependent virus mutants. The ultimate goal of a genetic analysis of a virus is to provide a genetic definition of the physical map location of each gene and the biological function of each gene product. This chapter deals with the "classical" approach to genetic analysis of orthopoxviruses.

Interest in poxviruses has been sustained primarily by the unusual biology of the infection: these are the only DNA-containing viruses which replicate entirely in the cytoplasm of infected cells (MOSS 1985; MOYER 1987). Because of their cytoplasmic site of replication, virtually all of the viral nucleic acid metabolism is controlled directly by viral genes. Thus, the genetic analysis of poxviruses provides tools for studying the control of eucaryotic transcription, mRNA processing, DNA replication, and DNA recombination. Furthermore, the virus undergoes a complex and fascinating cytoplasmic morphogenesis pathway involving de novo synthesis of a lipid-containing viral membrane, condensation of the viral nucleoprotein within this membrane, further morphological reorganization within this immature particle, and acquisition of a second cell-derived membrane. Morphogenesis must also be controlled primarily by virus genes and can therefore be studied directly through the use of genetics. Recent interest in poxviruses has been intensified by the discovery that vaccinia is an extraordinarily efficacious live recombinant vaccine vector (MACKETT and SMITH 1986; MOSS and FLEXNER 1987).

In the early 1960s, genetic analysis of rabbitpox virus established many techniques and concepts that provided the experimental framework for more recent studies (FENNER and SAMBROOK 1966; SAMBROOK et al. 1966; PADGETT and TOMKINS 1968). In fact, several mutants isolated during these earlier studies are still in use. For the most part, this review will focus on developments since 1978. These can be conceived of as proceeding in three stages. In the first stage, there was a burst of research which comprised the isolation and preliminary characterization of large numbers of poxvirus mutants. The second stage, still ongoing and involving many more laboratories, involves the detailed characterization of mutations affecting individual virus genes. The third stage, now in its

infancy, recognizes the need for the development of efficient techniques for targeted in vitro mutagenesis which results in conditionally lethal mutations of specific viral genes. An analysis of the progress in stages one and two is provided below in sections headed "Methods", "Mutants", and "Genome Organization". Stage three is discussed in the section headed "Directed Genetics". Three specific types of poxvirus mutants will be emphasized: temperature sensitive (ts) mutants, drug resistant mutants, and deletion/transposition mutants.

Research on DNA recombination is a direct outgrowth of genetic analysis of orthopoxviruses. We have therefore included in this review a section which analyzes our current understanding of recombination in poxviruses.

2 Methods

2.1 General Technical Considerations

2.1.1 Virus Strains and Genome Structure

Most of the recent genetic, biochemical, and biological analysis of orthopoxviruses has been carried out using the prototypical orthopoxvirus, vaccinia, and therefore vaccinia will provide the major focus for this review; however, much of our current thinking about host range and pathogenicity in orthopoxviruses is shaped by comparative studies of different orthopoxviruses: vaccinia, ectromelia, variola, monkeypox, and cowpox viruses.

Vaccinia contains a linear, nonpermuted, double-stranded DNA genome 186 kb in length, including 10 kb of inverted terminal repetition. This is enough information to encode approximately 150 average sized (40 kDa) proteins. It is somewhat ironic that despite the large physical size of the genome, several features of the virus' biology make the genome and the genetics of the virus relatively easy to understand, even when compared to very small viruses. First, vaccinia genes lack introns and, as a consequence, interpretation of DNA sequence data is greatly simplified relative to systems in which mRNA splicing is likely to occur. Simply stated, in every case that has been examined to date, an open reading frame (orf) in a DNA sequence corresponds to a virus gene. Each virus gene seems to contain its own transcriptional promoter, genes occur in both possible transcriptional orientations on the genome, and they are very closely spaced (see Sect. 4). Second, the high degree of autonomy imposed on the virus by its cytoplasmic site of replication greatly simplifies interpretation of biochemical data. Although we must never lose sight of the fact that an infection represents a complex interaction between the virus and its host, nonetheless, in the case of vaccinia, the majority of molecular biological changes observed after infection are directly attributable to the activity of virus genes rather than an indirect consequence of a virus-cell interaction. Lastly, recombinant DNA clones comprising the entire vaccinia genome are available, and to date at least 45% of the genome has been sequenced (Earl and Moss 1987).

Several different strains of vaccinia have been used for genetic analysis. For the most part the differences may be inconsequential, but it is important to recognize that in some cases there is insufficient data to permit an unambiguous direct comparison between results obtained with different virus strains. Recent genetic analyses of vaccinia have been conducted on four different strains: *WR* (Western Reserve), *IHD* (International Health Department), *Copenhagen*, and *MM*. Both the *WR* and the *IHD* strains originated from the New York Board of Health vaccine strain of vaccinia (PARKER et al. 1941). Therefore, although there is no published restriction map of the *IHD* strain to compare with maps of *WR* (DEFILIPPES 1984), they should be very closely related. The restriction map of *Copenhagen* reveals differences from *WR* (DRILLIEN and SPEHNER 1983). A recent report suggests that the *Hin*dIII map of *MM* is identical to *WR*, but detailed restriction enzyme analysis of *MM* has not been published (CHERNOS et al. 1985). It is noteworthy that until recently none of these viruses were plaque purified or maintained as low passage stocks through passage at low multiplicity. For example, *WR* was maintained for years by passage in mouse brain (FENNER 1958). For use in genetic studies, "wild type" strains were plaque purified from these stocks at different times and by different investigators (CONDIT and MOTYCZKA 1981; ENSINGER 1982). Therefore, polymorphisms exist between "*WR*" strains used by different investigators.

It has been noted that "outbreaks of rabbit pox have been reported only in laboratory stock" (DOWNIE and DUMBELL 1956). Although the restriction endonuclease cleavage map of rabbitpox is distinguishable from vaccinia, the virus is nevertheless very closely related to vaccinia as judged by immunological, biological, and restriction mapping criteria (DOWNIE and DUMBELL 1956; MACKETT and ARCHARD 1979).

Comparative hybridization and restriction endonuclease mapping of the genomes of several strains of cowpox, monkeypox, vaccinia, variola, and ectromelia viruses reveals that approximately half of the genome, positioned in the center of the map, is highly conserved throughout the orthopoxvirus family (MACKETT and ARCHARD 1979; ESPOSITO and KNIGHT 1985). Conversely, most of the variation which distinguishes individual members of the orthopoxvirus family occurs at the ends of the genome. This observation has been interpreted to mean that most of the genes which control the basic aspects of poxvirus biology are localized in the central conserved region of the poxvirus genome and that functions which control individual characteristics such as host range and pathogenicity map in the variable terminal regions. As we will see, genetic analysis of the viruses supports this view.

2.1.2 Virus Growth Properties

The growth properties of vaccinia under laboratory conditions contribute to its manageability as a genetic system. Vaccinia replicates well on a wide variety of continuous cell lines in monolayer and suspension culture. Under optimum conditions, one can expect a yield of at least 100 plaque forming units per cell. The

time course of growth in a one step growth experiment, from infection of plateau, is 24 to 48 h. The virus seems to plaque best on primate cells which in monolayer culture form close intercellular contacts. It forms plaques approximately 3 mm in diameter during a 3–4 day incubation at 37 °C. Plaque assays can be performed under a nutrient agar overlay or, since during plaque formation vaccinia stays very tightly cell-associated, plaque assays can be performed under liquid. Several genetic tests are greatly simplified technically by taking advantage of the ability of the virus to form discrete plaques under liquid. Temperatures ranging from 31 °C to 40 °C have proven satisfactory for work with ts mutants.

2.2 Mutagenesis

Although mutants of all the types discussed here can occur spontaneously during propagation of poxviruses, isolation of mutants can be simplified by artificial induction of mutations with mutagens. Several investigators have compared the effectiveness of a variety of mutagens in inducing poxvirus ts mutants (SAMBROOK et al. 1966; CHERNOS et al. 1978; DRILLIEN et al. 1982; DALES et al. 1978; ENSINGER 1982). An accurate assessment of mutagenesis may be obtained by measuring mutation at a specific genetic locus. This requires that mutation at the locus results in a selectable phenotype, and this is most easily accomplished by measuring acquisition of resistance to a specific drug. There are several drugs which inhibit vaccinia plaque formation in a monolayer without killing the monolayer (see Sect. 3), and any one of these would theoretically work in this assay. CONDIT and MOTYCZKA (1981) have compared the effectiveness of bromodeoxyuridine (BUdR), hydroxylamine (HA), and nitrosoguanidine (NTG) on vaccinia by assaying both virus killing and acquisition of resistance to phosphonoacetic acid (PAA). Both NTG and HA were found to be effective mutagens, whereas BUdR was found to be relatively ineffective. Temperature sensitive mutants were found at a level of approximately 0.5% in a population of NTG treated virus in which the fraction of virus resistant to PAA was 150-fold over background.

Theoretically, one should avoid the use of excessively high doses of mutagen in order to avoid creating multiple mutants, and one should ensure that each mutant is an independent isolate to avoid isolation of siblings. From a practical point of view, multiple mutants and siblings have seldom caused problems in any of the studies reported, and the effort required to avoid them is usually unjustified. For example, CONDIT et al. (1983) reported only two double mutants in a collection of 65 ts mutants, and, despite detailed analysis of a large fraction of the mutants in this collection, only one pair of sisters has been discovered (FATHI and CONDIT, in preparation).

2.3 Mutant Isolation

2.3.1 Temperature Sensitive Mutants

ts mutants can be isolated using either a brute force protocol or a more efficient plaque enlargement protocol (CONDIT and MOTYCZKA 1981; ENSINGER 1982). In the brute force approach, mutagenized virus is plated at a permissive temperature and individual plaques are picked at random for assay of temperature sensitivity. In the plaque enlargement protocol, plaques are grown at the permissive temperature, the plaque sizes are marked on the back of the dish, marked dishes are incubated at the nonpermissive temperature, and plaques which do not increase in size are picked and tested for temperature sensitivity. The advantage of the plaque enlargement technique is that greater than 90% of the mutants which are definitely not ts can be eliminated with this simple screen. With practise, about one-half of the plaques selected by this screen will be ts. A theoretical disadvantage of the technique, never proven, is that since one must recover live viruses from plaques which have been incubated at the nonpermissive temperature for several days, the procedure might select against some mutants having ts virions. Other mutant enrichment protocols have been tested. SAMBROOK et al. (1966) used an isatin-β-thiosemicarbazone (IBT) counter selection to enrich for ts mutants, and DALES et al. (1978) used a BUdR counter selection to enrich for mutants with defects in DNA replication.

Individual plaques can be scored for temperature sensitivity with a simple spot test (CONDIT and MOTYCZKA 1981; ENSINGER 1982). Individual isolates are usually re-plaque purified, grown, and tested for leakiness and reversion index. The test for leakiness is carried out by comparing virus replication following a high multiplicity (one step) infection at the permissive and nonpermissive temperatures. This provides a quantitative measure of the ability of mutant virus to replicate at the nonpermissive temperature. Reversion index is assayed by comparing the efficiency of plating at the permissive and nonpermissive temperatures. This provides a quantitative measure of the number of wild type viruses contained in a population of mutant viruses. The vast majority of mutants which are ts in the spot test are stable and have suitably low values of leakiness and reversion.

2.3.2 Drug Resistant Mutants

Mutants of vaccinia virus resistant to several different drugs have been characterized (see Sect. 3). In the case of one compound, IBT, drug dependent mutants have also been described (KATZ et al. 1973). From a technical perspective, drugs which might specifically inhibit poxvirus infection must be divided into two classes: those which do not have a toxic effect on a cell monolayer and those which kill a cell monolayer. In the first instance, drug resistant mutants can easily be selected from a population of viruses by plating directly on a monolayer in the presence of concentrations of drug which are inhibitory to the growth of wild type

virus. Since the drug will not kill the cells, resistant viruses can be identified as plaques growing on the drug treated monolayer. This protocol has been used successfully for the isolation of mutants resistant to PAA (Moss and Cooper 1982; Sridhar and Condit 1983), IBT (Katz et al. 1973), rifampicin (RIF) (Tartaglia and Paoletti 1985; Baldick and Moss 1987), and BUdR (Dubbs and Kit 1964). In the case of BUdR, which selects for virus mutants deficient in thymidine kinase (TK^-), the cells in the monolayer must also be TK^- or they will be killed by the drug treatment. Isolation of mutants resistant to cytotoxic drugs is less straightforward. In this instance, a virus stock must be enriched for drug resistant mutants by serial passage in the presence of the drug; individual plaques grown on untreated monolayers must then be tested for growth in a one step growth assay done in the presence of the drug. An example of this type of isolation can be found in the work of Villarreal et al. (1984), who have isolated α-amanitin (AM) resistant mutants of vaccinia. Enrichment by passage in the presence of drug may also be used during isolation of mutants resistant to noncytotoxic drugs.

2.3.3 Deletion/Transposition Mutants

A large class of poxvirus mutants has been described which contain either simple deletions or genome rearrangements which involve replacement of sequence from one terminus of the genome with sequence from the other terminus in an inverted orientation (see chapter by Turner and Moyer). Some of these mutants have phenotypes which can be used as a screen to aid in their isolation. This observation dates to work by Fenner and Sambrook (1966), who described white pock, host range mutants of rabbitpox. These authors found that, like other hemorrhagic poxviruses, rabbitpox spontaneously mutates to form white pocks on the chorioallantoic membrane of eggs and that many of these variants display an altered host range on cultured cell lines. The spontaneous mutation frequency to a white pock phenotype is high, about 1%. More recently, several groups have used either host range or pock color as a screen for isolation of new poxvirus deletion/transposition mutants (Moyer and Rothe 1980; Dumbell and Archard 1980; Esposito et al. 1981; Drillien et al. 1981; Archard et al. 1984; Pickup et al. 1984).

2.4 Complementation Analysis

Complementation analysis has proven to be an extraordinarily powerful technique for assigning vaccinia virus ts mutants to individual genes. Two types of complementation test have been used, a qualitative test (Condit and Motyczka 1981; Condit et al. 1983; Ensinger and Rovinsky 1983) and a quantitative test (Padgett and Tomkins 1968; Lake et al. 1979; McFadden and Dales 1980; Condit and Motyczka 1981; Drillien et al. 1982; Ensinger 1982;

CONDIT et al. 1983; CHERNOS et al. 1983). In the quantitative test, high multiplicity single or mixed infections with two mutant viruses are carried out at the nonpermissive temperature, the virus yields are determined by plaque titration, and a complementation index (CI) is calculated using the formula (FIELDS and JOKLIK 1969):

$$\frac{\text{yield } (A \times B)_{31^\circ} - \text{yield } (A \times B)_{40^\circ}}{\text{yield } (A)_{31^\circ} + \text{yield } (B)_{31^\circ}} = CI$$

Subscripts refer to the temperature at which the yield is titrated. The first term in the numerator represents the total yield of virus (mutants plus recombinants) from a mixed infection at the nonpermissive temperature. Recombinants are subtracted from this total with the second term. The denominator represents growth of individual mutants at the nonpermissive temperature, or leakiness. Although in theory this ratio should be equal to one for noncomplementing mutants and greater than one for mutants which complement, in practice the numbers are variable, and most authors have used a value of greater than or equal to two to indicate complementation. In the qualitative test, low multiplicity (less than 0.1 plaque forming units/cell) single or mixed infections with two mutant viruses are carried out at the nonpermissive temperature, infected dishes are stained, and complementation is scored directly, without further plaque titration, as plaque formation specific to mixed infections. Low multiplicities of infection must be used in both this test and the one step marker rescue test (described below) to avoid cytotoxic effects displayed by ts mutants at the nonpermissive temperature. A theoretical problem with the qualitative test is that it does not discriminate between complementation and recombination occurring at the nonpermissive temperature. In practice, this has not proven to be a problem (CONDIT and MOTYCZKA 1981).

It is noteworthy that complementation analysis can theoretically be performed between any two mutants for which a common nonpermissive condition can be found. For example, mixed infections at 40 °C in the absence of drug have been successfully used for complementation analysis of an IBT^d mutant with ts mutants of vaccinia (MEIS and CONDIT, unpublished work).

The qualitative and quantitative complementation tests seem to be equally accurate, and the qualitative test is technically much easier. The accuracy of the tests is revealed in the fact that many mutants have now been mapped to individual orfs by marker rescue analysis, and in the vast majority of cases complementation groups correlate precisely with orfs.

2.5 Recombination Analysis

A common approach to measure the frequency of DNA recombination between two ts mutations has been employed by several investigators (FENNER and COMBEN 1958; GEMMELL and CAIRNS 1959; PADGETT and TOMKINS 1968; BASILICO

and JOKLIK 1968; CHERNOS et al. 1978; LAKE et al. 1979; ENSINGER 1982; DRILLIEN et al. 1982; ESSANI and DALES 1983; FATHI et al., unpublished work). Cells are infected with two ts mutant virus at a multiplicity of infection of between 2.5 and 20 plaque forming units per cell, and the infected cells are harvested after 20–48 h incubation at the permissive temperature. Virus is released from the infected cells, and the progeny are titered under conditions which are either permissive or nonpermissive for propagation of the parental virus. For example, in a cross between two ts mutants, A and B, carried out at the permissive temperature of 31 °C, the recombination frequency (%R) is calculated from the formula:

$$\%R = \frac{(A \times B)_{40}}{(A \times B)_{31}} \times 2 - \frac{A_{40}}{A_{31}} - \frac{B_{40}}{B_{31}} \times 100$$

The numerator contains the titer of progeny virus determined under nonpermissive conditions (total recombinants) and the denominator contains the titer of virus under permissive conditions (total progeny virus). This ratio is corrected for the replication of each mutant virus at the nonpermissive temperature and is multiplied by two to account for the fact that for each wild type virus formed, a nonviable double mutant will be generated. In order to convert the ratio to a percentile, it is multiplied by 100. It is important to recognize that the application of this equation requires several assumptions: (a) each parental virus possesses a single ts mutation, (b) each parental virus replicates at the same rate as the recombinant progeny at the permissive temperature, and (c) neither mutation encodes a function which either directly or indirectly impairs DNA recombination at the permissive temperature.

2.6 Marker Rescue

By far the most potent weapon in the poxvirus geneticist's arsenal is marker rescue. This method permits the localization of virus mutations to physically defined regions of the genome by testing for conversion of mutant virus to a wild type phenotype by recombination with specific cloned restriction fragments of wild type virus DNA (CONDIT et al. 1983; ENSINGER and ROVINSKY 1983; DRILLIEN and SPEHNER 1983). Since poxvirus DNA is noninfectious, this test must be performed by first infecting cells with mutant virus, then transfecting with calcium phosphate precipitated cloned viral DNA restriction fragments. The assay may be carried out in one step or two. In the one step assay, monolayers are infected at a low multiplicity, transfected with DNA, incubated at the nonpermissive temperature, stained, and scored directly for plaque formation. In the two step assay, cells are infected at any desired multiplicity, transfected with DNA, incubated at the nonpermissive temperature, harvested, and wild type virus yields are assayed by plaque titration. For most purposes, the one step assay is preferred because it is faster and easier. Marker rescue is equally efficient with either double-stranded or single-stranded DNA (SETO et al. 1987).

New mutants can be rapidly mapped to a resolution of 5–10 kb by marker rescue using a library of overlapping wild type DNA clones which span the virus genome (THOMPSON and CONDIT 1986). Fine mapping can be done with subclones as small as 300 nucleotides (ENSINGER et al. 1985; SETO et al. 1987) and with nested M13 deletion subclones generated during DNA sequencing (THOMPSON et al. 1989).

3 Mutants

3.1 Deletion/Transposition Mutants

There exists a class of poxvirus mutants which, because of a simple deletion or a combination of a deletion and a transposition, contain genomes which lack large segments of DNA relative to wild type virus. These mutants are discussed in the accompanying chapter by TURNER and MOYER.

3.2 Drug Resistant and Dependent Mutants

Given the large size of the poxvirus genome and the autonomous nature of the replication cycle, it is perhaps not surprising that a relatively large number of specific inhibitors of poxvirus replication have been discovered. In some cases, foreknowledge of the precise nature of the target provided a strong incentive to look for drug resistant mutants. For example there was good reason to believe that BUdR, PAA, and hydroxyurea (HU) would target the viral tk, DNA polymerase, and ribonucleotide reductase, respectively, and therefore that mutants resistant to these drugs would aid in characterizing the genes encoding these enzymes. In other cases, the precise mechanism of action of a drug was not

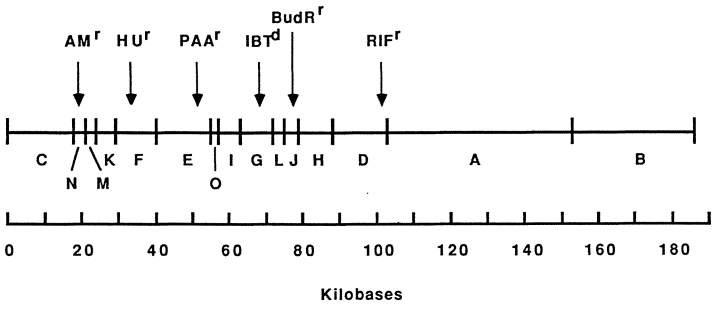


Fig. 1. Map position of vaccinia virus drug resistant and dependent mutants. A *Hin*dIII restriction endonuclease cleavage map is shown with a scale in kilobases. See text for references

known, but the biochemical effects of the drug provided a strong incentive to search for drug resistant mutants. For example, it was believed that mutants resistant to RIF, IBT, and AM might target genes involved in virus morphogenesis, control of gene expression, and nuclear interaction, respectively. Drug resistance, in particular PAA^r, has also been useful as a dominant selectable marker for monitoring tests of genetic protocols (CONDIT and MOTYCZKA 1981; FATHI et al. 1986). We will discuss below these six best-defined examples of drug resistance or dependence markers in vaccinia virus and comment on the potential for future research in this area. A summary of the map positions of the mutants is given in Fig. 1.

3.2.1 BUdR

BUdR has been used to target the vaccinia tk in precisely the same fashion as it has been used to study other cellular and viral tk. When present in the 5′ phosphorylated form, BUdR is toxic to a poxvirus infection (EASTERBROOK and DAVERN 1963; PENNINGTON 1976), presumably because of incorporation into viral DNA. Vaccinia mutants resistant to BUdR are uniformly defective in the viral tk because the unphosphorylated form of BUdR is nontoxic (DUBBS and KIT 1964). As noted above, experiments with BUdR must be carried out on TK^- cells so that BUdR will not be phosphorylated by the host tk. Since any mutation which inactivates the viral tk gene will result in a TK^- phenotype, the TK^- mutants which have been characterized include nonsense, frameshift, deletion, and insertion mutations (WEIR and MOSS 1983). As in other systems, TK^+ poxviruses are resistant to the action of methotrexate, so that a positive selection is available for both the TK^- and TK^+ alleles of the tk gene (WEIR et al. 1982). Marker rescue of TK^- mutants of vaccinia was one method used to map the vaccinia tk gene (WEIR et al. 1982). This gene has now been sequenced, and its expression and biochemistry are well-studied (WEIR et al. 1982; HRUBY and BALL 1982; BAJSZAR et al. 1983; WEIR and MOSS 1983; HRUBY et al. 1983). The vaccinia tk gene has been a preferred site for insertion of foreign genes into vaccinia, since the gene is nonessential, insertions can be detected by selecting for BUdR resistance, and TK^- viruses display reduced pathogenicity in animals (BULLER et al. 1985).

3.2.2 PAA

PAA is a pyrophosphate analogue which acts as a specific competitive inhibitor of DNA polymerases from a wide range of sources, including both eucaryotes, procaryotes, and a number of viruses (COZZARELLI 1977). Although the drug will inhibit DNA replication and cell division in a growing monolayer, it does not kill the cells, so that, for the purposes of virus plaque assay, a PAA treated confluent monolayer seems unaffected by the drug. PAA^r mutants of vaccinia encode a PAA resistant viral DNA polymerase (MOSS and COOPER 1982; SRIDHAR and CONDIT 1983). Marker rescue of PAA^r mutants of vaccinia was one method used

to physically map the viral DNA polymerase gene (SRIDHAR and CONDIT 1983; JONES and MOSS 1984). The DNA polymerase gene and missense PAA^r mutants of the gene have been sequenced, and the expression of the gene and the biochemistry of the enzyme are well understood (CHALLBERG and ENGLUND 1979; TRAKTMAN et al. 1984; EARL et al. 1986; TRAKTMAN et al. 1989). Although two independently isolated PAA^r mutants of vaccinia both contain the same mutational change (EARL et al. 1986; TRAKTMAN et al. 1989), PAA^r mutations which map at other sites in the DNA polymerase gene have been isolated (CONDIT, unpublished work). Two ts mutants of the viral DNA polymerase have also been described (SRIDHAR and CONDIT 1983).

3.2.3 HU

Vaccinia virus synthesizes a novel ribonucleotide reductase during infection of primate cells (SLABAUGH and MATHEWS 1984; SLABAUGH et al. 1984). Recent DNA sequence analysis indicates that the vaccinia ribonucleotide reductase is homologous to the enzymes characterized from other systems and is a heterodimer with subunits of 86 kDa and 37 kDa (SLABAUGH et al. 1988; TENGELSEN et al. 1988; SCHMITT and STUNNENBERG 1988). One of the approaches used to identify the vaccinia gene, that encodes the small subunit of ribonucleotide reductase was to isolate and characterize virus mutants resistant to HU (SLABAUGH and MATTHEWS 1984; SLABAUGH et al. 1988), which in other systems inactivates a tyrosyl free radical in this subunit (REICHARD and EHRENBERG 1983). The HU^R mutants of vaccinia are particularly interesting because they maintain resistance by amplification of expression of the small subunit of the viral enzyme. The amplification is accomplished through gene dosage: resistant mutants contain tandem repeats, up to 15 copies, of the gene which encodes the 37 kDa ribonucleotide reductase subunit. Thus, the map position of this gene could be determined in part by restriction endonuclease analysis of viral DNA from HU^R mutants. As might be expected, the HU^R mutant phenotype is unstable in the absence of selection, presumably because intragenomic or intergenomic recombination between tandemly repeated sequences will cause a loss of repeated sequence and therefore reduce the amplification of the gene product (see Sect. 5).

3.2.4 RIF

RIF is a specific inhibitor of vaccinia virus growth (FOLLET and PENNINGTON 1973). Although RIF was initially characterized as an inhibitor of bacterial RNA polymerase, it has no apparent effect on the vaccinia RNA polymerase. Instead, RIF inhibits vaccinia virus morphogenesis. The biochemistry of RIF action and the role of the RIF sensitive virus gene product in a poxvirus infection are not yet understood in detail. In a vaccinia-infected, RIF treated cell, viral gene expression and nucleic acid metabolism proceed normally, but morphogenesis is interrupted at a stage when replicated viral nucleoprotein is beginning to condense in the

cytoplasm and viral membranes are beginning to enclose the nucleoprotein. The viral membranes which form appear abnormal: rather than assuming a curved but rigid-looking cup-shaped morphology, they are irregular in shape and lack the normal spicules. Two independently isolated RIF^r mutations have been mapped to a single virus gene, *D13L*, using marker rescue (TARTAGLIA and PAOLETTI 1985; BALDICK and MOSS 1987). Three ts mutants which affect this same gene have been described (CONDIT et al. 1983; SETO et al. 1987). Both the wild type gene and the two missense RIF^r mutations have been sequenced (TARTAGLIA and PAOLETTI 1985; NILES et al. 1986; WEINRICH and HRUBY 1986; BALDICK and MOSS 1987). The gene is expressed late in infection, and the 62 kDa protein product of the gene is a virion component (TARTAGLIA et al. 1986; BALDICK and MOSS 1987; WEINRICH et al. 1985; LEE-CHEN and NILES 1988b; MINER and HRUBY 1989). Recent studies using anti D13L antibody show that RIF prevents the association of the D13L protein with virosomes in the cytoplasm (MINER and HRUBY 1989). Further studies of RIF action, the behavior of ts mutants in *D13L*, and of the *D13L* gene product itself should provide insight into poxvirus morphogenesis, particularly viral membrane biogenesis.

3.2.5 IBT

IBT is a potent and specific inhibitor of poxvirus growth (BAUER 1955). It has no apparent cytotoxic effects on cells in culture, and it does not inhibit the growth of other viruses. Although the precise mechanism of action of IBT is currently completely obscure, the drug has a novel and fascinating effect on poxvirus-infected cells. It causes the breakdown of ribosomal and viral RNA late during infection resulting in a cessation of viral protein synthesis (WOODSON and JOKLIK 1965; PENNINGTON 1977; COOPER et al. 1979; PACHA and CONDIT .1985). This effect is strikingly similar to the phenotype of infection at the nonpermissive temperature with the ts mutant, *ts22* (PACHA and CONDIT 1985). It therefore seems likely that the drug inhibits the product of the gene affected by *ts22*, though there is at yet no direct evidence to support this hypothesis. Both IBT dependent and IBT resistant mutants of vaccinia virus have been isolated (KATZ et al. 1973; FATHI et al. 1986). The IBT^r locus has not yet been mapped, but the IBT^d locus has been mapped by marker rescue to the right half of the *Hin*dIII G fragment (FATHI et al. 1986). Interestingly, this is approximately 50 kb distant from the *ts22* locus, which resides in the *Hin*dIII A fragment (THOMPSON and CONDIT 1986). Several lines of evidence indicate that IBT^d mutants affect the same gene affected by a ts mutant, *ts56*. IBT^d and *ts56* map to the same DNA fragments using marker rescue (though this has not yet been resolved to a single orf; THOMPSON and CONDIT 1986; MEIS and CONDIT, unpublished), IBT^d and *ts56* are noncomplementing at 40 °C in the absence of drug (MEIS and CONDIT, unpublished), and *ts56* has an IBT^d phenotype at 40 °C (PACHA 1986). Further studies of IBT, IBT resistant and dependent mutants, *ts56*, *ts22*, and their interrelationships are underway (see Sect. 3.3). It is clear that IBT and the related mutants are keys to a complex and novel aspect of RNA metabolism in poxvirus infected cells.

3.2.6 AM

Although the emphasis in describing poxviruses is usually placed on the cytoplasmic site of replication and an independence from nuclear functions, there is a requirement for an active nucleus during infection by wild type vaccinia virus (MOYER 1987). If the nucleus is removed from cells, or inactivated by UV irradiation or AM treatment prior to infection, vaccinia replication is inhibited. The inhibition is characterized by slightly reduced viral protein and nucleic acid synthesis accompanied by incomplete morphogenesis and incomplete processing of virion structural proteins. The observation that AM would mimic the effects of enucleation and UV irradiation prompted VILLARREAL et al. (1984) to seek AM resistant mutants of the virus in hopes that these mutants would shed light on the interaction between the virus and the cell nucleus. AM^r mutants were isolated which would replicate to 40% of wild type titers on enucleated cells (VILLARREAL et al. 1984). For subsequent marker rescue mapping studies, to circumvent problems associated with AM cytotoxicity, VILLARREAL and HRUBY (1986) isolated AM^r-ts vaccinia mutants in which the ts and AM^r lesions were very tightly linked, if not identical. The ts mutation maps to a region containing only two orfs near the *Hin*dIII N/M junction on the vaccinia physical map, and rescued AM^r-ts mutants have an AM^s phenotype (VILLARREAL and HRUBY 1986; TAMIN et al. 1988). At the nonpermissive temperature, the AM^r-ts mutant shows normal viral DNA and protein synthesis and impaired processing of virion precursor proteins, suggestive of a morphogenesis defect.

3.2.7 Other Drugs

There is no reason to believe that the list of useful drug resistance markers in poxviruses is complete. Several other replication inhibitors have been investigated including 2′-O-methyl adenosine (RACZYNSKI and CONDIT 1983); the DNA polymerase inhibitor, aphidicolin (DEFILIPPES 1984); the guanine methyltransferase inhibitor, sinefungin (PUGH et al. 1978); and topoisomerase inhibitors such as novobiocin (TRAKTMAN, personal communication). Although mutants resistant to 2′-O-methyl adenosine (RACZYNSKI and CONDIT 1983) and sinefungin (CONDIT, unpublished) have been isolated, progress in characterizing the mutants has been hampered by drug cytotoxicity and variable or weak effects of the drug on wild type virus. A systematic survey of potential inhibitors would very likely result in characterization of additional useful resistance loci in the virus.

3.3 Temperature Sensitive Mutants

By far the largest and most actively studied class of poxvirus mutants is ts mutants. By definition, these mutations target genes which are essential for virus growth on the cell line used for mutant isolation. Mutants which display host range at the nonpermissive temperature have also been identified (DRILLIEN et al. 1982). The

beauty of ts mutations is that with a single set of technical protocols a wide variety of essential genes can be identified.

During the last 25 years, six major collections of ts mutants of vaccinia and rabbitpox have been assembled by TOMKINS and co-workers (SAMBROOK et al. 1966; PADGETT and TOMKINS 1968; LAKE and COOPER 1980), DALES and co-workers (STERN et al. 1977; DALES et al. 1978; LAKE et al. 1979; MCFADDEN et al. 1980; SCHÜMPERLI et al. 1980; MCFADDEN and DALES 1980; SILVER and DALES 1982; ESSANI et al. 1982; ESSANI and DALES 1983), CHERNOS and co-workers (CHERNOS et al. 1978, 1983, 1985), DRILLIEN and co-workers (DRILLIEN et al. 1982; DRILLIEN and SPEHNER 1983), ENSINGER and co-workers (ENSINGER 1982; ENSINGER and ROVINSKY 1983; ENSINGER et al. 1985; ENSINGER 1987), and CONDIT and co-workers (CONDIT and MOTYCZKA 1981; CONDIT et al. 1983; SRIDHAR and CONDIT 1983; PACHA and CONDIT 1985; THOMPSON and CONDIT 1986; SETO et al. 1987; THOMPSON et al. 1989; HOODA-DHINGRA et al. 1989). A total of more than 350 ts mutants have been reported. A comprehensive survey of each of the collections is beyond the scope of this review. Nonetheless, an accurate overview of ts mutants in vaccinia can be presented by first focusing in detail on the features of one collection which now includes mutants isolated by CONDIT and co-workers and some of the mutants isolated by ENSINGER and co-workers. A comparison with results from separate mutant collections will be presented where appropriate. We will then present a summary of the basic features of five other collections. Recombination mapping will be described in more detail in Sect. 5.

3.3.1 Overview

A summary of the ts mutant collection described by CONDIT and co-workers is given in Table 1 and Fig. 2. Included in this summary are several ts mutants isolated by ENSINGER (ENSINGER 1982; ENSINGER and ROVINSKY 1983), which have been integrated into the collection through studies on the organization of

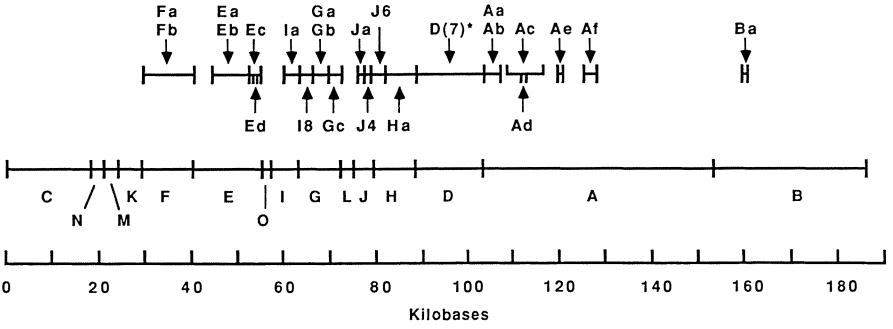


Fig. 2. Physical map of vaccinia virus ts mutants. A *Hin*dIII restriction endonuclease cleavage map is shown with a scale in kilobases. Map positions of complementation groups (Table 1) are shown above the restriction map. The relative map position within six pairs of complementation groups (Fa and Fb, Ea and Eb, Ec and Ed, Ga and Gb, Aa and Ab is unknown. The designation D(7)* indicates that seven complementation groups map in the *Hin*dIII D fragment; see Fig. 3

Table 1. Vaccinia virus temperature sensitive mutants

Group[a]	Mutants[b, c]	Phenotype/Function[d]
Fa	12, 15, *28*, 54, 61	Normal
Fb	(14), *30*, 48, (59)	Normal
Ea	*52*	Normal
Eb	*19*	Normal
Ec	*49*	Normal
Ed	**42, NG26**	DNA^-/DNA polymerase/PAA^{r}[e]
Ia	*16*, 34, (59)	Normal
I8	**10, 18, 38, 39, 44**	Normal/virion structure[f]
Ga	*56*	Defective late
Gb	*60*	Normal
Gc	*11*, 41	Normal
Ja	*45*	Normal
J4	**7, 20**	Defective late/RNA pol (22 kDa)[g]
J6	**51, 53, 65**	Defective late/RNA pol (147 kDa)[h]
Ha	*1*, 31, 55, 58	Normal
D2	**E52, E94**	Normal
D3	**5, 35**	Normal/virion structure[i]
D5	17, 24, **E69**	DNA^-
D6	**46, E93**	Normal
D7	**21, E45**	Defective late
D11	*36*, 50, **E17**	Defective late/ATPase[j]
D13	33, 43, **E101**, (14)	Normal/rif/virion structure[k]
Aa	*8*, *26*	Normal
Ab	*63*,	Defective late
Ac	*40*	Normal
Ad	4, **22**, 23	Abortive late/RNA stability[l]
Ae	**27, 29, 32, 47, 62**	Defective late/RNA pol (135 kDa)[m]
Af	*6*, 9	Normal
Ba	2, 3, **25**	DNA^-
X	57	Normal
Y	37	Normal
Z	13, 64	Normal

[a] Each line in the table represents a complementation group. The names of the groups indicate their map position: the first letter denotes a *Hin*dIII fragment; where the map position is known, the number of an orf is given, otherwise a letter is used. The group X, Y, and Z are unmapped

[b] Preliminary mapping: CONDIT et al. (1983); ENSINGER and ROVINSKY (1983); THOMPSON and CONDIT (1986); SETO et al. (1987)

[c] Numbers without a preceeding letter represent mutants isolated by CONDIT and co-workers; numbers preceded by an E represent mutants isolated by Ensinger and co-workers. The number in italics denote mutants which are mapped by marker rescue; numbers in boldface indicate mutants mapped to an open reading frame

[d] Preliminary phenotypes described by CONDIT and MOTYCZKA (1981) and CONDIT et al. (1983)

[e] SRIDHAR and CONDIT (1983); JONES and MOSS (1984); TRAKTMAN et al. (1984, 1989)

[f] FATHI and CONDIT (in preparation)

[g] THOMPSON et al. (1989)

[h] ENSINGER et al. (1985); THOMPSON et al. (1989)

[i] DYSTER (unpulished work)

[j] RODRIGUEZ et al. (1986); BROYLES and MOSS (1987)

[k] TARTAGLIA and PAOLETTI (1985); TARTAGLIA et al. (1986); BALDICK and MOSS (1987); MINER and HRUBY (1989)

[l] PACHA and CONDIT (1985); PACHA (1986)

[m] HOODA-DHINGRA et al. (in preparation)

the *Hin*dIII D fragment of vaccinia (see Sect. 4; SETO et al. 1987). The evolution of the collection involves conceptual and technical contributions from many different laboratories. The characteristics can be conveniently divided into genetic analysis and phenotypic analysis, each of which is presented separately below.

3.3.2 Genetic Analysis

The 72 mutants, all isolated in the *WR* strain of vaccinia, have been assigned to 32 complementation groups (CONDIT et al. 1983; SETO et al. 1987). Although both the quantitative and the qualitative complementation tests were used, most of the assignments were based on the qualitative test. All but three of the complementation groups have been assigned a physical map position based on marker rescue with cloned wild type DNA fragments which span virtually the entire vaccinia genome (CONDIT et al. 1983; ENSINGER and ROVINSKY 1983; THOMPSON and CONDIT 1986; SETO et al. 1987). The map positions of several of the mutants have been refined to a single orf by marker rescue with small DNA fragments and, in a few cases, the precise nucleotide change causing temperature sensitivity has been determined (see Table 1 for specific references).

Both complementation analysis and marker rescue mapping have proven to be extraordinarily accurate, and the results from these analyses correlate extremely well with DNA sequence analysis. In nearly all cases where mutants have been mapped to individual orfs, mutants in the same complementation group map to the same orf and mutants in different complementation groups map to different orfs (see Sect. 4).

A striking feature of the physical map of ts mutants is that the mutants are clustered in the 100 kb central conserved region of the viral genome. Similar observations have been made by ENSINGER and ROVINSKY (1983) and DRILLIEN and SPEHNER (1983) on independent mutant collections. This observation is consistent with the idea that conservation of this region throughout the orthopoxvirus family represents clustering of housekeeping functions essential for replication of all poxviruses. These would include most of the functions for nucleic acid and protein metabolism and virion morphogenesis.

Estimates based on the distribution of ts mutants into complementation groups, the amount of DNA which can be deleted from the genome, and the degree of mutant saturation in well characterized regions of the genome (see Sect. 4) suggest that the collection described in Table 1 represents approximately 30%–50% of the possible ts complementation groups in vaccinia. We therefore conclude that the virus contains approximately 75–100 genes which are essential for growth in culture.

3.3.3 Phenotypic Analysis

During the preliminary analysis of their mutant collection, CONDIT and co-workers assayed representatives of each complementation group in the collection

for their ability to synthesize viral DNA and protein at the nonpermissive temperature (CONDIT and MOTYCZKA 1981; CONDIT et al. 1983). DNA synthesis was assayed by measuring [^{3}H] thymidine incorporation and protein synthesis was assayed by ^{35}S pulse-labeling followed by gel electrophoresis and autoradiography. A variety of phenotypes were detected, and these were divided into four major groups: normal, DNA$^-$, defective late, and abortive late. The current tally (Table 1) lists 23 complementation groups with a normal phenotype, 7 with a defective late phenotype, 3 with a DNA$^-$ phenotype, and 1 with an abortive late phenotype. In general, mutants in the same complementation group display the same phenotype, but some variation of phenotype exists within groups. This heterogeneity is particularly pronounced in the defective late group. Since the initial characterization, a more detailed examination has been made of at least one complementation group representing each of the four phenotypes (see below). It is noteworthy that, with the exception of the abortive late phenotype, each of these phenotypes has been observed during characterization of other mutant collections. A more detailed description of each of these phenotypes is presented below.

3.3.3.1 The Normal Phenotype

At the nonpermissive temperature, mutants with a normal phenotype display viral DNA and protein synthesis which is indistinguishable from wild type virus. It was originally hypothesized that the genes affected in mutants with this phenotype would encode proteins important for virion structure or morphogenesis. This hypothesis is supported by more detailed analysis of three different normal complementation groups, those affecting genes *18R* (FATHI and CONDIT, in preparation), *D3R* (LEE-CHEN and NILES 1988b; DYSTER and NILES, unpublished work), and *D13L*, the target of RIF (TARTAGLIA et al. 1986; BALDICK and MOSS 1987; WEINRICH et al. 1985; LEE-CHEN and NILES 1988b; MINER and HRUBY 1988). In each case, the mutants affect a gene which is transcribed late during infection (orf *18R* is also transcribed early) and which encodes a virion structural component. Mutants in this group are ideal candidates for further analysis by electron microscopy and by assays for proteolytic processing of virion components.

3.3.3.2 The DNA$^-$ Phenotype

Mutants with a DNA$^-$ phenotype are incapable of [^{3}H] thymidine incorporation into viral DNA at the nonpermissive temperature. Since during vaccinia infection late protein synthesis is coupled to DNA replication, DNA$^-$ mutants are also defective in the synthesis of late viral proteins. The DNA$^-$ complementation group which is best understood is that affecting the viral DNA polymerase, group Ec (Table 1; SRIDHAR and CONDIT 1983; TRAKTMAN et al. 1984, 1989). Several different investigators have isolated mutants which affect gene *D5R* (Table 1). Both the gene and the mutants have been extensively characterized, but

the precise role of this gene in DNA replication is not yet understood (NILES et al. 1986; ROSEMAN and HRUBY 1987; EVANS and TRAKTMAN 1987). Characterization of DNA$^-$ group Ad is in progress (TRAKTMAN, personal communication).

If ts mutants have been isolated in one-third of the essential genes in vaccinia, then, based on the results to date, we might expect ultimately to find approximately nine complementation groups with a DNA$^-$ phenotype. In addition, it should be noted that the DNA$^-$ phenotype is defined by the most stringent possible criterion, [^{3}H] thymidine incorporation. It is possible that viral mutants in many gene products involved in DNA metabolism may have much more subtle effects on DNA replication and could therefore appear as mutants with a defective late or even a normal phenotype. For example, resolution of concatemeric intermediates in viral DNA replication seems to be a late viral function, evidenced by the fact that many defective late mutants are incapable of resolving concatemers at the nonpermissive temperature (MERCHLINSKY and MOSS 1989; DELANGE 1989). It is also possible that some replication functions could be duplicated by and therefore substituted by the host. For example, the viral ribonucleotide reductase in a nonessential viral gene (SLABAUGH, personal communication), presumably because its function can be complemented by the host enzyme.

3.3.3.3 The Defective Late Phenotype

Mutants with a defective late phenotype show normal [^{3}H] thymidine incorporation at the nonpermissive temperature but are defective to varying degrees in the synthesis of late viral proteins. It is critical to understand that this is a very heterogeneous phenotype. Some mutants show a delayed onset of late protein synthesis which ultimately reaches almost normal levels, some show a properly timed onset of protein synthesis which persists at abnormally low levels, some mutants show a delayed onset of late protein synthesis which persists at abnormally low levels, and some show virtually no late viral protein synthesis. The heterogeneity is not only evident when complementation groups are compared, but mutants within a complementation group may differ in the precise characteristics of late viral protein synthesis (ENSINGER 1987; THOMPSON et al. 1989; HOODA-DHINGRA et al. 1989). In almost all cases, the time course of early viral protein synthesis is related to the time course of late viral protein synthesis; shut off of early viral protein synthesis is delayed in mutants which show a delayed onset of late viral protein synthesis. This observation is consistent with the notion that a shutoff of early viral protein synthesis is controlled by one or more late viral gene products.

Mutants representing several defective late complementation groups have now been characterized in detail. At least three groups affect genes which encode viral RNA polymerase subunits (ENSINGER 1987; THOMPSON et al. 1989; HOODA-DHINGRA et al. 1989; HOODA-DHINGRA and CONDIT, in preparation), one group affects a nucleic acid dependent ATPase (RODRIGUEZ et al. 1986; BROYLES and MOSS 1987; SETO et al. 1987), and one may affect a function related to the RNA

metabolism pathway affected by IBT (MEIS and CONDIT, unpublished work). Thus, all of the defective late mutants studied so far affect nucleic acid metabolism functions, and most if not all affect functions involved in RNA metabolism.

Given the large number of RNA metabolism enzymes which are contained in the vaccinia virion, one might expect to find mutants which are defective in synthesis of early proteins at the nonpermissive temperature; however no "defective early" mutants have yet been described, and 13 ts mutants (from two separate mutant collections) affecting three different virion RNA polymerase subunits all have defective late phenotypes. These observations strongly suggest that mutants in other virion enzymes (note *D11* in Table 1) may yield a defective late phenotype.

3.3.3.4 The Abortive Late Phenotype

The term "abortive late" describes an infection in which early viral protein synthesis and DNA replication appear normal and late viral protein synthesis begins normally, but labeling of all proteins ceases abruptly approximately 8 h after infection. Three mutants comprising a single complementation group display this phenotype. DNA sequence analysis, transcription analysis, and marker rescue show that the complementation group maps to a gene which is transcribed both early and late during infection and which could encode a protein of 56 kDa (PACHA et al. in preparation). PACHA and CONDIT (1985) have shown that at the same time that protein synthesis aborts during a *ts22* infection, rRNA and viral mRNA are degraded. It is possible that during a normal infection, the *ts22* gene product prevents RNA degradation. The drug IBT induces the same phenotype in wild type virus-infected cells, suggesting that it acts on the same pathway as the *ts22* gene product. Inspired by the similarity between this phenotype and rRNA degradation characteristic of the interferon-induced, 2-5A pathway, COHRS et al. (1989) have measured 2-5A concentrations in *ts22* infected cells. Their results show that intracellular 2-5A concentrations are abnormally high under conditions in which the abortive late phenotype is observed. Together these results suggest that an interaction with the 2-5A pathway is a normal part of a vaccinia infection. The fact that IBT^d mutants map in a gene distinct from *ts22* suggests that the interaction may involve more than one viral gene.

3.3.4 Independent Mutant Collections

The first collection of orthopoxvirus ts mutants was isolated in rabbitpox (SAMBROOK et al. 1966). Eighteen of the mutants isolated by SAMBROOK et al. (1966) were grouped into 14 complementation groups using a quantitative assay (PADGETT and TOMKINS 1968). Recombination mapping of some of the mutants was attempted (see Sect. 5), but physical mapping of the mutants has not been reported. LAKE and COOPER (1980) assayed the mutants for DNA replication, viral protein synthesis, and posttranslational cleavage of viral proteins. Of the 18 mutants assayed, 2 were DNA^-, 1 was defective late, and, of the remaining

"normal" mutants, 6 were defective in posttranslational cleavage of viral proteins.

DALES and co-workers have isolated 90 ts mutants of the *IHD-W* strain of vaccinia (DALES et al. 1978). Although complementation and recombination analysis has been reported on selected subsets from this collection (LAKE et al. 1979; MCFADDEN and DALES 1980; ESSANI and DALES 1983), comprehensive physical mapping and complementation analysis of the mutants have not been reported. The mutants were assayed for viral DNA replication and for morphogenesis defects using electron microscopy. The microscopic examination of the mutants revealed a fascinating array of morphogenetic defects which the authors grouped into 17 classes. Since complementation analysis and physical mapping data for this collection is currently limited, the groups defined by electron microscopy cannot yet be correlated with complementation groups. Detailed analysis of one morphogenetic group indicates that it comprises several genes (LAKE et al. 1979; ESSANI and DALES 1983). Seven DNA^- mutants, comprising at least four complementation groups, have been charcterized in greater detail using temperature shift protocols. One of the DNA^- mutants, 6389, maps to gene *D5L* (TRAKTMAN, personal communication).

CHERNOS and co-workers have assembled a collection of 80 ts mutants in the *MM* strain of vaccinia (CHERNOS et al. 1978, 1983, 1985). Although complementation and recombination analysis have been reported on selected subsets from this collection, comprehensive physical mapping and complementation analysis of the mutants have not been reported. Phenotypic analysis of the collection reveals 9 DNA^- mutants and 19 mutants which were weakly defective in DNA replication ($DNA^{+/-}$). Three of the DNA^- mutants have been mapped to the *Hin*dIII D and E fragments by restriction endonuclease analysis of vaccinia-ectromelia recombinants. CHERNOS et al. (1983) have also described an interesting phenotype characterized by DNA^+ mutants which produce low yields of virus if they are incubated first at the nonpermissive temperature, then shifted to a permissive temperature in the presence of cytosine arabinoside.

DRILLIEN and colleagues have assembled a collection of 42 mutants in the Copenhagen strain of vaccinia (DRILLIEN et al. 1982; DRILLIEN and SPEHNER 1983). Genetic characterization of this group includes extensive complementation analysis and mapping by recombination and marker rescue. The mutants fall into 23 complementation groups, 17 of which have been positioned on the genome by marker rescue. DRILLIEN et al. (1982) report that some of the mutants display a host range phenotype at the nonpermissive temperature. A biochemical phenotype for the mutants has not been reported.

ENSINGER and co-workers have described a collection of 100 mutants of the *WR* strain of vaccinia (ENSINGER 1982; ENSINGER and ROVINSKY 1983). Of these, 62 have been assigned to 28–32 groups by complementation analysis and physically mapped on the genome by marker rescue. A recombination map of the mutants was constructed from 22 mutants representing 12 complementation groups (see Sect. 5). Six DNA^- mutants, which seem to affect two different genes, have been identified in the *Hin*dIII D (*D5R*) and A fragments. Fine mapping

analysis has been carried out on four complementation groups which map to the *Hin*dIII L, J, and H fragments (ENSINGER et al. 1985). One of these groups has a defective late phenotype and maps to a single orf affecting the 147 kDa viral RNA polymerase subunit (ENSINGER 1987).

4 Genome Organization

DNA sequence information is now available for approximately 45% of the vaccinia genome, and in many parts of the genome the sequence information has been correlated with a transcription map. In some areas the DNA sequence and transcription data have been integrated with genetics and with biochemical information relating to the function of individual poxvirus genes. A summary of this information has been compiled by EARL and MOSS (1987).

In one region of the vaccinia genome, the *Hin*dIII D fragment, a deliberate effort has been made to integrate detailed biochemical and genetic information throughout a continuous segment of viral DNA containing several genes (NILES et al. 1986; SETO et al. 1987; LEE-CHEN and NILES 1988a, b; LEE-CHEN et al. 1988; NILES and SETO 1988). The characterization of this fragment was initiated in an effort to understand the general features of the organization of the vaccinia genome at the level of DNA sequence. These studies can therefore be used as a model to describe the general features of poxvirus genome organization. The *Hin*dIII D fragment was a particularly appropriate choice for a detailed study because: (1) it is part of the central, conserved region of the genome and therefore is likely to contain many genes which are common to all poxviruses and essential for viral replication (MACKETT and ARCHARD 1979; ESPOSITO and KNIGHT 1985); (2) it was known to contain a large number of ts mutants representing all but the abortive late phenotype (CONDIT et al. 1983; ENSINGER and ROVINSKY 1983); and (3) translational mapping studies (BELLE ISLE et al. 1981) indicated that the fragment contained both early and late viral genes.

To understand in detail the molecular and genetic characteristics of this region of viral DNA, the DNA has been sequenced (NILES et al. 1986; WEINRICH and HRUBY 1986), a transcription map of the region has been derived (LEE-CHEN and NILES 1988a, b; LEE-CHEN et al. 1988; NILES and SETO 1988; WEINRICH et al. 1985; WEINRICH and HRUBY 1986), and ts mutants from two collections have been mapped to high resolution within the fragment (SETO et al. 1987; EVANS and TRAKTMAN 1987; ROSEMAN and HRUBY 1987). A description of the organization of the fragment is present below and is summarized in Fig. 3. Analysis of the DNA sequence from *Hin*dIII D reveals 13 orfs and all other biochemical and genetic analyses performed to date supports the conclusion that each of these orfs corresponds to a viral gene. There is very little space between orfs (a maximum of 42 bp), and some orfs overlap. This suggests that the transcription signals for a given gene are frequently imbedded in the coding region of the adjacent genes.

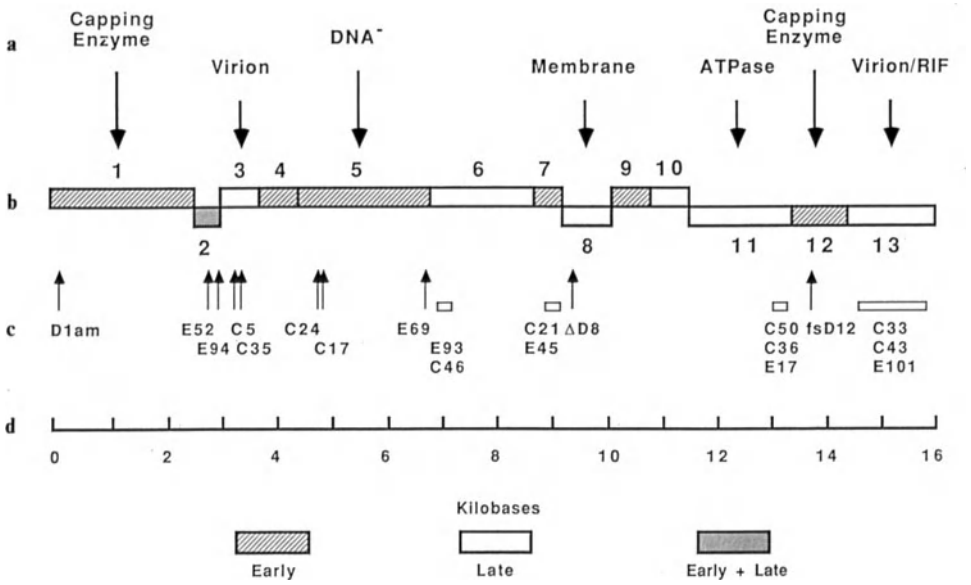


Fig. 3a–d. Genome organization in the *Hin*dIII D fragment. **a** Function of gene products or phenotype of ts mutants, where known. **b** *Numbered boxes* denote individual genes; those above center are transcribed rightwards, below center are transcribed leftwards. **c** Mutant map positions: *Arrows* indicate sequenced mutants, *open boxes* denote the approximate map position of unsequenced mutants. Numbers preceded by a C indicate mutants isolated by Condit and co-workers, numbers preceded by an E denote mutants isolated by Ensinger and co-workers. An amber mutant isolated in gene *D1* (*D1am*, CONDIT unpublished work) and a frameshift mutant isolated in gene *D12* (*fsD12*, LEE-CHEN and NILES, unpulished work) prove that these genes are essential: the mutants replicate only in the presence of wild type virus. The deletion in gene *D8* (*D8*) replicates independently (NILES and SETO 1988). **d** Scale in kilobases

Transcription analysis shows that the fragment contains both early and late genes, and these are arranged in both possible transcriptional orientations. Sixteen ts mutants have been grouped into seven complementation groups, and each complementation group maps to a single gene. In addition, frameshift mutagenesis of gene *D8R* shows that this gene is nonessential for vaccinia replication (NILES and SETO 1988), and frameshift and nonsense mutagenesis of genes *D1R* (CONDIT, unpublished work) and *D12L* (LEE-CHEN and NILES, unpublished work) show that they are essential. Recent fine mapping of a ts mutant from the mutant collection of DALES et al. (1978) suggests that it affects gene *D12L* (DELANGE, personal communication). Using a variety of methods, functions have been assigned to several orfs, as summarized in Fig. 3. Genetic and biochemical studies are continuing in an effort to define the precise role of each of the *Hin*dIII D fragment genes in a poxvirus infection. In other areas of the genome where high resolution information is available, the organization is similar to the *Hin*dIII D fragment (see EARL and MOSS 1987).

The characterization of the *Hin*dIII D fragment leads to important generalizations about the organization of the poxvirus genome. Genes seem to be arranged

in the DNA such that transcriptional interference between genes is minimized. Specifically, genes are situated such that early transcription termination signals are not found in the body of other early genes and with one exception, potential interference between divergent promoters is avoided by using promoters that are expressed at different times during infection (LEE-CHEN et al. 1988). Aside from these facts, the organization of the fragment does not immediately suggest a logic with respect to clustering of functions or coordination of gene expression. For example, the two subunits of the viral capping enzyme (MORGAN et al. 1984; NILES et al., in preparation) are both synthesized early during infection, transcribed from opposite DNA strands, and separated by 12 kb of DNA. The sequence between the two genes encodes both early and late functions used for processes ranging from DNA replication to virion morphogenesis.

The integration of mutant phenotype studies with biochemical analysis is rewarding. For example, it is significant that normal and DNA^- mutants affect genes which are transcribed late and early, respectively, and that defective late mutants affect genes which are transcribed either early (*D7R*) or late (*D11L*). Particularly noteworthy is the convergence of data is gene *D13L*, for which there now exist ts mutants, drug resistance mutants, DNA sequence and transcription information, and immunocytochemical data relating to the synthesis and function of the gene product (Table 1).

The status of the genetic analysis of vaccinia can be evaluated by inspection of the map of the *Hin*dIII D fragment. Of 13 genes in this fragment, mutational analysis has shown that 1 gene is nonessential and 9 are essential. If we assume that the remaining 3 genes are essential, then, by combining mutants from two ts mutant collections, we can conclude that ts mutants in approximately one-half of the essential genes have been identified. It is interesting that the integration of a total of 12 complementation groups from two independent collections distills down to a total of 7 unique groups, an increase of only 16% for each collection. Although in some areas there is a very satisfying convergence of biochemical and genetic data, some very interesting genes, for example gene *D1*, the large subunit of the mRNA capping enzyme, have so far escaped mutagenesis. In summary, the good news is that for some genes both genetic and biochemical tools are available to probe the biological role of the gene product in vaccinia replication. The bad news is that the brute force isolation of ts mutants in the poxvirus genome has reached a point of diminishing returns. Methods must be developed which will permit the targeted mutation of specific essential poxvirus genes to conditional lethality.

5 DNA Recombination

DNA recombination in poxvirus-infected cells is of particular interest to the molecular biologist for several reasons. Since poxviruses replicate in the cytoplasm, in order for genetic recombination to occur, the virus must either

encode its own recombination enzymes or it must recruit the enzymes from the nucleus of the infected cell. In either case, this affords the opportunity to both identify a set of eucaryotic recombination factors and to investigate the mechanism of DNA recombination in a eucaryotic environment. Furthermore, recombination between plasmid and viral DNA sequences serves as a cornerstone of current poxvirus genetic technology. A deeper understanding of the recombination pathway would serve to enhance its efficiency and to increase its utility. In spite of the fact that genetic recombination in poxviruses was first demonstrated by FENNER and COMBEN in 1958, we have progressed only a short distance in our understanding of the molecular basis of this process.

In this segment, we will first discuss past observations derived from virus/virus recombination studies culminating in the derivation of genetic maps of viral ts mutations. We will follow this with a description of recent attempts to refine the relationship between the observed recombination frequency and the physical distance between two ts mutations, which serves to explain discrepancies in the original recombination mapping experiments. Finally, we will discuss results derived from new approaches designed to investigate specific steps in the recombination pathway.

5.1 Recombination Mapping

A basic tenet of molecular genetics is the belief that, to a first approximation, the probability of a recombination event between two mutations is proportional to the distance between the mutations. This has been borne out in the construction of genetic maps of a wide variety of organisms, including several bacteriophage and some eucaryotic viruses. The validity of this concept in poxvirus-infected cells has been tested by several investigators and differing results have been obtained. After analysis of the results of several poxvirus recombination studies, it is apparent that, although limited genetic maps can be constructed by a series of two factors crosses between mutations in different genes, the relationship between intergenic recombination frequency and physical distance is tenuous at best. In contrast, however, a reliable proportionality exists between the intragenic recombination frequency and the distance between mutations up to about 700 bp.

Recombination frequencies were measured in several laboratories by two or three factor crosses in attempts to construct genetic maps. PADGETT and TOMKINS (1968) placed 18 rabbitpox viruses mutants into 14 complementation groups and measured recombination frequencies between most pairs of mutants. Although they observed recombination in each cross, they were unable to construct a reliable genetic map based on their results. CHERNOS et al. (1978) initially formed a limited recombination map which placed 7 mutants from their collection of 57 ts mutations into a linear order. They subsequently reported an attempt to arrange a set of DNA^- and $DNA^{+/-}$ mutants into a linear map (CHERNOS et al. 1983). LAKE et al. (1979) and ESSANI and DALES (1983) sorted a set

of five ts mutants, which share a common morphogenic phenotype, into either three or four complementation groups and generated a linear genetic map based on two and three factor crosses. In each of the cases described above, no physical evidence was presented to corroborate the genetic order determined by recombination frequency measurements. As a result, the reliability of each genetic map is uncertain.

More detailed genetic studies have been reported by ENSINGER and DRILLIEN, who tested their genetic maps by carrying out marker rescue analysis on the same set of ts mutations.

ENSINGER (1982) has reported the isolation of 100 ts mutants of the *WR* strain of vaccinia. She initially placed 30 of these mutants into 17 complementation groups and, through a set of two factor crosses, placed mutants in 12 groups into a linear recombination map. ENSINGER and ROVINSKY (1983) and ENSINGER et al. (1985) went on to refine the genetic map by carrying out marker rescue analysis on these mutants. They found that ts mutants in eight of the groups in the original map assignment were in the correct order, two were reversed in position, and two mutants actually belonged to a single complementation group. Based on their marker rescue analysis, they also were able to assign map positions to five additional complementation groups which did not fit into their genetic map.

DRILLIEN et al. (1982) isolated 42 ts mutants from the *Copenhagen* strain of vaccinia virus and sorted the mutants into 23 complementation groups. They generated a linear genetic map based on the results of two factor crosses between mutants from 14 of the 23 groups. The available recombination data did not permit the other nine groups to be placed into their genetic map. DRILLIEN and SPEHNER (1983) also carried out physical mapping analyses using marker rescue in an attempt to test the gene order determined by recombination mapping. They were able to confirm the map positions of the mutants in 10 of the 14 groups originally assigned and, in addition, they reported the map locations of 8 additional ts mutations.

DRILLIEN et al. (1982) also described the results of detailed intragenic recombination frequency measurements made by a series of two factor crosses among five ts mutants in each of two different complementation groups. In one case an unambiguous map was generated that contained five ts mutants; in the other, a map was constructed that located four of five mutants unambiguously. Although the overall map assignments were convincing, they did not provide additional physical evidence which would corroborate the location of the ts mutations.

Both ENSINGER and DRILLIEN have noted that the recombination frequency measurements display a significant degree of variability. In addition, each points out that while the frequencies can often reach 30%–50%, crosses exhibiting recombination frequencies greater than 20% were of little value in making map assignments. Furthermore, each has noted that the observed intragenic recombination frequencies, derived from crosses between mutants in the same gene, were substantially lower than is found intergenic crosses.

With the availability of the sequence of large segments of vaccinia virus DNA and the assignment of a set of ts mutations to specific base changes, it has been possible to assess viral DNA recombination with greater precision. In an attempt to further define the relationship between the measured recombination frequency and the physical distance between markers, FATHI et al. (unpublished) determined the recombination frequencies between ts mutants whose precise locations had been mapped in the *Hin*dIII D fragment (SETO et al. 1987; ROSEMAN and HRUBY 1987; DYSTER and NILES, unpublished work; EVANS and TRAKTMAN, personal communication) or gene *I8R* (FATHI and CONDIT, in preparation). Through a complete set of two factor crosses, they have determined the recombination frequencies between mutants which are separated by distances of 129–10 600 bp. Two patterns can be observed from the results of this analysis (Fig. 4). In intragenic crosses between mutants in genes *D2L*, *D3R*, *D5R*, and *I8R*, the recombination frequency is linearly dependent upon the distance separating the mutations up to about 700 bp, above which a plateau value of about 4% is reached. This observation is in agreement with the genetic results of DRILLIEN et al. (1982) who demonstrated that it is possible to construct consistent intragenic recombination maps based on two factor crosses.

The relationship between the observed recombination frequency and the distance separating mutations in intergenic crosses between mutants in different complementation groups was unexpected. As is shown in Fig. 4, FATHI et al. (unpublished work) have been unable to demonstrate a length dependence on recombination frequencies for intergenic crosses over a distance between 328 to greater than 10 000 bp. Since these results are derived from crosses between

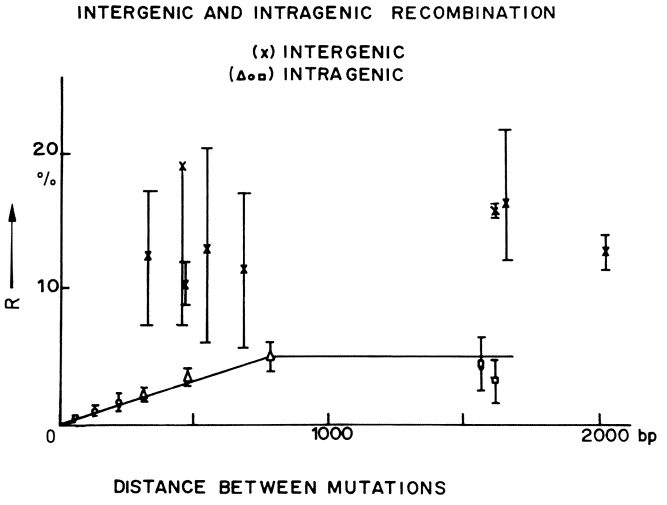


Fig. 4. Distance dependence of intergenic and intragenic recombination frequencies. X = the mean frequency for intergenic crosses; *open triangles*, *circles*, and *squares* = the mean frequency for intragenic crosses. Standard deviations are indicated with error bars

mutants representating six complementation groups that exhibit several phenotypes, it is unlikely that they are due to marker effects within this subset of vaccinia genes.

Superficially, it might appear that the genetic mapping results reported by ENSINGER (1982) and DRILLIEN et al. (1982) are incompatible with the observations of FATHI et al. (unpublished work), but upon further analysis it is clear that they are not. In the two factor crosses carried out by FATHI et al. (unpublished work) between mutations separated by less than 11 kb, they observed intergenic recombination frequencies from 11.0 +/− 4.9% to 22.5 +/− 17.7%, averaging about 13%. ENSINGER and DRILLIEN each would have considered two mutants displaying a recombination frequency of this magnitude to be closely linked. In fact, when one considers the entire poxvirus genome of 186 000 bp, the distances over which recombination was measured by FATHI et al. (unpublished work) are rather short, and the mutations are close together. Although reasonable recombination maps of the entire poxvirus genome have been constructed, it is apparent from the sum of the intergenic recombination results that it is unreasonable to attempt to identify the map position of a mutation by determining the recombination frequency between it and some known mutation in a different gene.

The results displayed in Fig. 4 demonstrate that there is a distance dependence on intragenic recombination frequency measurements that is not observed in intergenic crosses. In attempting to explain the observed difference, one must consider the following: In order for the %R to be dependent on the distance between two markers, DNA recombination must be the rate limiting step in the generation of wild type progeny virus. If recombination events between the markers are frequent, the distance relationship will reach a plateau. This distance dependence holds true for intragenic crosses where the markers are separated by up to about 700 bp (Fig. 4), resulting in an apparent recombination rate about 0.006% per base pair. Since this linear relationship is not apparent in intergenic crosses as close as 328 bp, we must deduce that, in this set of intergenic crosses, the probability of DNA crossover is not the rate limiting step in generating mature wild type progeny.

What then is the difference in the infected cell between intergenic and intragenic crosses? In intergenic crosses, a wild type allele exists in the infected cell for each gene. This is not true in an intragenic cross in which each virus carries an alteration in the same gene. This suggests that, in an intergenic cross, some form of genetic complementation occurs at the permissive temperature and that this complementation alters the rate limiting step from DNA crossover to some other step in wild type virus production.

It has often been noted with some surprise that the apparent recombination rate in vaccinia-infected cells is high. In an intragenic cross, a rate of 0.006% per base pair can be calculated (Fig. 4). This compares favorably with intragenic recombination rates measured for a bacteriophage T4 tRNA gene, 0.014% (COMER 1977); the bacteriophage T4 lysozyme gene, 0.043% (RAVIN and ARTEMIEV 1974); the lambda p_{rm} region, 0.013%; and the bacteriophage lambda

cy cII region, 0.056% (GUSSIN et al. 1980). In an intergenic cross, however, the minimum recombination frequency is at least 0.043% per base pair, based on the results in Fig. 4. Since this result is not derived from the slope of the line in the linear range of the curve this rate must be considered a lower estimate.

5.2 Recent Advances in Vaccinia Virus Recombination Studies

5.2.1 Virus/Virus Recombination

In order to investigate the pathway of viral DNA recombination in vaccinia-infected cells, BALL (1987) constructed a chimeric virus that possesses the vaccinia tk gene sandwiched between tandemly arranged identical bacteriophage lambda DNA sequences. He employed this virus to investigate both intragenomic and intergenomic DNA recombination in virus-infected cells by measuring the retention of the tk gene and the structure of the viral genome in virus propagated under selective or nonselective conditions. He found that the chimeric TK^+ virus contained a mixture of TK^+ and TK^- virus which could be identified by replication of the virus under TK^+ or TK^- selective conditions. Virus passaged under TK^+ selection possessed multiple tk genes arranged in tandem which must have been derived from intermolecular DNA recombination events between parental TK^+ virus. In addition, when virus was propagated for several passages under nonselective conditions, the tk gene was rapidly lost. In order to determine if intramolecular DNA recombination could account for at least a part of this loss, a circular DNA product was sought and found. This product of intramolecular DNA recombination appeared within 3.5 h after infection, concomitant with the initiation of DNA replication, and its appearance was inhibited by actinomycin D, cycloheximide, and HU, demonstrating that RNA and protein synthesis and DNA replication are required. With the hope of identifying viruses that exhibited impaired DNA recombination, BALL analyzed the genomes of viruses which maintained tk activity after 13 rounds of passage in the absence of selection. No TK^+ viruses that possessed an altered DNA recombination phenotype were found. It may be that recombination functions are essential for virus replication and the selection protocol will have to be modified to select for conditional lethal mutations which exhibit an altered recombination phenotype.

5.2.2 Plasmid/Virus Recombination

The ability of homologous plasmid DNA sequences to recombine with viral DNA is the basis for genetic engineering and genetic mapping in vaccinia (NAKANO et al. 1982; MACKETT et al. 1982). Although this process is an essential element in our biochemical and genetic methodology, it is poorly understood at the molecular level.

SPYROPOULOS et al. (1988) investigated the pathway of plasmid insertion into viral DNA by constructing chimeric plasmids containing the *E. coli*

β-galactosidase gene and monitoring incorporation of the plasmid DNA into the viral genome. They observed that recombinant virus could be identified by 12 h post-infection, after DNA replication is well underway, and that recombinant progeny continued to accumulate for up to 48 h, representing about 2.5% of the virus population. Through a comparison of the ability of linear and circular plasmids of different constructions to form TK^+ β-gal$^+$ virus, they concluded that circular DNA could recombine in either a single or double recombination event, while linear DNA required a double recombination event to insert both loci, as expected. In progeny virus derived from a single crossover of the circular plasmid DNA, the tk gene flanking sequences are duplicated. As observed with the tk recombinants described by BALL (1987) and the HU^R mutants described by SLABAUGH et al. (1988), this duplication results in an unstable virus genome that permits both the generation of tandem duplications from intermolecular recombination and loss of the duplicated gene by either intermolecular or intramolecular crossover events.

5.2.3 Plasmid/Plasmid Recombination

In an attempt to develop a system to study the mechanism of the recombination event in cells infected with myxoma virus, Shope fibroma virus (SFV) or vaccinia virus, EVANS et al. (1988) transfected infected cells with two plasmids that differed in the position of a single restriction endonuclease cut site. By identifying recombination products by Southern transfer, they have been able to demonstrate that recombination occurs in each case, although the extent of recombination in vaccinia-infected cells is lower than that observed in SFV-infected cells by fivefold. In SFV-infected cells, recombinants can first be observed at about 8 h post-infection, concomitant with the onset of DNA replication. Furthermore, they found that the addition of PAA decreases but does not eliminate plasmid recombination. This observation suggests that, although DNA replication is required for maximal levels of recombination to be attained, ongoing replication is not essential for plasmid DNA recombination. Perhaps this heterologous recombination system can be exploited to elucidate the components of the viral DNA recombination pathway.

5.3 Summary

DNA recombination in poxvirus-infected cells is an efficient process which exhibits apparent intramolecular recombination frequencies of 0.006% per base pair of the same order of magnitude as is found in bacteriophage systems. Genetic mapping of ts viral mutants is possible, but the generation of a reliable intergenic recombination map is impractical and of little apparent value in light of our ability to precisely map mutations by marker rescue. Furthermore, since there is a marked variability in the observed intergenic recombination frequency measurements and no obvious linear relationship between recombination

frequency and the distance between mutations, intergenic recombination frequency measurements are an unsatisfactory tool in poxvirus recombination studies. Intragenic recombination frequencies, however, display a linear dependence on the distance separating the mutations up to about 700 bp, so that frequency measurements of this sort can be of value. The difference observed between intergenic and intragenic recombination frequencies suggests that genetic complementation occurs in intergenic crosses at the permissive temperature. The role that this apparent complementation plays in increasing the recombination frequency is unclear.

DNA recombination appears to require DNA synthesis in intramolecular viral DNA recombination (BALL 1987); however, in plasmid/plasmid recombination studies, EVANS et al. (1988) have shown that while inhibition of viral DNA polymerase decreases the level of recombination, it does not eliminate it. It is not clear if replication is needed to increase the amount of DNA in the infected cell or if replication intermediates serve also as recombination substrates. It is presumed that vaccinia encodes at least some of the enzymes required for viral DNA recombination, although no viral gene product has yet been shown to participate directly in the recombination event.

Genetic recombination studies in poxvirus-infected cells are in their infancy. With the development of virus and plasmid recombination systems that are easy to analyze we can anticipate rapid progress in identifying the cellular and viral components involved in the recombination pathway.

6 Directed Genetics

From the foregoing discussion it is obvious that the next major step in genetic analysis of poxviruses is the development of techniques for targeted in vitro mutagenesis to conditional lethality of specific essential viral genes. Although some relevant technical progress has been made toward this goal, to date there are no published examples describing in vitro construction of conditionally lethal poxvirus mutants.

Directed mutagenesis in poxviruses can be broken down into two major technical considerations: (1) in vitro mutagenesis of cloned viral DNA fragments and (2) incorporation of mutant genes into the viral genome. Progress in each of these areas will be considered separately below.

6.1 Incorporation of Mutant Genes into the Viral Genome

As described in Sect. 2.6, the poxvirus genome can be altered in a directed fashion by homologous recombination with sequences transfected into infected cells. In most instances, alterations have been carried out on nonessential genes, and

the engineering is often assisted by insertion of a dominant selectable marker (FRANKE et al. 1985; FALKNER and MOSS 1988) or a sequence which provides an efficient screen for recombinant virus (PANICALI and PAOLETTI 1982; CHAKRABARTI et al. 1985; SPYROPOULOS et al. 1988). The primary impediment to constructing mutations in essential genes is that the desired phenotype is lack of growth, and therefore a direct selection for the desired mutants is impossible. Furthermore, in some of the mutagenesis procedures described below, the sequence of the desired mutant virus cannot be predicted, so that a biochemical screen for mutants is also impossible. Thus, any mutagenesis protocol must focus on optimizing either the efficiency of insertion of markers, or the screen for viruses containing mutations, or both.

A procedure has been described which results in insertion of unselected markers into the vaccinia genome with frequencies as high as 30% (KIENY et al. 1984; FATHI et al. 1986). Briefly, the protocol involves infection of cells with a defective helper virus, followed by cotransfection with full length wild type viral DNA and a cloned viral DNA fragment containing the desired mutation. Recent experiments have shown that the efficiency of the protocol is directly proportional to the length of the viral sequence in the cloned viral fragment (CONDIT and TRAKTMAN, unpublished work). This procedure is efficient enough so that if the mutant clone is homogeneous in sequence composition and is known to encode a conditionally lethal mutation, the mutant viruses can be isolated from a cotransfection experiment simply by testing individual plaques for conditional lethality (FATHI et al. 1986).

Alternative efficient insertion protocols can be envisioned which take advantage of insertion of dominant selectable markers. For example, if plasmids containing the *E. coli* gpt gene and a mutant vaccinia gene were recombined into the viral genome, selection for gpt would yield some viruses with a duplication of the viral gene (SPYROPOULOS et al. 1988; FALKNER and MOSS 1988). Subsequent resolution of the duplications would yield some viruses containing only the mutant gene. This strategy has been applied successfully in yeast (SHORTLE et al. 1984).

6.2 In Vitro Mutagenesis

There are three kinds of conditional lethality which can be considered for devising an in vitro mutagenesis protocol: (1) host range, (2) suppressible nonsense mutation, and (3) temperature sensitivity. The protocols for creating these mutations are quite different from each other; therefore, the pros and cons of each are best discussed separately.

6.2.1 Host Range

In other viral systems, considerable success has been achieved in creating host range virus mutants using in vitro techniques (GOLDSTEIN and WELLER 1988;

CARMICHAEL et al. 1988). In general, the protocol involves construction of a permissive host cell line which expresses an essential viral gene and inactivation of the corresponding gene in the virus. The primary disadvantage of the protocol is that a different permissive cell line must be engineered for each conditionally lethal virus mutant to be created. In the case of poxviruses, there are now numerous examples of inactivation of virus genes using in vitro techniques, so that this would not be an impediment to construction of host range mutants; however, since vaccinia genes have evolved for expression and function in a cytoplasmic environment, it seems likely that in many cases efficient expression of vaccinia genes in the cell nucleus would be compromised by the presence of inappropriate signals affecting mRNA synthesis, processing, and transport. There are currently no published examples of correct expression of a poxvirus gene in a nuclear environment. Given the precedent that several procaryotic genes have been successfully expressed in the eucaryotic cell nucleus, it is probably best to approach construction of vaccinia host range mutants on a gene by gene basis.

6.2.2 Nonsense Suppression

Nonsense suppression is in fact a specific type of host range that has two important theoretical advantages for in vitro mutagenesis. First, a single nonsense suppressing cell line could theoretically be used for suppression of nonsense mutations in a wide variety of viral genes. Second, the precise nucleotide change to conditional lethality in a virus gene can be predicted based solely on DNA sequence, even when nothing else is known about the structure, synthesis, or function of the gene product. The primary impediment to the development of nonsense suppressible mutants in animal viruses has been the lack of a suitable nonsense suppressing host cell line. The recent results of SEDIVY et al. (1987) strongly suggest that nonsense suppressing tRNAs are toxic to mammalian cells. These authors were able to circumvent this problem by constructing a cell line which is capable of inducible synthesis of a suppressing rRNA, and they were successful in constructing a poliovirus nonsense mutant which would plaque only on induced nonsense suppressing cells. So far, attempts to adapt this system to use with poxviruses have been unsuccessful. Although suppression of an amber mutant β-galactosidase during infection with a recombinant vaccinia virus can be demonstrated, and although viral genomes have been constructed which contain nonsense mutations in either the viral DNA polymerase or the large subunit of the viral mRNA capping enzyme, plaque formation on induced suppressing cells by vaccinia mutants bearing nonsense mutations in essential virus genes has not been demonstrated (CONDIT, unpublished work). The probability that vaccinia amber mutants cannot form plaques under controlled conditions in which polio amber mutants will form plaques suggests that some feature of vaccinia biology destroys suppression during infection in this system. The potential advantages of nonsense suppression justify further investigation of this system.

6.2.3 Temperature Sensitivity

Like nonsense suppression, temperature sensitivity has the advantage that a single set of technical protocols could theoretically be used to create conditionally lethal mutants in a wide variety of essential virus genes. The primary problem with trying to create ts mutations in vitro is that our ability to predict from sequence what type of mutational change will cause a ts phenotype is severely limited. Two types of mutagenesis protocols can be envisioned. First, it may be possible to randomly mutagenize cloned viral DNA fragments in vitro (SMITH 1985) in the hope that one of the random assortment of mutations which would be created would lead to temperature sensitivity. A problem with this approach is that the efficiency of insertion of an appropriate mutation is compromised by the fact that the mutagenized gene exists as a heterogeneous mixture of sequence alterations. An alternative protocol for mutation to temperature sensitivity involves in-frame insertion of one or two amino acids into viral genes (BARANY 1985; BERNSTEIN and BALTIMORE 1988; TULLIS et al. 1988). Although laborious, this procedure could be used to create individual mutant clones which are homogeneous in sequence and these could be tested individually for their ability to produce ts virus.

7 Summary and Conclusions

Genetic analysis of orthopoxviruses has contributed substantially to our understanding of the functional organization of the poxvirus genome, and individual mutants provide invaluable tools for future studies of poxvirus biology. Deletion and transposition mutants, localized primarily in the termini of the genome, may be particularly useful for studying virus host range and pathogenicity. Numerous drug resistant and dependent mutants provide keys to understanding a wide variety of virus genes. A large number of well-characterized ts mutants, clustered in the center of the virus genome, are taking on an increasingly important role in research on the function of essential poxvirus genes.

Genetic characterization of orthopoxviruses has progressed rapidly during the past decade, and one can reasonably anticipate a time when mutants will be available for the study of any poxvirus gene. Considerable progress toward this goal can be achieved through organized attempts to integrate and further characterize existing mutant collections and through the continued isolation and characterization of deletion, drug resistant, and ts mutants using established techniques. The most exciting possibility is that soon techniques will be available for directed mutagenesis to conditional lethality of any essential poxvirus gene.

References

Archard LC, Mackett M, Barnes DE, Dumbell KR (1984) The genome structure of cowpox virus white pock variants. J Gen Virol 65: 875–886

Bajszar G, Wittek R, Weir JP, Moss B (1983) Vaccinia virus thymidine kinase and neighboring genes: mRNAs and polypeptides of wild-type virus and putative nonsense mutants. J Virol 45: 62–72

Baldick JCJ, Moss B (1987) Resistance of vaccinia virus to rifampicin conferred by a single nucleotide substitution near the predicted NH2 terminus of a gene encoding an Mr 62,000 polypeptide. Virology 156: 138–145

Ball LA (1977) High-frequency homologous recombination in vaccinia virus DNA. J Virol 61: 1788–1795

Barany F (1985) Two-codon insertion mutagenesis of plasmid genes by using single-stranded hexameric oligonucleotides. Proc Natl Acad Sci USA 82: 4202–4206

Basilico C, Joklik W (1968) Studies on a temperature-sensitive mutant of vaccinia virus strain WR. Virology 36: 668–677

Bauer DJ (1955) The antiviral and synergic actions of isatin thiosemicarbazones and certain phenoxypyrimidines in vaccinia infection in mice. Br J Exp Pathol 36: 105–110

Belle Isle H, Venkatesan S, Moss B (1981) Cell-free translation of early and late mRNAs selected by hybridization to cloned DNA fragments derived from the left 14 million to 72 million daltons of the vaccinia virus. Virology 112: 306–317

Bernstein HD, Baltimore D (1988) Poliovirus mutant that contains a cold-sensitive defect in viral RNA synthesis. J Virol 62: 2922–2928

Broyles SS, Moss B (1987) Identification of the vaccinia virus gene encoding nucleoside triphosphohydrolase I, a DNA-dependent ATPase. J Virol 61: 1738–1742

Buller RML, Smith GL, Cremer K, Notkins AL, Moss B (1985) Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. Nature 317: 813–815

Carmichael EP, Kosovsky MJ, Weller SK (1988) Isolation and characterization of herpes simples virus type 1 host range mutants defective in viral DNA synthesis. J Virol 62: 91–99

Chakrabarti S, Brechling K, Moss B (1985) Vaccinia virus expression vector: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol Cell Biol 5: 3403–3409

Challberg MD, Englund PT (1979) Purification and properties of the deoxyribonucleic acid polymerase induced by vaccinia virus. J Biol Chem 254: 7812–7819

Chernos VI, Belanov EF, Vasilieva NN (1978) Temperature sensitive mutants of vaccinia virus I: isolation and preliminary characterization. Acta Virol 22: 81–90

Chernos VI, Chelyapov NV, Antonova TP, Vasiljeva NN, Mitina IV (1983) Biochemical and genetic characterization of vaccinia virus temperature-sensitive mutants with DNA- and DNAf-phenotypes. Arch Virol 77: 209–221

Chernos VI, Antonova TP, Senkevich TG (1985) Recombinants between vaccinia and ectromelia viruses bearing the specific pathogenecity markers of both parents. J Gen Virol 66: 621–626

Cohrs RJ, Condit RC, Pacha RF, Thompson CL, Sharma OK (1989) Modulation of 2-5A dependent RNAse by a temperature sensitive mutant of vaccinia virus. J Virol 63: 948–951

Comer MM (1977) Correlation between genetic and nucleotide distances in the bacteriophage T4 transfer RNA gene. J Mol Biol 113: 267–271

Condit RC, Motyczka A (1981) Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. Virology 113: 224–241

Condit RC, Motyczka A, Spizz G (1983) Isolation, characterization, and physical mapping of temperature-sensitive mutants of vaccinia virus. Virology 128: 429–443

Cooper J, Moss B, Katz E (1979) Inhibition of vaccinia virus protein synthesis by isatin-B-thiosemicarbazone: characterization and in vitro translation of viral mRNA. Virology 96: 381–392

Cozzarelli NR (1977) The mechanism of action of inhibitors of DNA synthesis. Annu Rev Biochem 46: 641–668

Dales S, Milovanivitch V, Pogo B, Weintraub S, Huima T, Wilton S, McFadden G (1978) Biogenesis of vaccinia: isolation of conditional lethal mutants and electron microscopic characterization of their phenotypically expressed defects. Virology 84: 403–428

DeFilippes FM (1982) Restriction enzyme mapping of vaccinia virus DNA. J Virol 43: 136–149

DeFilippes FM (1984) Effect of aphidicolin on vaccinia virus: isolation of an aphidicolin-resistant mutant. J Virol 52: 474–482
DeLange AM (1989) Identification of temperature-sensitive mutants of vaccinia virus that are defective in conversion of concatemeric replicative intermediates to the mature linear DNA genome. J Virol 63: 2437–2444
Downie AM, Dunbell KR (1956) Poxviruses. Ann Rev Microbiol 10: 237–253
Drillien R, Spehner D (1983) Physical mapping of vaccinia virus temperature-sensitive mutations. Virology 131: 385–393
Drillien R, Koehren F, Kirn A (1981) Host range deletion mutant of vaccinia virus defective in human cells. Virology 111: 488–499
Drillien R, Spehner D, Kirn A (1982) Complementation and genetic linkage between vaccinia virus temperature-sensitive mutants. Virology 119: 372–381
Dubbs DR, Kit S (1984) Isolation and properties of vaccinia mutants deficient in thymidine kinase-inducing activity. Virology 22: 214–225
Dumbell KR, Archard LC (1980) Comparison of white pock (h) mutants of monkeypox virus with parental monkeypox and variola-like viruses isolated from animals. Nature 286: 29–32
Earl P, Moss B (1987) Vaccinia virus. In: O'Brien 55 (ed) Genetic maps. Cold Spring Harbor, New York, pp 116–123
Earl PL, Jones EV, Moss B (1986) Homology between DNA polymerases of poxviruses, and adenoviruses: nucleotide sequence of the vaccinia virus DNA polymerase gene. Proc Natl Acad Sci USA 83: 3659–3663
Easterbrook KB, Davern CI (1963) The effect of 5-bromodeoxyuridine on the multiplication of vaccinia virus. Virology 19: 509–520
Ensinger MJ (1982) Isolation and genetic characterization of temperature-sensitive mutants of vaccinia virus WR. J Virol 43: 778–790
Ensinger MJ (1987) Phenotypic characterization of temperature-sensitive mutants of vaccinia virus with mutations in a 135,000-Mr subunit of the virion-associated DNA-dependent RNA polymerase. J Virol 61: 1842–1850
Ensinger MJ, Rovinsky M (1983) Marker rescue of temperature-sensitive mutations of vaccinia WR: correlation of genetic and physical maps. J Virol 48: 419–428
Ensinger MJ, Weir JP, Moss B (1985) Fine structure marker rescue of temperature-sensitive mutations of vaccinia virus within a central conserved region of the genome. J Virol 56: 1027–1029
Esposito JJ, Knight JC (1985) Orthopoxvirus DNA: A comparison of restriction profiles and maps. Virology 143: 230–251
Esposito JJ, Cabradilla CC, Nakano JH, Obijeski JF (1981) Intragenomic sequence transposition in monkeypox virus. Virology 109: 231–243
Essani K, Dales S (1983) Biogenesis of vaccinia: Analysis by three factor crosses reveals mutual influence on stability of drug resistance and temperature sensitivity when both markers occur in some recombinant virus isolates. Intervirology 20: 101–107
Essani K, Dugre R, Dales S (1982) Biogenesis of vaccinia: Involvement of spicules of the envelope during virion assembly examined by means of conditional lethal mutants and serology. Virology 118: 279–282
Evans E, Traktman P (1987) Molecular genetic analysis of a vaccinia virus gene with an essential role in DNA replication. J Virol 61: 3152–3162
Evans DH, Stuart D, McFadden G (1988) High levels of genetic recombination among cotransfected plasmid DNAs in poxvirus-infected mammalian cells. J Virol 62: 367–375
Falkner FG, Moss B (1988) *Escherichia coli* gpt gene provides dominant selection for vaccinia virus open reading frame expression vectors. J Virol 62: 1849–1854
Fathi Z, Sridhar P, Pacha RF, Condit RC (1986) Efficient targeted insertion of an unselected marker into the vaccinia virus genome. Virology 155: 97–105
Fenner F (1958) The biological characters of several strains of vaccinia, cowpox and rabbitpox viruses. Virology 5: 502–529
Fenner F, Comben BM (1958) Genetic studies with mammalian poxviruses: 1. Demonstration of recombination between two strains of vaccinia virus. Virology 5: 530–548
Fenner F, Sambrook J (1966) Conditional lethal mutants of rabbitpox virus II. Mutants (p) that fail to multiply in PK-2a cells. Virology 28: 600–609
Fields BN, Joklik WK (1969) Isolation and preliminary genetic and biochemical characterization of temperature-sensitive mutants of reovirus. Virology 37: 335–342
Follet EAC, Pennington TH (1973) The mode of action of rifamycins and related compounds on poxvirus. Adv Virus Res 18:105–142

Franke CA, Rice CM, Strauss JH, Hruby DE (1985) Neomycin resistance as a dominant selectable marker for selection and isolation of vaccinia virus recombinants. Mol Cell Biol 5: 1918–1924
Gemmell A, Cairns J (1959) Linkage in the genome of an animal virus. Virology 8: 381–383
Goldstein DJ, Weller SK (1988) An ICP6::lacZ insertional mutagen used to demonstrate that the UL52 gene of herpes simplex virus type 1 is required for virus growth and DNA synthesis. J Virol 62: 2970–2977
Gussin GN, Rosen ED, Wulff DL (1980) Mapability of very close markers of bacteriophage lambda. Genetics 86: 1–24
Hooda-Dhingra U, Thompson CL, Condit RC (1989) Detailed phenotypic characterization of five temperature sensitive mutants in the 22 kilodalton and the 147 kilodalton subunits of the vaccinia virus DNA-dependent RNA polymerase. J Virol 63: 714–729
Hruby DE, Ball LA (1982) Mapping and identification of the vaccinia virus thymidine kinase gene. J Virol 43: 403–409
Hruby DE, Maki RA, Miller DB, Ball LA (1983) Fine structure analysis and nucleotide sequence of the vaccinia virus thymidine kinase gene. Proc Natl Acad Sci USA 80: 3411–3415
Jones EV, Moss B (1984) Mapping of the vaccinia virus DNA polymerase gene by marker rescue and cell-free translation of selected RNA. J Virol 49: 72–77
Katz E, Margalith E, Winer B, Lazar A (1973) Characterization and mixed infection of three strains of vaccinia virus: wildtype, IBT-resistant and IBT-dependent mutants. J Gen Virol 21: 469–475
Kieny MP, Lathe R, Drillien R, Spehner D, Skory S, Schmitt D, Wiktor T, Koprowski H, Lecocq JP (1984) Expression of rabies virus glycoprotein from a recominant vaccinia virus. Nature 312: 163–166
Lake JR, Cooper PD (1980) Deletions of the terminal sequences in the genomes of white pock (u) and host-restricted (p) mutants of rabbitpox virus. J Gen Virol 48: 135–147
Lake JR, Silver M, Dales S (1979) Biogenesis of vaccinia: complementation and recombination analysis of one group of conditional-lethal mutants defective in envelope self-assembly. Virology 96: 9–20
Lee-Chen GJ, Niles EG (1988a) Transcription and translation map of the thirteen genes in the vaccinia virus *Hin*dIII D fragment. Virology 163: 52–63
Lee-Chen GJ, Niles EG (1988b) Map positions of the 5′ ends of eight mRNAs synthesized from the late genes in the vaccinia virus *Hin*dIII D fragment. Virology 163: 80–92
Lee-Chen GJ, Bourgeois N, Davidson K, Condit RC, Niles EG (1988) Structure of the transcription initiation and termination sequences of seven early genes in the vaccinia virus *Hin*dIII D fragment. Virology 163: 64–79
Mackett M, Archard LC (1979) Conservation and variation in orthopoxvirus genome structure. J Gen Virol 45: 683–701
Mackett M, Smith GL (1986) Vaccinia virus expression vectors. J Gen Virol 67: 2067–2082
Mackett M, Smith GL, Moss B (1982) Vaccinia virus: a selectable eukaryotic cloning and expression vector. Proc Natl Acad Sci USA 79: 7415–7419
McFadden G, Dales S (1980) Biogenesis of vaccinia: preliminary characterization of conditional lethal mutants of vaccinia virus defective in DNA synthesis. Virology 103: 68–79
McFadden G, Essani K, Dales S (1980) A new endonuclease restriction site which is at the locus of a temperature-sensitive mutation in vaccinia virus is associated with true and pseudoreversion. Virology 101: 277–280
Merchlinsky M, Moss B (1989) Resolution of vaccinia virus DNA concatemer junctions requires late gene expression. J Virol 63: 1595–1603
Miner JN, Hruby DE (1989) Rifamipicin prevents virosome localization of L65, an essential vaccinia virus polypeptide. Virology 170: 227–237
Morgan JR, Cohen LK, Roberts BE (1984) Identification of the DNA sequences encoding the large subunit of the mRNA-capping enzyme of vaccinia virus. J Virol 52: 206–214
Moss B (1985) "Replication of poxviruses. In: Fields BN, Karpi DM, Chanock RM, Melnick JL, Roizman B, Shope RE (eds) Virology. Raven, New York, pp 685–704
Moss B, Cooper N (1982) Genetic evidence for vaccinia virus-encoded DNA polymerase: isolation of a phosphonoacetate-resistant enzyme from the cytoplasm of infected cells. J Virol 43: 673–678
Moss B, Flexner C (1987) Vaccinia virus expression vectors. Ann Rev Immunol 51: 305–324
Moss B, Winters E, Cooper JA (1981) Deletion of a 9,000-base-pair segment of the vaccinia virus genome that encodes nonessential polypeptides. J Virol 40: 387–395
Moyer RW (1987) The role of the host cell nucleus in vaccinia virus morphogenesis. Virus Res 8: 173–191

Moyer RW, Graves RL (1981) Mechanism of cytoplasmic orthopoxvirus DNA replication. Cell 27: 391–401

Moyer RW, Rothe CT (1980) The white pock mutants of rabbit poxvirus I Spontaneous host range mutants contain deletions. Virology 102: 119–132

Moyer RW, Graves RL, Rothe CT (1982) The white pock (u) mutants of rabbit poxvirus. III Terminal DNA sequence duplication and transposition in rabbit poxvirus. Cell 22: 545–553

Nakano E, Panicali D, Paoletti E (1982) Molecular genetics of vaccinia virus: demonstration of maker rescue. Proc Natl Acad Sci USA 79: 1593–1596

Niles EG, Seto J (1988) Vaccinia virus gene D8 encoded a trans-membrane protein which is not essential for virus replication in culture. J Virol 62: 3772–3778

Niles EG, Condit RC, Caro P, Davidson K, Matusick L, Seto J (1986) Nucleotide sequence and genetic map of the 16-kb vaccinia virus *Hin*dIII D fragment. Virology 153: 96–112

Pacha R (1986) Biochemical and genetic characterization of a temperature-sensitive mutant of vaccinia virus with an abortive late phenotype. PhD thesis

Pacha RF, Condit RC (1985) Characterization of a temperature-sensitive mutant of vaccinia virus reveals a novel function that prevents virus-induced breakdown of RNA. J Virol 56: 395–403

Padgett BL, Tomkins JKV (1968) Conditional lethal mutants of rabbitpox virus III. Temperature sensitive (ts) mutants: physiological properties, complementation, and recombination. Virology 36: 161–167

Panicali D, Paoletti E (1982) Construction of poxviruses as cloning vectors: Insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc Natl Acad Sci USA 79: 4927–4931

Parker RF, Bronson LH, Green RH (1941) Further studies of the infectious unit of vaccinia. J Exp Med 74: 263–281

Pennington TH (1976) Effect of 5-bromodeoxyuridine on vaccinia virus-induced polypeptide synthesis: selective inhibition of the synthesis of some post-replicative polypeptides. J Virol 18: 1131–1133

Pennington TH (1977) Isatin-β-thiosemicarbazone causes premature cessation of vaccinia virus-induced late post-replicative polypeptide synthesis. J Gen Virol 35: 567–571

Pickup DJ, Ink BS, Parsons BL, Hu W, Joklik WK (1984) Spontaneous deletions and duplications of sequences in the genome of cowpox virus. Proc Natl Acad Sci USA 81: 6817–6821

Pugh CSG, Borchardt RT, Stone HO (1978) Sinefungin, a potent inhibitor of virion mRNA(guanine-7-)-methyltransferase, mRNA(nucleoside-2′-)-methyltransferase and viral multiplication. J Biol Chem 253: 4075–4077

Raczynski P, Condit RC (1983) Specific inhibition of vaccinia virus growth by 2′-O-methyladenosine: isolation of a drug-resistant virus mutant. Virology 128: 458–462

Ravin VK, Artemiev MI (1974) Fine structure of the lysozyme gene of bacteriophage T4B. Mol Gen Genetics 128: 359–365

Reichard P, Ehrenberg A (1983) Ribonucleotide reductase—a radical enzyme. Science 221: 514–519

Rodriguez JF, Kahn JS, Esteban M (1986) Molecular cloning, encoding sequence, and expression of vaccinia virus nucleic acid-dependent nucleoside triphosphatase gene. Proc Natl Acad Sci USA 83: 9566–9570

Roseman NA, Hruby DE (1987) Nucleotide sequence and transcript organization of a region of the vaccinia virus genome which encodes a constitutively expressed gene required for DNA replication. J Virol 61: 1398–1406

Sambrook JF, Padgett BL, Tomkins JKN (1966) Conditional lethal mutants of rabbitpox virus I Isolation of host cell-dependent and temperature-dependent mutants. Virology 28: 592–599

Schmitt JFC, Stunnenberg HG (1988) Sequence and transcriptional analysis of the vaccinia virus *Hin*dIII I fragment. J Virol 62: 1889–1897

Schumperli D, McFadden G, Wyler R, Dales S (1990) Location of a new endonuclease restriction site associated with a temperature-sensitive mutation of vaccinia virus. Virology 101: 281–285

Sedivy JM, Capone JP, RajBhandary UL, Sharp PA (1987) An inducible mammalian amber suppressor: propagation of a poliovirus mutant. Cell 50: 825–833

Seto J, Celenza LM, Condit RC, Niles EG (1987) Genetic map of the vaccinia virus *Hin*dIII D fragment. Virology 160: 110–119

Shortle D, Novick P, Botstein D (1984) Construction and genetic characterization of temperature-sensitive mutant alleles of the yeast actin gene. Proc Natl Acad Sci USA 81: 4889–4893

Silver M, Dales S (1982) Biogenesis of vaccinia: interrelationship between post-translational cleavage, virus assembly, and muturation. Virology 117: 341–356

Slabaugh MB, Mathews CK (1984) Vaccinia virus-induced ribonucleotide reductase can be distinguished from host cell activity. J Virol 52: 501–506
Slabaugh MB, Johnson TL, Matthews CK (1984) Vaccinia virus induces ribonucleotide reductase in primate cells. J Virol 52: 507–514
Slabaugh M, Roseman RD, Christopher M (1988) Vaccinia virus-encoded ribonucleotide reductase: sequence conservation of the gene for the small subunit and its amplification in hydroxyurea-resistant mutants. J Virol 62: 519–527
Smith M (1985) In vitro mutagenesis. Ann Rev Genet 19: 423–462
Spyropoulos DD, Roberts BE, Panicali D, Cohen LK (1988) Delineation of the viral products of recombination in vaccinia virus-infected cells. J Virol 62: 1046–1054
Sridhar P, Condit RC (1983) Selection for temperature-sensitive mutations in specific vaccinia virus genes: isolation and characterization of a virus mutant which encodes a phosphonoacetic acid-resistant, temperature-sensitive DNA polymerase. Virology 128: 444–457
Stern W, Pogo BGT, Dales S (1977) Biogenesis of poxviruses: analysis of the morphogenetic sequence using a conditional lethal mutant defective in envelope self assembly. Proc Natl Acad Sci USA 74: 2162–2166
Tamin A, Villarreal EC, Weinrich SL, Hruby DE (1988) Nucleotide sequence and molecular genetic analysis of the vaccinia virus N/M region encoding the genes responsible for resistance to α-amanitin. Virology 165: 141–150
Tartaglia J, Paoletti E (1985) Physical mapping and DNA sequence analysis of the rifampicin resistance locus in vaccinia virus. Virology 147: 394–404
Tartaglia J, Piccini A, Paoletti E (1986) Vaccinia virus rifampicin-resistance locus specifies a late 63,000 Da gene product. Virology 150: 45–54
Tengelsen LA, Slabaugh MB, Bibler JK, Hruby DE (1988) Nucleotide sequence and molecular genetic analysis of the large subunit of ribonucleotide reductase encoded by vaccinia virus. Virology 164: 121–131
Thompson CL, Condit RC (1986) Marker rescue mapping of vaccinia virus temperature-sensitive mutants using overlapping cosmid clones representing the entire virus genome. Virology 150: 10–20
Thompson CL, Hooda-Dhingra U, Condit RC (1989) Fine structure mapping of five temperature sensitive mutations in the 22 kilodalton and the 147 kilodalton subunits of the vaccinia virus DNA-dependent RNA polymerase. J Virol 63: 705–713
Traktman P, Kelvin M, Pacheco S (1989) Molecular genetic analysis of vaccinia virus DNA polymerase mutants. J Virol 63: 841–846
Traktman P, Sridhar P, Condit RC, Roberts BE (1984) Transcriptional mapping of the DNA polymerase gene of vaccinia virus. J Virol 49: 125–131
Tullis GE, Labieniec-Pintel L, Clemens KE, Pintel D (1988) Generation and chararcterization of a temperature-sensitive mutation in the NS-1 gene of the autonomous parvovirus minute virus of mice. J Virol 62: 2736–2744
Villarreal EC, Hruby DE (1986) Mapping and genomic location of the gene encoding α-amanitin resistance in vaccinia virus mutants. J Virol 57: 65–70
Villarreal EC, Roseman NA, Hurby DE (1984) Isolation of vaccinia virus mutants capable of replicating independently of the host cell nucleus. J Virol 51: 359–366
Weinrich SL, Hruby DE (1986) A tandemly-oriented late gene cluster within the vaccinia virus genome. Nucleic Acids Res 14: 3003–3016
Weinrich SL, Niles EG, Hruby DE (1985) Transcriptional and translational analysis of the vaccinia virus late gene L65. J Virol 55: 450–457
Weir JP, Moss B (1983) Nucleotide sequence of the vaccinia virus thymidine kinase gene and the nature of spontaneous frameshift mutations. J Virol 46: 530–537
Weir JP, Bajszar G, Moss B (1982) Mapping of the vaccinia virus thymidine kinase gene by marker rescue and by cell-free translation of selected mRNA. Proc Natl Acad Sci USA 79: 1210–1214
Woodson B, Joklik WK (1965) The inhibition of vaccinia virus multiplication by isatin-β-thiosemicarbazone. Proc Natl Acad Sci USA 54: 946–953

Regulation of Orthopoxvirus Gene Expression

B. Moss

1 Introduction

Poxviruses differ from the other major groups of DNA viruses with regard to their use of the cytoplasm, rather than the nucleus, as the site of transcription. Consistent with this strategy, poxviruses encode their own DNA-dependent

Laboratory of Viral Diseases, Building 4, Room 229, National Institutes of Health, Bethesda, MD 20892 USA

Current Topics in Microbiology and Immunology, Vol. 163

RNA polymerase as well as many—if not all—of the additional enzymes and factors needed to synthesize functional capped, methylated, and polyadenylated mRNA. Furthermore, all of the factors necessary for transcription of the early class of genes are packaged within the virus particle allowing viral mRNA to form within minutes after infection. A cascade of events follows involving DNA replication and the synthesis of an assortment of trans-acting factors and leading to the expression of successive classes of viral genes.

Poxviruses provide an excellent opportunity to combine biochemical and genetic approaches for the investigation of transcriptional and posttranscriptional regulation. Determining the similarities and differences between the mechanisms used by poxviruses and their hosts is an intriguing aspect of this research. In addition, the extensive use of poxviruses as expression vectors demands a thorough understanding of their molecular biology. The purpose of this chapter is to summarize and interpret current information regarding the regulation of orthopoxvirus gene expression.

2 Early Gene Expression

2.1 The Virus Particle

The virus particle is an obvious starting point for a discussion of poxvirus transcription. In addition to providing a mechanism for virus spread, the virion contains a virtual factory for the synthesis of early mRNA. The genome and all of the enzymes involved in transcription are located within the core. Structures with unknown function, called lateral bodies, fit into concavities of the core and the entire particle is enveloped by a lipoprotein bilayer. Orthopoxvirus genomes consist of a linear duplex DNA molecule of about 200 000 bp with hairpin loops at each end (Baroudy et al. 1982; Geshelin and Berns 1974). The DNA is largely composed of unique sequences, although there is a 10 kbp inverted terminal repetition (Garon et al. 1978; Wittek et al. 1978) within which are blocks of short tandem repeats and several genes (Wittek et al. 1980; Wittek and Moss 1980). Most essential genes map within the highly conserved central region of the genome, whereas many of the genes that are dispensable for replication in tissue culture are nearer the ends. The apparent absence of introns, the short promoter sequences (to be discussed), and the relatively small sizes of many open reading frames (ORFs) account for the packing of an estimated 150–200 genes into the orthopoxvirus genome. A more exact count will have to await completion of DNA sequencing. Both strands of the DNA are transcribed, yet extensive overlap of ORFs is uncommon. ORFs frequently occur in head to tail tandem arrays with some clusters containing predominantly early genes and others late genes (Earl and Moss 1989).

The virion is composed of a large number of polypeptides; more than 100 have been resolved by two-dimensional polyacrylamide gel electrophoresis (ESSANI and DALES 1979). Although there are more than a dozen known enzymes within the core, they account for only a minor portion of the total protein. Four polypeptides (74 kDa, 62 kDa, 25 kDa, and 11 kDa) which may have structural roles account for about 70% of the mass of the core (BERTHOLET et al. 1985; ROSEL and MOSS 1985; VAN MEIR and WITTEK 1988; WEIR and MOSS 1985). There are no histones, but several viral proteins are strongly bound to the DNA and apparently help to maintain it in a folded supercoiled state (ICHIHASHI et al. 1984; SOLOSKI and HOLOWCZAK 1981). The 11 kDa and 25 kDa proteins have strong affinity for DNA (KAO et al. 1981; YANG and BAUER 1988).

Activation of the transcriptional machinery accompanies or closely follows virus entry into the cytoplasm (KATES and MCAUSLAN 1967a; MUNYON and KIT 1966). The penetration process involves fusion of viral and cellular membranes (CHANG and METZ 1976; DALES and KAJIOKA 1964). Concomitantly, release of phospholipid and about half of the virion protein occurs in a process known as the first stage of uncoating (JOKLIK 1964b). This step is not blocked by inhibitors of either RNA or protein synthesis and results in the formation of structures resembling cores (HOLOWCZAK 1972; SAROV and JOKLIK 1972). A second stage of uncoating, measured by the susceptibility of the genome to DNase (not necessarily synonymous with release of naked DNA from the core structure), begins after a lag period of 0.5–2 h, depending on the multiplicity of infection and requires an additional 1–2 h for 50% of the DNA to become DNase sensitive. The second stage of uncoating never goes to completion and, unlike the first, is dependent on RNA and protein synthesis and is prevented by ultraviolet irradiation of the virus (JOKLIK 1964a).

Transcription is not prevented by inhibitors of protein or DNA synthesis; on the contrary, viral RNA formation is enhanced and prolonged when second stage uncoating of the core is prevented by protein synthesis inhibitors suggesting that transcription actually occurs within the latter structure (WOODSON 1967). Sequestration of the RNA polymerase and early transcription factors within the core could explain the very low efficiency of transcription of plasmids, containing early promoters, that are transfected into cells (COCHRAN et al. 1985a). In the presence of DNA replication inhibitors, the initial rate of RNA synthesis is proportional to the number of virus particles in the inoculum, but uncoating does occur and under these conditions early RNA synthesis gradually decreases. Therefore, the activation and decline of early transcription may correspond to the first and second stages of uncoating, respectively.

2.2 Early mRNA

Early viral mRNAs, like those of their eukaryotic hosts, are capped (BOONE and MOSS 1977) and polyadenylated (KATES and BEESON 1970a). The cap structure is required for binding of vaccinia virus mRNA to ribosomes (MUTHUKRISHNAN

et al. 1978) and probably for mRNA stability, whereas the function of the 3′ poly(A) tail is still poorly understood. The early viral mRNAs are of discrete length (COOPER et al. 1981a; WITTEK et al. 1980) and evidence against functional polycistronic precursors was provided by ultraviolet target size determinations in vivo (COOPER et al. 1981b) and in vitro (see below). In addition, there is no evidence for RNA splicing. Early mRNAs generally have short untranslated leaders since DNA sequences corresponding to the start sites, as determined by nuclease digestion of RNA:DNA hybrids and/or by reverse transcriptase extension of DNA primers, are usually located not far upstream of the ORFs (LEE-CHEN et al. 1988; MAHR and ROBERTS 1984a; ROSEL et al. 1986; VENKATESAN et al. 1980; WEIR and MOSS 1983). Some early mRNAs that are transcribed from genes that have a TAAAT sequence at the RNA start site have a variable length 5′ poly(A) leader that is probably generated by RNA polymerase slippage (to be discussed later).

Quantitative RNA:DNA hybridization analysis indicated that about one-half of the vaccinia virus genome is transcribed prior to DNA replication and that there is a wide variation in the abundance of individual early RNAs (BOONE and MOSS 1978; KAVERIN et al. 1975; ODA and JOKLIK 1967; PAOLETTI and GRADY 1977). There is some evidence for a second or delayed class of early genes that is expressed only after viral protein synthesis has occurred (KATES and MCAUSLAN 1967a). No support for such a class, however, has come from recent transcriptional studies. The stability of early mRNA has been measured following the addition of actinomycin D to block further transcription; half-life values of several hours were obtained (ODA and JOKLIK 1967; SEBRING and SALZMAN 1967).

2.3 Early Promoters

Nucleotide sequence analysis indicated that the DNA segments preceding the ORFs of early genes have a high percentage of A and T residues. The promoter function of such DNA segments was demonstrated by fusing them to a reporter gene encoding an easily assayable product. The chimeric DNA was then recombined into the vaccinia genome and expression of the reporter was determined (MACKETT et al. 1982). Further studies with recombinant vaccinia viruses indicated that the promoters of early genes extend only about 30 bp upstream of the RNA start sites (COCHRAN et al. 1985b; COUPAR et al. 1987; MARS and BEAUD 1987; VASSEF 1987; VASSEF et al. 1985; WEIR and MOSS 1987a). Thus far, no evidence for specific regulatory sequences further upstream has been reported.

One early promoter has been subjected to a detailed mutational analysis that included every possible single nucleotide substitution and many multiple substitutions (DAVISON and MOSS 1989a). The effects of these mutations on promoter function were measured by expression of the β-galactosidase reporter gene and by transcriptional analyses. The results indicated the presence of a critical region, located between nucleotides -13 to -28 (relative to the RNA

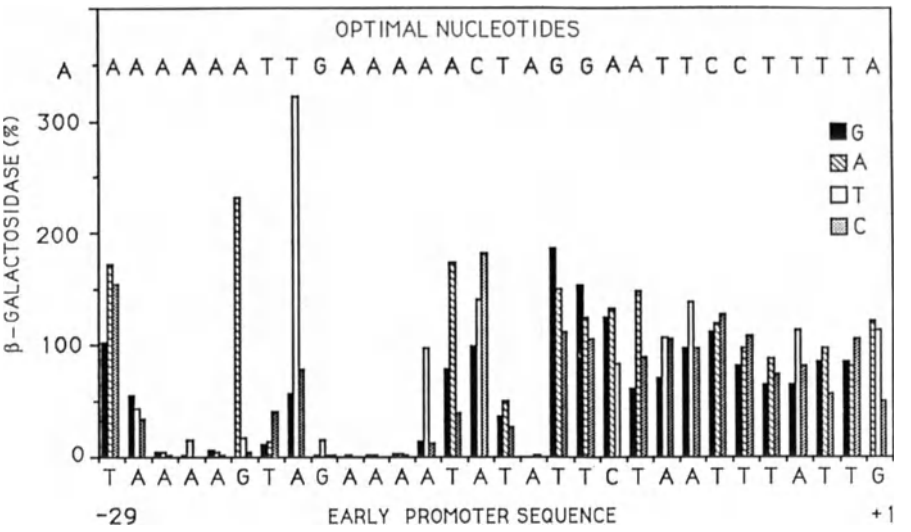


Fig. 1. Effect of single nucleotide substitutions on the activity of an early promoter. A series of recombinant vaccinia viruses that contain the early promoter of the 7.5 kDa gene, or derivatives with single nucleotide substitutions, regulating expression of β-galactosidase were used to infect cells in the presence of cytosine arabinoside, an inhibitor of DNA replication (DAVISON and MOSS 1989a). The activities obtained with the mutated promoters are given relative to the 100% value obtained with the parental promoter. The nucleotide substitutions found to be optimal at each position are shown near the top

start site at + 1), in which most single nucleotide substitutions have a severe functional effect (Fig. 1). A few substitutions, however, increased expression several-fold. Adenylate residues are essential at several locations within the critical region and optimal at all positions except − 14, − 15, and − 21, − 22, − 23. At − 21 a G residue is needed and Ts are best at − 22 or − 23. An analysis of multiple substitutions within the critical region indicated that most behaved independently, although some of the up mutations shown in Fig. 1 could partially compensate for potentially detrimental nucleotides at other positions (DAVISON and MOSS 1989a).

Like the TATA box of higher eukaryotic RNA polymerase II promoters, the critical region specifies the distance downstream at which transcription initiation occurs. Since vaccinia virus mRNAs start with a purine, pyrimidine substitutions at the site of initiation shift the latter by a few nucleotides upstream or downstream indicating some degree of flexibility of the RNA polymerase (DAVISON and MOSS 1989a). The region of about 11 bp between the critical region and the RNA start site is very tolerant of single nucleotide substitutions (Fig. 1) although the effect of more drastic changes was not evaluated.

The optimal nucleotide at each position of the critical region, determined by mutagenesis, corresponded closely with the predominant one obtained by lining up a large series of early genes. Interestingly, however, most natural promoters do

not have optimal nucleotides in all positions and in some cases single nucleotide substitutions were shown to increase activity tenfold (DAVISON and MOSS 1989a). Variability in promoter strength may provide one way of regulating the level of gene expression.

2.4 The Termination Signal

The rather discrete size of early transcripts suggested the existence of specific transcription termination or RNA 3′ processing mechanisms (VENKATESAN et al. 1981). The sequence AAUAAA, which invariably appears about 20 nucleotides before the polyadenylation site of eukaryotic mRNA (PROUDFOOT and BROWNLEE 1976), is rarely present near the 3′ ends of poxvirus mRNAs. The occurrence of clusters of T residues preceding the 3′ ends of early mRNAs was noted during an analysis of cDNA copies of an early mRNA (YUEN and MOSS 1986). Using an in vitro transcription system, (described below) the signal was identified as TTTTTNT in which N can be the only variable nucleotide (ROHRMANN and MOSS 1985; YUEN and MOSS 1987). Termination occurs approximately 20–50 bp past this T motif. The T motif is present near the ends of most but not all early genes; in the latter situations downstream signals are used and mRNAs with coterminal ends are formed. Termination is less than 100% efficient, accounting for the formation of some extended early RNAs (BAJSZAR et al. 1983). Occasionally, overlapping or tandem TTTTTNT signals occur possibly increasing the extent of termination. Consistent with their role in termination, TTTTTNT sequences are rarely found in early genes at locations other than the ends although there are some exceptions (EARL et al. 1986; LEE-CHEN et al. 1988; PATEL and PICKUP 1989; PLUCIENNICZAK et al. 1985). It remains to be determined whether, in exceptional cases, the T motifs are unrecognized as termination signals because of an unappreciated structural context or whether they cause attenuation of expression and represent still another regulatory mechanism. Since the presence of the T motif within a foreign gene introduced into vaccinia virus leads to premature transcription termination, it is unlikely that any other cis-acting signal is required (EARL et al., 1990). The sequence TTTTTNT also may form part of some mRNA transcription termination signals in eukaryotic organisms (HENIKOFF and COHEN 1984; SATO et al. 1988).

2.5 In Vitro Transcription by Permeabilized Virions

The formation of RNA when orthopoxvirus particles were incubated in vitro with ribonucleoside triphosphates provided the first evidence for a transcription system within a virus (KATES and MCAUSLAN 1967b; MUNYON et al. 1967). The in vitro mRNAs produced in this manner closely resembled those made intracellularly with respect to size, 5′ cap structure, 3′ poly(A), and translatability

(COOPER and MOSS 1978; KATES and BEESON 1970a; PELHAM et al. 1978; WEI and MOSS 1975). Whether synthesized in vitro or in vivo, the 5′ ends of the mRNAs are invariably purines which may be followed by any nucleotide (BOONE and MOSS 1977; KEITH et al. 1980). One minor difference is that mRNA made in vitro by virus cores contains a cap I structure which consists of a terminal 7–methylguanosine connected via a 5′ to 5′ triphosphate bridge to a 2′–O–methylnucleoside, whereas viral RNA isolated from the cytoplasm of infected cells may contain two successive 2′–O–methylated nucleosides to form a cap II structure, and the N^6 position of the penultimate 2′–O–methyladenosine may be methylated to form a dimethylated adenosine residue. These additional modifications apparently are carried out in the cytoplasm by host enzymes.

High ATP concentrations are needed for RNA synthesis by virus particles (KATES and MCAUSLAN 1967b; MUNYON et al. 1967). ATP hydrolysis is necessary for initiation of transcription (GERSHOWITZ et al. 1978) and elongation (SHUMAN et al. 1980). Following a lag of about 1.5 min, RNA synthesis becomes linear; the maximum rate of RNA chain elongation is 17 nucleotides per second and there are at least 40 RNA chain growing points per particle (KATES and BEESON 1970b). The RNA accumulates transiently in the core and is actively extruded by a process that requires high concentrations of ATP with a hydrolyzable β–γ bond (KATES and BEESON 1970b; VEOMETT and KATES 1973). Recent studies confirm that the average time for synthesis and release of mRNAs is about 2.2 min (SHUMAN and MOSS 1989).

Elongated RNAs that are not polyadenylated and which fail to be extruded from the core are produced under certain reaction conditions. These include: high virus concentration and ATP depletion (PAOLETTI 1977b; PAOLETI and LIPINSKAS 1978a), addition of ATP analogs (GERSHOWITZ et al. 1978), and heating of virus (HARPER et al. 1978). Evidence that these long RNAs could be chased into mRNA size molecules in a reaction that required nucleoside triphosphates and that an endoribonuclease extracted from virions cleaved the isolated elongated RNAs into shorter molecules suggested an RNA processing model (GERSHOWITZ et al. 1978; PAOLETTI 1977a; PAOLETTI and LIPINSKAS 1978b). Other data, derived from ultraviolet target size determinations, argued against functional polycistronic mRNA (BOSSART et al. 1978; PELHAM 1977). Evidence that the 5′ ends of mRNAs made by virus cores correspond to start sites rather than sites of cleavage came from incorporation of [β-^{32}P]GTP into the cap structures (VENKATESAN and MOSS 1981). The cap itself is formed by transfer of a GMP moiety from GTP to the 5′ di- or triphosphate end of mRNA (MOSS et al. 1976). More recently, it was shown that BrUTP, which specifically interferes with termination but not initiation or elongation (see below), blocked RNA extrusion from cores and led to the accumulation of long transcripts (SHUMAN and MOSS 1989). Taken together, the data support the conclusion that most or all early mRNAs made by virus particles are formed by termination rather than processing.

Studies with virus cores also suggested that a specific 3′ terminal RNA sequence is not required for polyadenylation since RNAs, prematurely terminated

because of omission of a ribonucleoside triphosphate or ultraviolet cross-linking of the DNA template were polyadenylated (GERSHOWITZ and MOSS 1979; PAOLETTI et al. 1980).

2.6 A Soluble Template-Dependent Transcription System

While permeabilized virions or intact virus cores provide an in vitro transcription system that seems to closely mimic in vivo RNA synthesis, the particulate nature and the exclusive use of the endogenous template precluded many types of experiments. The development of a soluble template-dependent transcription system from vaccinia virus cores (GOLINI and KATES 1985; ROHRMANN and MOSS 1985) led to the identification of the termination signal and opened the way to the isolation of trans-acting factors. Enzymatically active extracts were prepared by disrupting virus particles with detergent and then removing the endogenous DNA by passage through a DEAE-cellulose column. Cloned segments of vaccinia virus DNA, containing early promoters were added back as templates. When the template contained an entire early gene, the RNAs had both correct 3′ and 5′ ends (ROHRMANN et al. 1986). Termination, rather than processing, was considered for two reasons. First, the correct size transcripts appeared within 1 min of the start of RNA synthesis and before any longer products could be detected. Secondly, longer transcripts made in vitro with bacteriophage T7 RNA polymerase were not processed when added to the extracts indicating the absence of endonuclease activity. By introducing mutagenized templates, it was demonstrated that the sequence TTTTTNT was necessary and sufficient for termination which occurred downstream of this signal (YUEN and MOSS 1987).

The RNAs made in the template-dependent extract were also polyadenylated (ROHRMANN and MOSS 1985). This modification did not depend on correct termination since even prematurely truncated trancripts were polyadenylated, consistent with prior results obtained in vivo and with intact virus cores. Furthermore, added nonviral RNAs were polyadenylated and even competed with nascent RNA for poly(A) polymerase.

2.7 Enzymes and Transcription Factors

2.7.1 DNA-Dependent RNA Polymerase

An RNA polymerase is present within the core of infectious poxvirus particles. The purified enzyme is incapable of transcribing double-stranded DNA, even if a vaccinia virus promoter is present, but can copy single-stranded DNA templates in the presence of Mn^{2+} as the divalent cation (BAROUDY and MOSS 1980; SPENCER et al. 1980). The enzyme has associated polypeptides of approximately 147, 132, 34, 22, 21, 20, and 17 kDa and sediments with an apparent molecular mass of nearly 500 kDa (BAROUDY and MOSS). The RNA polymerase is virus-

encoded and the genes for most of the subunits have been mapped and sequenced (BROYLES and MOSS 1986; JONES et al. 1987; MORRISON et al. 1985; PATEL and PICKUP 1989; AHN et al. 1990; AMEGADZIE, COLE, EARL, GERSHON, HOLMES, JONES and Moss, unpublished work). The 147 and 132 kDa subunits have extensive similarities in sequence to the corresponding large prokaryotic and eukaryotic subunits, although the resemblance is greater to the latter. Missing from the 147 kDa subunit, however, is the highly phosphorylated heptapeptide repeat present at the C-terminal of the eukaryotic RNA polymerase II large subunit (ALLISON et al. 1985; CORDEN et al. 1985).

2.7.2 VETF: An Early Transcription Factor

In contrast to the inability of highly purified vaccinia virus RNA polymerase to transcribe double-stranded DNA molecules, less pure forms of RNA polymerase, isolated either by DEAE-cellulose chromatography or by sucrose gradient centrifugation, were found to accurately transcribe vaccinia virus early genes (BROYLES and MOSS 1987b). This difference might have been due to denaturation of the purified enzyme, loss of a subunit, or a need for additional factors. To evaluate the latter possibility, a complementation assay was devised in which column fractions were added to preparations of purified RNA polymerase and a suitable DNA template containing a cloned vaccinia virus gene. In this manner, an early transcription factor, VETF, was identified and purified by conventional and affinity chromatography steps (BROYLES et al. 1988). VETF is active with several different early promoters indicating that it is a general early transcription factor. VETF binds specifically to functional early promoters but not to promoters with single nucleotide substitutions in the critical region that also prevent transcription (YUEN et al. 1987). Interaction of the transcription factor with the critical region also was demonstrated by DNA footprinting (YUEN et al. 1987) and methylation interference (BROYLES and MOSS, unpublished work). The DNA binding protein analyzed in another study (WILSON et al. 1988) is probably identical to VETF, although transcription assays were not performed.

VETF has a DNA-dependent ATPase that might be responsible for the ATP hydrolysis requirement for initiation of RNA synthesis (BROYLES and MOSS 1988). The same ATP analogs inhibit both the ATPase activity of VETF and transcription by permeabilized virions. It will be interesting to see whether the ATPase activity is associated with DNA unwinding. Free VETF binds specifically to early promoters in vitro suggesting that the RNA polymerase might recognize the promoter DNA-VETF complex; however, VETF has been isolated in association with RNA polymerase raising the alternative possibility that the protein-protein interaction precedes attachment to DNA (BROYLES and MOSS 1987b).

Highly purified VETF contains two polypeptides of about 82 kDa and 77 kDa and sediments with an S value of 7.6 consistent with a dimer structure (BROYLES et al. 1988). From partial protein sequence data, the ORFs corresponding to both subunits were identified within the vaccinia virus genome (GERSHON

and Moss, 1990). Both have late promoters, and the DNA sequence for the smaller of the two subunits contains a motif previously associated with ATP binding or ATPase activity consistent with the properties of VETF.

RNA polymerase, VETF, and a DNA template with an early promoter appear to represent the minimal components necessary for initiation and elongation of RNA molecules, but further studies may reveal additional factors associated with those components or new factors that increase the rate of RNA synthesis or are required under different assay conditions.

2.7.3 Capping and Methylating Enzymes

The enzyme responsible for capping vaccinia virus early mRNA is a 127 kDa multifunctional complex composed of 97 and 33 kDa subunits (Martin et al. 1975). An additional role of the capping enzyme in transcription termination will be discussed later. The enzyme complex carries out several successive reactions: removal of the terminal or γ—phosphate from an RNA chain, transfer of the GMP moiety of GTP to the now diphosphate—ended RNA, and transfer of a methyl group from S—adenosylmethionine to the 7 position of the added guanosine which stabilizes the final product against reversal by pyrophosphate (Martin and Moss 1975; Martin and Moss 1976; Tutas and Paoletti 1977; Venkatesan et al. 1980). The enzyme is specific for GTP as the donor and for an RNA molecule with either a di- or triphosphate end as the acceptor. The ability of short oligoribonucleotides to serve as cap acceptors suggests that capping might occur shortly after transcription initiation and is consistent with the finding of capping enzyme associated with the transcription complex (Broyles and Moss 1987b). Rapid capping of nascent transcripts would ensure that this modification occurs before the loss of the essential 5′ terminal phosphates and might help to stabilize the nascent transcript against exonucleolytic degradation.

The large capping enzyme subunit reacts with GTP to form a covalent lysine—GMP intermediate (Roth and Hurwitz 1984; Shuman and Hurwitz 1981; Shuman et al. 1980). The isolated enzyme-guanylate complex can then transfer the guanylate moiety to the 5′ end of a polyribonucleotide. A similar mechanism is utilized by eukaryotic capping enzymes (Venkatesan and Moss 1982). The RNA triphosphatase and the guanylyltransferase domains reside in a 59 kDa tryptic fragment from the 97 kDa subunit of the vaccinia RNA guanylyltransferase (Shuman 1989).

The genes encoding the large and small subunits of capping enzyme have been identified (Morgan et al. 1984; Niles et al. 1989), and their coexpression in *E. coli* has resulted in the generation of both guanylyltransferase and methyltransferase activities (Guo and Moss, 1990). The large subunit expressed by itself has guanylyltransferase activity (Guo and Moss, 1990; Niles, Shuman, personal communications).

A separate RNA (nucleoside—2′)methyltransferase catalyzes the transfer of a methyl group from S—adenosylmethionine to the ribose of the nucleotide adjacent to the 7-methylguanosine (Barbosa and Moss 1978b). Neither capped

polyribonucleotides lacking the 7-methyl group, i.e., G(5′)pppA-, nor uncapped polyribonucleotides are methyl acceptors indicating that 2′–O–methylation is the final step in the formation of m^7G(5′)pppNm (BARBOSA and MOSS 1978a). The gene encoding this 38 kDa enzyme has not yet been identified nor has the role of ribose methylation for either poxvirus or eukaryotic mRNAs been determined.

2.7.4 Termination Factor

The existence of a specific termination factor was deduced from the fact that partially purified RNA polymerase preparations were capable of initiating and elongating RNA chains but failed to terminate them. Using a complementation assay, protein fractions that restored termination were found (SHUMAN et al. 1987). The copurification of the termination factor with capping enzyme was totally unexpected and led to an initial consideration that the cap structure was required for termination. Further experiments revealed, however, that termination was not inhibited if capping of RNA was prevented. The likely conclusion, that termination is a separate function of the capping enzyme, needs to be confirmed either genetically or by use of expression vectors.

The termination signal precedes the site of termination raising the question of whether DNA or RNA is actually recognized by the transcription apparatus. The high degree of sequence specificity for termination (YUEN and MOSS 1987) suggested a way of answering this question. Replacement of UTP with iodo- and bromo-derivatives had little effect on transcription except that termination was completely abrogated implying that the RNA is recognized (SHUMAN and MOSS 1988a). Apparently, only nascent RNA is recognized by the transcription complex (BROYLES and MOSS 1987b), which includes RNA polymerase, capping enzyme, and VETF, since added poly(U) does not compete for termination.

2.7.5 Poly(A) Polymerase

Poly(A) polymerases with molecular mass of approximately 80 kDa and containing 55 and 33 kDa subunits have been isolated from vaccinia virus cores (MOSS et al. 1975) and infected cells (NEVINS and JOKLIK 1977a). The purified enzyme exhibits no primer specificity and can add adenylate residues to the 3′ ends of RNA, synthetic polyribonucleotides, or even DNA fragments. The enzyme is selective for ATP, although it can inefficiently polymerize other ribonucleoside triphosphates (SHUMAN and MOSS 1988b). Kinetic studies revealed that polyadenylation is a biphasic reaction. The genes encoding the poly(A) polymerase subunits have been identified (GERSHON and MOSS, unpublished work) indicating that this enzyme also is virus encoded.

2.7.6 Nucleic Acid-Dependent Nucleoside Triphosphatases

This major ATPase activity in vaccinia virus cores belongs to a 61 kDa protein, nucleoside triphosphate phosphohydrolase I (NPH I). The phosphohydrolytic

activity is specific for ATP or dATP and requires a DNA or synthetic polydeoxyribonucleotide cofactor (PAOLETTI et al. 1974b; PAOLETTI et al. 1974a). Neither entirely single-stranded nor perfect double-stranded DNA are efficient cofactors. The NPH I gene has been identified; it contains a late promoter and an ORF capable of encoding a 631 amino acid polypeptide with a motif frequently found in proteins that bind or hydrolyze ATP (BROYLES and MOSS 1987a; RODRIGUEZ et al. 1986). The identity of the gene was confirmed by expression of the ATPase in *E. coli* (BROYLES and MOSS, unpublished work). Conditionally lethal temperature sensitive mutants that map to the NPH I ORF are defective in intermediate and late gene expression suggesting that the enzyme is involved in transcription (DELANGE 1989; KUNZI and TRAKTMAN 1989).

Another enzyme, with a molecular mass of 68 kDa, nucleoside triphosphate phosphohydrolase II (NPH II), is less specific in its nucleic acid cofactor requirement and hydrolyzes all four nucleoside triphosphates (PAOLETTI et al. 1974b; PAOLETTI et al. 1974). NPH I and NPH II are immunologically distinct (PAOLETTI et al. 1974a), but the latter gene has not yet been identified.

2.7.7 DNA Topoisomerase

DNA topoisomerases modify the topological state of DNA by breaking and rejoining DNA phosphodiester bonds; they are classified as type 1 or type 2 enzymes depending on whether they cut one or two DNA strands. The vaccinia virus DNA topoisomerase relaxes either positively or negatively supercoiled DNA in the absence of an energy cofactor and has the properties of a cellular type 1 enzyme, except for its resistance to camptothecin (BAUER et al. 1977; FOGELSONG and BAUER 1984; SHAFFER and TRAKTMAN 1987; SHUMAN et al. 1988). Among all eukaryotic viruses, only poxviruses are known to encode their own topoisomerase. The ORF was identified by N-terminal protein sequence analysis (SHUMAN and MOSS 1987) and confirmed by expression in *E. coli* (SHUMAN et al. 1988). The 314 amino acid polypeptide has homology to the three times larger type I yeast topoisomerase. Unsuccessful attempts to disrupt the topoisomerase gene and isolate a mutant virus suggest that it has an essential role in the life cycle of poxviruses (SHUMAN et al. 1989). Although the topoisomerase is not required for transcription of linear DNA templates in vitro (BROYLES and MOSS 1987b; BROYLES et al. 1988), this does not rule out a transcriptional role with more topologically constrained natural templates in vivo. Of course other or additional roles related to DNA packaging and replication are also possible and even likely.

2.7.8 Additional Enzymes

Several other enzymes, with unknown biological functions have been isolated from vaccinia virus cores. Their location, however, make a transcriptional role worthy of consideration. A protein kinase, with a native molecular mass of 62 kDa, phosphorylates serine and threonine residues of two virion-associated

phosphate acceptor proteins of 11.7 and 38.5 kDa (KLEIMAN and MOSS 1975a; KLEIMAN and MOSS 1975b). The kinase is stimulated by protamine and other basic proteins. A deoxyribonuclease (POGO and DALES 1969a; POGO and O'SHEA 1977; ROSEMOND-HORNBEAK and MOSS 1974; ROSEMOND-HORNBEAK et al. 1974) with ligase activity (LAKRITZ et al. 1985; MERCHLINSKY et al. 1988; REDDY and BAUER 1989) may have a role in resolution or debranching of replicating forms of vaccinia virus DNA. 5′-phosphate polynucleotide kinase (SPENCER et al. 1978), endoribonuclease (PAOLETTI and LIPINSKAS 1978b), and alkaline protease (ARZOGLOU et al. 1978) activities have been briefly described but not characterized or shown to be virus-specific.

3 Late Transcription

3.1 Late Genes

The initiation of poxvirus DNA replication is closely followed by dramatic changes in gene expression that can be most simply seen by pulsing cells with radioactive amino acids at intervals after infection and examining the labeled polypeptides by polyacrylamide gel electrophoresis (CARRASCO and BRAVO 1986; MOSS and SALZMAN 1968; PENNINGTON 1974). The above analyses indicated a decline in the synthesis of early proteins and a rather abrupt onset in synthesis of late proteins. Synchronization, with reversible inhibitors of DNA replication, suggested the existence of at least two temporal classes of late proteins. The proteins made at late times include most of the structural proteins and many of the enzymes packaged within the virus particle (BAUER et al. 1977; MOSS et al. 1973; PAOLETTI et al. 1974a; POGO and DALES 1969b). Although some late ORFs are located near the ends of the genome, most occur tandemly in clusters in the central region (BELLE ISLE et al. 1981; LEE-CHEN and NILES 1988; ROSEL et al. 1986).

3.2 Late mRNA

RNA: DNA hybridization studies revealed that most of the genome is transcribed at late times (BOONE and MOSS 1978; ODA and JOKLIK 1967; PAOLETTI and GRADY 1977). In addition, late RNA is able to self–anneal or anneal with early RNA to form ribonuclease–resistant hybrids (BOONE et al. 1979; COLBY et al. 1971; VARICH et al. 1979). An explanation for the above properties was provided by the observation that late transcripts lack defined 3′ ends and that an mRNA coding for a single polypeptide varies many-fold in length (COOPER et al. 1981a; MAHR and ROBERTS 1984b). These RNAs start at late genes but continue downstream through early genes and other late genes. Since both DNA strands

are transcribed in this manner, self-annealing can lead to duplex formation, at least in vitro. Evidently, the early termination sequence TTTTTNT goes unrecognized since it occurs frequently in late genes (YUEN and MOSS 1987). Possibilities that may account for the failure of termination include differences in RNA polymerase structure at early and late times, separation of the capping enzyme/termination factor from the transcription complex during formation of the poly(A) leader, and the formation of an anti-terminator. A discrete size major cowpox virus transcript appears to be an exception to the general rule of length heterogeneity of late RNA (PATEL and PICKUP 1987). Whether the 3′ end of cowpox RNA results from termination or cleavage, however, has not been determined.

The 5′ ends of late mRNA are predominantly m^7GpppAm and m^7GpppAmAm, suggesting that the RNAs start with a pppApA-sequence which is subsequently capped (BOONE and MOSS 1977). The finding of consecutive 5′-terminal A residues was consistent with the nuclease S1 mapping of the 5′ ends of several late RNA transcripts within a TAAAT sequence (ROSEL et al. 1986; WEIR and MOSS 1987b). Further analysis by reverse transcriptase primer extension and cDNA cloning of vaccinia (BERTHOLET et al. 1987; SCHWER et al. 1987) and cowpox (PATEL and PICKUP 1987) late mRNAs, however, revealed a poly(A) tract of approximately 35 nucleotides at their 5′ ends. This unusual structure was confirmed by nuclease S1 digestion of hybrids formed by annealing of DNA containing synthetic poly(dT) tracts to late mRNAs. Direct examination of the capped 5′ ends of a purified late message demonstrated heterogeneity in poly(A) length from a few A residues to more than 30 (AHN and MOSS 1989). Even more complex structures, consisting of noncontiguously encoded mRNAs connected 3′ to 5′ by a poly(A) tract, were also suggested by cDNA cloning and electron microscopy (BERTHOLET et al. 1987). cDNA structures of this type could arise artifactually if a reverse transcript of an mRNA with a 5′ poly(A) leader were separated from the template and then served as a primer upon reannealing to the 3′ poly(A) of another physically unconnected mRNA. Therefore, the biological significance of the apparently fused RNAs is uncertain.

The function of the 5′ poly(A) leader is a mystery. One possible role might be to resist the potentially deleterious effects on translation caused by read-through complementary antisense RNA made late in infection. The capped 5′ poly(A) leader might provide a binding site for the 40S ribosomal subunit, which would then move unimpeded by antisense RNA to the first AUG where ribosome assembly and translation occur. Since most early mRNAs do not have poly(A) leaders, their translation might be selectively inhibited by antisense RNA.

3.3 Late Promoters

A conserved TAAAT motif, corresponding to the RNA start site and poly(A) leader, appears close to the translation initiation codon of most late ORFs (BERTHOLET et al. 1985; ROSEL and MOSS 1985; ROSEL et al. 1986; WEIR and MOSS

1984). In many ORFs, the TAAAT sequence is followed by a G forming the first methionine codon. No other obvious conserved sequence is present in the promoter region except for upstream runs of A or T residues.

The dependence on DNA replication for activation of late promoters is retained even when they are transposed into the middle of an early gene (BERTHOLET et al. 1985; WEIR and MOSS 1984) or when transfected as a plasmid into an infected cell (COCHRAN et al. 1985a; SHEPARD et al. 1989). Deletion mutagenesis, similar to that used to define the minimal length of early promoters, was also carried out with late promoters. A gradual decrease in expression of a reporter gene occurred as 5′ to 3′ deletions were made, but significant activity remained with only 15–20 bp upstream of the RNA start site (BERTHOLET et al. 1986; WEIR and MOSS 1987b). The importance of the conserved TAAAT region was suggested by mutagenesis studies (BERTHOLET et al. 1986; HANNGI et al. 1986). Reports of specific regulatory elements more than 30 bp upstream of the RNA initiation site (MINER et al. 1988) remain to be confirmed.

Additional mutational analysis suggested that the late promoter may be considered in terms of three regions: an upstream sequence of about 20 bp with some consecutive T or A residues separated by a region of about 6 bp from a highly conserved TAAAT element within which transcription initiates (DAVISON and MOSS 1989b). The profound effects on β-galactosidase expression, obtained by single base substitutions around the RNA start site of one late promoter, are shown in Fig. 2. All mutations within the A triplet of TAAAT had a drastic

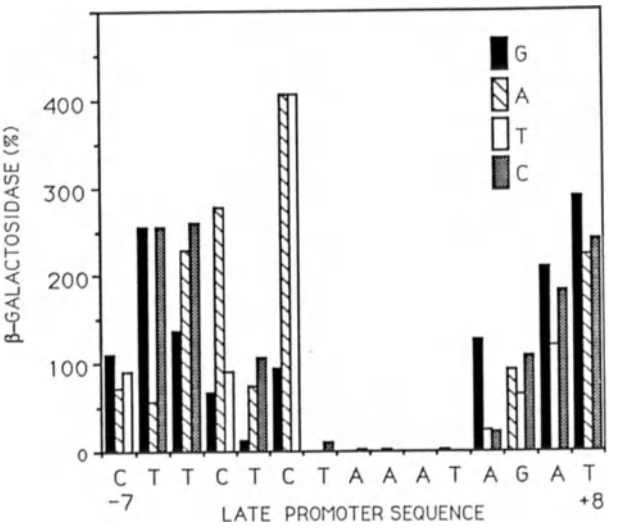


Fig. 2. Effect of single nucleotide substitutions on the activity of a late promoter. A series of recombinant vaccinia viruses that contain single nucleotide substitutions of the P28 promoter, regulating expression of β-galactosidase, were used to infect cells (DAVISON and MOSS 1989b). The activities obtained with the mutated promoter are given relative to the 100% value obtained with the parental promoter

effect. Insertion of a fourth A also had a negative effect although there are some natural late vaccinia promoters with TAAAAT (KOTWAL et al. 1989). All substitutions of the flanking T residues also had a negative effect; the degree depended inversely on the strength of the promoter as determined by upstream sequences (DAVISON and MOSS 1989b). Similar effects were noted when RNA was analyzed by primer extension (DAVISON and MOSS 1989b). It is important to point out that the above in vivo studies do not distinguish between effects on transcription and on stability. This is not a trivial point since some of the mutations, particularly those within the TAAAT element, affect the formation of the poly(A) leader. Mutations within the TAAAT also affect transcription in vitro under conditions in which a poly(A) leader is not required for stability (WRIGHT and MOSS 1987). Therefore, despite the previous caveat, it seems likely that the TAAAT is a part of the promoter.

Nucleotide changes around the TAAAT also influenced expression (DAVISON and MOSS 1989b). At the position immediately downstream of the TAAAT, a G is optimal for promoter activity and an A is second best. The most and least favorable single nucleotide substitutions within 6 bp upstream and 3 bp downstream of TAAAT increased expression fourfold and decreased expression eightfold, respectively. The region further upstream is essential for efficient late promoter function and usually contains runs of A or T residues. T runs have a much greater activating effect than A runs, suggesting that the requirement is not merely for a region with a low energy of denaturation. T is present at -10 or -11 in 16 of 22 promoters examined and at both positions in 14 of them. A synthetic promoter, stronger than any natural one, was constructed with runs of 18–20 T residues (DAVISON and MOSS 1989b). Significantly, initiation always occurred within the A triplet, regardless of promoter strength.

Some genes have tandem early and late promoters and are active throughout infection. One example is the P7.5 compound promoter which has separate early and late RNA start sites about 50 bp apart (COCHRAN et al. 1985b). The upstream late promoter is active when the downstream early promoter is not, arguing against the existence of a putative early promoter repressor protein which might interfere with both late and early transcription in the above case.

3.4 Formation of the 5′ Poly(A) Leader

Mutagenesis studies have provided support for an RNA slippage model in which three consecutive A residues are necessary for formation of the 5′ poly(A) leader in late mRNA. Thus, the 5′ poly(A) leader was absent or limited to a few nucleotides when one of the three A residues was mutated (DAVISON and MOSS 1989b). Although these mutations drastically decreased the amount of RNA, the start site did not appear to change. Sequences around the AAA also affected the length of the poly(A) leader. The length was diminished when the T residues flanking the AAA were mutated (DAVISON and MOSS 1989b). A decrease in poly(A) length was

noted when mutations further downstream were made (DE MAGISTRIS and STUNNENBERG 1988). In vitro transcription studies, to be discussed later, also support a slippage model. The heterogeneity in length of the leader implies that, after each addition of an A residue by RNA polymerase, there is a probability of proceeding forwards past the triple A or backward to add another A residue. Apparently this probability may be affected by the next nucleotide. Slippage has previously been described for bacterial and bacteriophage polymerases (CHAMBERLIN 1976; KASSAVETIS et al. 1986; MARTIN et al. 1988) but is usually suppressed when nucleoside triphosphates complementary to downstream sequences are present.

Although the poly(A) leader appears to be characteristic of late RNAs, it is not restricted to the transcripts of this temporal class. A TAAATG sequence is present at the start of several early genes, including one of the small subunits of RNA polymerase and DNA polymerase, and leaders with 6–12 A residues were found on these early mRNAs (AHN and MOSS, 1990; INK and PICKUP, 1990). In addition, mutagenesis of an early promoter to create a TAAAT sequence resulted in the formation of an mRNA with a short poly(A) leader (B. INK and D. PICKUP, 1990). These data suggest that the vaccinia virus RNA polymerase itself has an intrinsic ability to slip when confronted with a TAAAT sequence at the RNA start site. The more characteristic occurrence of the poly(A) leader on late mRNA, compared to early mRNA, may reflect the role of TAAAT as an important functional element in late not early promoters. Such a situation does not eliminate the possibility that poly(A) also has a role in mRNA stability or translation.

3.5 A Soluble Template-Dependent Transcription System

Extracts prepared from purified vaccinia virus particles are specific for added templates containing early genes implying that either different or additional proteins are needed for late transcription. Presumably, these factors are made after infection and are present in infected cells. In vitro transcription systems made from vaccinia virus-infected cells (FOGELSONG 1985; PUCKETT and MOSS 1983) are capable of recognizing vaccinia virus late promoters and of synthesizing RNA with a 5′ poly(A) leader (SCHWER and STUNNENBERG 1988; WRIGHT and MOSS 1987). Interestingly, whole cell extracts prepared at 2 h or later after infection are no longer able to transcribe genes with eukaryotic RNA polymerase II promoters, although transcription of RNA polymerase III promoters is inhibited to a much lesser extent (PUCKETT and MOSS 1983).

The inability of the extracts to transcribe a late promoter with a mutation within TAAAT confirmed the specificity of the transcription system (WRIGHT and MOSS 1987). In addition, as short defined templates were used, the in vitro studies ruled out the possibility of cis-splicing as a mechanism of poly(A) formation. To some extent, the length of the poly(A) head was dependent on the amount of

exogenous ATP added suggesting that the leader sequence was formed de novo in the extract (SCHWER and STUNNENBERG 1988).

Curiously, although early genes are not expressed in cells at late times of infection, extracts of such cells are capable of transcribing genes of both classes (WRIGHT and MOSS 1989; WRIGHT and MOSS 1987). This apparent lack of specificity might be due to the extraction of factors from progeny virus particles or to unmasking or activating VETF.

3.6 Late Transcription Factors

Attempts have been made to isolate specific factors needed for in vitro transcription of late promoters (WRIGHT and MOSS 1989). Three fractions were obtained by phosphocellulose chromatography of a cytoplasmic extract of infected cells. Although none of the fractions alone was capable of transcribing a template containing a late promoter, specific transcriptase activity, with the ability to produce the novel poly(A) leader, could be reconstituted by mixing the first two column fractions and optimal activity was obtained with all three. Each of the activities are heat labile, resistant to micrococcal nuclease, and present only in extracts of infected cells. One of the factors, VLTF-1, was partially purified through several additional columns and was found to sediment with an approximate molecular mass of 45 kDa. The second fraction contained RNA polymerase, VETF, and a putative late transcription factor. A protein that elutes from phosphocellulose at high salt and which binds to two different sites, 5′ and 3′ to the transcription start of a late promoter, has been described (MINER and HRUBY 1989). Whether the latter is related to the third transcription stimulatory factor (WRIGHT and MOSS 1989), which also elutes from phosphocellulose at high salt, is not known.

The finding of VETF in one of the phosphocelluose fractions needed for late transcription raises the possibility that it is a general transcription factor. Arguing against this is the fact that VETF has DNA-dependent ATPase activity which is apparently not required for late transcription since the latter can fully utilize a derivative of ATP with a nonhydrolyzable γ phosphate in place of ATP (SCHWER and STUNNENBERG 1988; WRIGHT and MOSS 1989). It does seem likely, however, that the RNA polymerases used for early and late transcription are similar or at least share subunits, since the phenotype of conditionally lethal mutations in the large subunit and in a small subunit include decreased expression of late genes (ENSINGER 1987; HOODA-DHINGRA et al. 1989). Although not compared directly, the sizes of the polypeptides associated with RNA polymerase isolated from infected cells (NEVINS and JOKLIK 1977a) and virus cores (BAROUDY and MOSS 1980) are generally similar except for some small differences. Whether the latter are due to different methods of purification and analysis or represent significant differences or modifications of the enzymes is unknown.

3.7 Intermediate Genes

Although the existence of at least two classes of late genes was suspected from the time course of polypeptide synthesis during a normal infection and after synchronizing cells with a reversible inhibitor of DNA replication (Moss and Salzman 1968; Opperman and Koch 1976; Pennington 1974), direct evidence for intermediate genes was not obtained until recently (Vos and Stunnenberg 1988). It was found that intermediate genes can be expressed in the absence of DNA replication when transfected into cells (Vos and Stunnenberg 1988), whereas late genes require DNA replication for expression regardless of whether they are genomic or present in transfected plasmids (Cochran et al. 1985a). One interpretation of the above results is that the trans-acting factors for intermediate expression are present prior to replication and hence can transcribe transfected DNA. In support of this hypothesis, extracts of virus cores are incapable of transcribing intermediate genes, whereas extracts from cells infected in the presence of hydroxyurea are able to transcribe intermediate genes but not late genes (Vos and Stunnenberg 1988). Two intermediate genes, *I3* and *I8*, were identified. The former has a compound promoter with both early and intermediate RNA start sites. Similarities in sequence between the regions upstream of the RNA start sites of *I3* and *I8* were pointed out and neither contains the TAAAT motif. A role for intermediate genes in a transcriptional regulatory cascade was postulated (Vos and Stunnenberg 1988) although no information regarding regulatory functions of *I3* and *I8* was provided. The *I8* ORF encodes a protein of 78 kDa that is apparently a structural protein; ts mutants in this gene affect virion stability (Fathi and Condit, personal communication). The number of intermediate genes is not yet known and individual members of this class may have a variety of functions as do members of the early and late classes.

4 Role of DNA Replication in Gene Regulation

How does DNA replication activate the switch from early to late gene expression? Do intermediate genes add to the complexity or provide a key to the solution? One recent approach may have provided insights into this process. It was discovered that DNA replication is not necessary for expression of a transfected reporter gene regulated by a late promoter, provided purified vaccinia virus genomic DNA was also transfected into the infected cell (Keck et al. 1990). These data eliminated the possibility that special replicative forms of vaccinia virus DNA are needed to trigger the switch. The role of the transfected genomic DNA was determined by substituting cloned vaccinia virus DNA fragments. Starting with cosmids and then using smaller and smaller plasmids, it was found that the requirement could be satisfied with a minimum of three ORFs encoding polypeptides of 17, 26, and 30 kDa.

Transfection of plasmids containing these three ORFs into infected cells was both necessary and sufficient for expression of a transfected late gene (but not a late gene within the genome of the infecting virus) in the presence of an inhibitor of DNA replication. Moreover, point mutations within any of the three ORFs abrogated expression from the late promoter.The three ORFs, therefore, are trans-activators of late gene expression and are excellent candidates for the late transcription factors identified by biochemical methods (WRIGHT and MOSS 1989). Sequence comparisons showed no marked similarities of these ORFs to other entries in DNA or protein databases. Transcription studies indicate that the three ORFs are regulated as intermediate genes. In this respect, however, the three trans-activating genes do not map near intermediate genes *I3* or *I8* nor were the latter required for transactivation under the experimental conditions used.

An outstanding question that remains concerns the inability of virion DNA to act as a template for intermediate and late gene expression. Perhaps the simplest explanation is that virion proteins sequester the input DNA from newly synthesized enzymes and factors required for the expression of later classes of genes. An alternative explanation is that expression of the late classes of genes is prevented by specific repressor proteins.

5 Posttranscriptional Regulation

Transcription is clearly an important but not necessarily the only mechanism used by orthopoxviruses to regulate gene expression. For example, mRNA stability also may play a role. The decreasing stability of mRNA during the course of infection (ODA and JOKLIK 1967; SEBRING and SALZMAN 1967) should accelerate changes in mRNA populations when superimposed on transcriptional regulatory processes. Perturbations in mRNA stability may account for the late abortive phenotype of one class of temperature sensitive mutants (COHRS et al. 1989; CONDIT and MOTYCZKA 1981; PACHA and CONDIT 1985) and, in part, for the action of the drug isatin-β-thiosemicarbazone (COOPER et al. 1970; WOODSON and JOKLIK 1965).

Translational mechanisms also have been proposed as regulators of poxvirus gene expression. A translational suppressor was suggested by experiments describing the modulation of cowpox thymidine kinase synthesis by inhibitors of transcription and translation (MCAUSLAN 1963a; MCAUSLAN 1963b). The report of a disproportion between the amount of early proteins made in cells and the amount of functional mRNA that can be isolated at late times also supports this idea (HRUBY and BALL 1981; VASSEF et al. 1982). As discussed previously, the characteristic 5′ poly(A) leader of late mRNA may facilitate translation in the presence of antisense transcripts. Nevertheless, evidence that translational regulation plays a significant role in orthopoxvirus gene expression remains circumstantial.

6 Interactions with the Host Transcription Apparatus

Perhaps the most controversial area of orthopoxvirus gene regulation regards the role of the cell nucleus and the host RNA polymerase II in the virus growth cycle. The presence of poxvirus DNA and RNA in the nucleus of cells has been reported (ARCHARD 1983; BOLDEN et al. 1979; GAFFORD et al. 1976; LACOLLA and WEISSBACH 1957) but considered to be artifactual by others (MINNEGAN and MOYER 1985). In enucleated cells, virus maturation is severely inhibited, but synthesis of early and late proteins and DNA replication occur although at reduced levels (PENNINGTON and FOLLETT 1974; PRESCOTT et al. 1971). A similar phenomenon occurs when infected cells are treated with α-amanitin (HRUBY et al. 1979; SILVER et al. 1979) which inhibits RNA polymerase II but not vaccinia virus-associated RNA polymerase (BAROUDY and MOSS 1980; NEVINS and JOKLIK 1977b; SPENCER et al. 1980). Evidence that the target of α-amanitin was RNA polymerase II was provided in a report that infectious virus was formed in cells with an α-amanitin resistant RNA polymerase in the presence of drug (SILVER et al. 1979). Nevertheless, other reports describe a mutant vaccinia virus that can replicate in enucleated or α-amanitin treated cells (TAMIN et al. 1988; VILLARREAL and HRUBY 1986; VILLARREAL et al. 1984). The presence of some vaccinia virus RNA polymerase in the nucleus has been reported (WING et al. 1984), whereas other studies demonstrate that cellular RNA polymerase II (or at least the large subunit) comes out of the nucleus during vaccinia virus infection, associates with cytoplasmic regions that accumulate viral proteins, and is even packaged in virus particles (MORRISON and MOYER 1986; WILTON and DALES 1986). Still lacking, however, is direct evidence that RNA polymerase II itself or any part of it is necessary for transcription of any vaccinia gene or that either enucleation or α-amanitin inhibit such transcription. Another possibility, that a cellular gene product with a rapid turnover is required for virus maturation, has been given little consideration.

7 Summary: The Gene Regulation Cycle

The regulation of poxvirus gene expression can be thought of in the context of a cycle that starts with entry of the virus into the cell and ends with the assembly of mature infectious progeny (Fig. 3). The virion is programmed to express early genes: it contains a multisubunit, virus-encoded, DNA-dependent RNA polymerase; a specific transcription factor, VETF, that recognizes early promoters; and other enzymes involved in mRNA formation. The mRNA is actively extruded from the virus core and then associates with the ribosomes where early viral proteins, including RNA polymerase subunits, enzymes required for DNA replication, and putative factors for specific transcription of intermediate genes

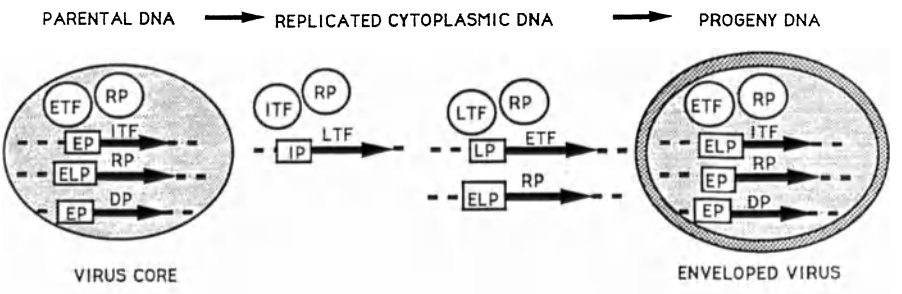


Fig. 3. Vaccinia virus gene regulation cycle. *EP*, *IP*, *LP*, early, intermediate and late promoters, respectively; *ELP*, early and late tandem promoters; *ETF*, *ITF*, *LTF* early, intermediate, and late transcription or transactivation factors, respectively; *RP*, RNA polymerase; *DP*, *DNA* polymerase. *Heavy arrows*, ORFs; *circles* protein factors. For simplicity, genes encoding only one subunit of RNA polymerase and factors are shown

are made. The parental DNA may not serve as a template for intermediate gene expression because of its location in the core and consequent sequestration by structural virion components or because of specific repressors that block transcription of the intermediate genes. In regard to the latter mechanism, the apparently small number of intermediate genes could provide a convenient bottleneck for repressor control. The virion DNA must become accessible to DNA polymerase, since replication begins within a few hours of infection. The cycle now enters its second phase in which the replicated DNA provides the template for transcription of intermediate genes including the three ORFs identified as trans-activators of late gene expression. In the third phase of the cycle, late genes encoding structural proteins, virion enzymes, and early transcription factors are expressed. Since transcription of early genes does not occur late in infection, when early transcription factors are synthesized, the factors may be activated during or after virus assembly. A variety of additional mechanisms such as promoter occlusion by the long, heterogenous late RNAs, hybridization of late antisense RNA to early mRNA, mRNA stability, and translational controls also may contribute to the negative regulation of early gene expression. The viral membrane, which forms during the initial steps in particle assembly, could provide a barrier to ribonucleotides thereby preventing the synthesis of early RNA. The membrane barrier is maintained during virus release and disrupted during the subsequent infection step when the virus fuses with the cell. This model invokes a cascade in which early transcription factors are made at late times in the previous round of infection, intermediate transcription factors are made early in the new infection, and late transcription factors are made at intermediate times.

Acknowledgments. I wish to thank members of Laboratory of Viral Diseases for discussions and B. Ahn, B. Amegadzie, J. Baldick, N. Cole, P. Gershon, and J. Keck for permission to cite their unpublished data.

References

Ahn B-Y, Moss B (1989) Capped poly(A) leader of variable lengths at the 5′ ends of vaccinia virus late mRNAs. J Virol 63: 226–232

Ahn B-Y, Jones EV, Moss B (1990) Identification of the vaccinia virus gene encoding an 18 kilodalton subunit of RNA polymerase and demonstration of a 5′ poly(A) leader on its early transcript. J Virol 64: in press

Allison LA, Moyle M, Shales M, Ingles CJ (1985) Extensive homology among the largest subunits of eukaryotic and prokaryotic RNA polymerases. Cell 42: 599–610

Archard LC (1983) Synthesis of full-length, virus genomic DNA by nuclei of vaccinia-infected HeLa cells. J Gen Virol 64: 2561–2575

Arzoglou P, Drillien R, Kirn A (1978) Evidence for an alkaline protease in vaccinia virus. Virology 95: 211–214

Bajszar G, Wittek R, Weir JP, Moss B (1983) Vaccinia virus thymidine kinase and neighboring genes: mRNAs and polypeptides of wild type virus and putative nonsense mutants. J Virol 45: 62–72

Barbosa E, Moss B (1978a) mRNA (nucleoside-2′-)-methyltransferase from vaccinia virus. Characteristics and substrate specificity. J Biol Chem 253: 7698–7702

Barbosa E, Moss B (1978b) mRNA (nucleoside-2′-)-methyltransferase from vaccinia virus. Purification and physical properties. J Biol Chem 253: 7692–7697

Baroudy BM, Moss B (1980) Purification and characterization of a DNA-dependent RNA polymerase from vaccinia virions. J Biol Chem 255: 4372–4380

Baroudy BM, Venkatesan S, Moss B (1982) Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. Cell 28: 315–324

Bauer WR, Ressner EC, Kates J, Patzke J (1977) A DNA nicking-closing enzyme encapsidated in vaccinia virus: partial purification and properties. Proc Natl Acad Sci USA 74: 1841–1845

Belle Isle H, Venkatesan S, Moss B (1981) Cell-free translation of early and late mRNAs selected by hybridization to cloned DNA fragments derived from the left 14 million to 72 million daltons of the vaccinia virus genome. Virology 112: 306–317

Bertholet C, Drillien R, Wittek R (1985) One hundred base pairs of 5′ flanking sequence of a vaccinia virus late gene are sufficient to temporally regulate transcription. Proc Natl Acad Sci USA 82: 2096–2100

Bertholet C, Stocco P, Van Meir E, Wittek R (1986) Functional analysis of the 5′ flanking sequence of a vaccinia virus late gene. EMBO J 5: 1951–1957

Bertholet C, Van Meir E, ten Heggeler-Bordier B, Wittek R (1987) Vaccinia virus produces late mRNAs by discontinuous synthesis. Cell 50: 153–162

Bolden A, Pedrali-Noy G, Weissbach A (1979) Vaccinia virus infection of the HeLa cells II. Disparity between cytoplasmic and nuclear viral specific RNA. Virology 94: 138–145

Boone RF, Moss B (1977) Methylated 5′ terminal sequences of vaccinia virus mRNA species made in vivo at early and late times after infection. Virology 79: 67–80

Boone RF, Moss B (1978) Sequence complexity and relative abundance of vaccinia virus mRNAs synthesized in vivo and in vitro. J Virol 26: 554–569

Boone RF, Parr RP, Moss B (1979) Intermolecular duplexes formed from polyadenylated vaccinia virus RNA. J Virol 30: 365–374

Bossart W, Nuss DL, Paoletti E (1978) Effect of UV irradiation on the expression of vaccinia virus gene products synthesized in a cell-free system coupling transcription and translation. J Virol 26: 673–680

Broyles SS, Moss B (1986) Homology between RNA polymerases of poxviruses, prokaryotes, and eukaryotes: nucleotide sequence and transcriptional analysis of vaccinia virus genes encoding 147-kDa and 22-kDa subunits. Proc Natl Acad Sci USA 83: 3141–3145

Broyles SS, Moss B (1987a) Identification of the vaccinia virus gene encoding nucleoside triphosphate phosphohydrolase I, a DNA-dependent ATPase. J Virol 61: 1738–1742

Broyles SS, Moss B (1987b) Sedimentation of an RNA polymerase complex from vaccinia virus that specifically initiates and terminates transcription. Mol Cell Biol 7: 7–14

Broyles SS, Moss B (1988) DNA-dependent ATPase activity associated with vaccinia virus early transcription factor. J Biol Chem 263: 10761–10765

Broyles SS, Yuen L, Shuman S, Moss B (1988) Purification of a factor required for transcription of vaccinia virus early genes. J Biol Chem 263: 10754–10760

Carrasco L, Bravo R (1986) Specific proteins synthesized during the viral lytic cycle in vaccinia virus-infected HeLa cells: analysis by high-resolution two-dimensional gel electrophoresis. J Virol 58: 569–577

Chamberlin MJ (1976) RNA polymerase. Cold Spring Harbor Laboratory, NY

Chang A, Metz DH (1976) Further investigations on the mode of entry of vaccinia virus into cells. J Gen Virol 32: 275–282

Cochran MA, Mackett M, Moss B (1985a) Eukaryotic transient expression system dependent on transcription factors and regulatory DNA sequences of vaccinia virus. Proc Natl Acad Sci USA 82: 19–23

Cochran MA, Puckett C, Moss B (1985b) In vitro mutagenesis of the promoter region for a vaccinia virus gene: evidence for tandem early and late regulatory signals. J Virol 54: 30–37

Cohrs RJ, Condit RC, Pacha RF, Thompson CL, Sharma OK (1989) Modulation of ppp(A2′p)nA-dependent RNase by a temperature sensitive mutant of vaccinia virus. J Virol 63: 948–951

Colby C, Jurale C, Kates JR (1971) Mechanism of synthesis of vaccinia virus double-stranded ribonucleic acid in vivo and in vitro. J Virol 7: 71–76

Condit RC, Motyczka A (1981) Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. Virology 113: 224–241

Cooper JA, Moss B (1978) Transcription of vaccinia virus mRNA coupled to translation in vitro. Virology 88: 149–165

Cooper JA, Moss B, Katz E (1970) Inhibition of vaccinia virus late protein synthesis by isatin-β-thiosemicarbazone. Characterization and in vitro translation of viral mRNA. Virology 96: 381–392

Cooper JA, Wittek R, Moss B (1981a) Extension of the transcriptional and translational map of the left end of the vaccinia virus genome to 21 kilobase pairs. J Virol 39: 733–745

Cooper JA, Wittek R, Moss B (1981b) Hybridization selection and cell-free translation of mRNAs encoded within the inverted terminal repetition of the vaccinia virus genome. J Virol 37: 284–294

Corden JL, Cadena DL, Ahearn JM, Dahmus ME (1985) A unique structure at the carboxyl terminus of the largest subunit of eukaryotic RNA polymerase II. Proc Nat Acad Sci USA 82: 7934–7938

Coupar BEH, Boyle DB, Both GW (1987) Effect of in vitro mutations in a vaccinia virus early promoter region monitored by herpes simplex virus thymidine kinase expression in recombinant vaccinia virus. J Gen Virol 68: 2299–2309

Dales S, Kajioka R (1964) The cycle of multiplication of vaccinia virus in Earle's strain L cells. I Uptake and penetration Virology 24: 278–294

Davison AJ, Moss B (1989a) The structure of vaccinia virus early promoters J Mol Biol 210: 749–769

Davison AJ, Moss B (1989b) The structure of vaccinia virus late promoters. J Mol Biol 210: 771–784

de Magistris L, Stunnenberg HG (1988) Cis-acting sequences affecting the length of the poly(A) head of vaccinia virus late transcripts. Nucleic Acids Res 16: 3141–3156

DeLange AM (1989) Identification of temperature-sensitive mutants of vaccinia virus that are defective in conversion of concatemeric replicative intermediates to the mature linear DNA genome. J Virol 63: 2437–2444

Earl PL, Hügin AW, Moss B (1990) Removal of cryptic poxvirus transcription termination signals from the human immunodeficiency virus type 1 envelope gene enhances expression and immunogenicity of a recombinant vaccinia vins. J Virol 64: 2448–2451

Earl PL, Moss B (1989) Vaccinia virus. Genetic maps 1989. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Earl PL, Jones EV, Moss B (1986) Homology between DNA polymerases of poxviruses, herpesviruses, and adenoviruses: nucleotide sequence of the vaccinia virus DNA polymerase gene. Proc Natl Acad Sci USA 83: 3659–3663

Ensinger MJ (1987) Phenotypic characterization of temperature-sensitive mutants of vaccinia virus with mutations in a 125000-Mr subunit of the virion-associated DNA-dependent RNA polymerase. J Virol 61: 1842–1850

Essani K, Dales S (1979) Biogenesis of vaccinia: evidence for more than 100 polypeptides in the virion. Virology 95: 385–394

Fogelsong PD (1985) In vitro transcription of a cloned vaccinia virus gene by a soluble extract prepared from vaccinia virus-infected HeLa cells J Virol 53: 822–826

Fogelsong PD, Bauer WR (1984) Effects of ATP and inhibitory factors on the activity of vaccinia virus type 1 topoisomerase. J Virol 49: 1–8

Gafford LG, Mitchell EB, Randall CC (1976) Virus-specific RNA and DNA in nuclei of cells infected with fowlpox virus. Virology 69: 1–14

Garon CF, Barbosa E, Moss B (1978) Visualization of an inverted terminal repetition in vaccinia virus DNA. Proc Natl Acad Sci USA 74: 4863–4867
Gershon PD, Moss B (1990) Early transcription factor subunits are encoded by vaccinia virus late genes. Proc Natl Acad Sci USA 87: in press
Gershowitz A, Moss B (1979) Abortive transcription products of vaccinia virus are guanylylated, methylated and polyadenylylated. J Virol 31: 849–853
Gershowitz A, Boone RF, Moss B (1978) Multiple roles for ATP in the synthesis and processing of mRNA by vaccinia virus: specific inhibitory effects of adenosine (β,γ-imido) triphosphate. J Virol 27: 399–408
Geshelin P, Berns KI (1974) Characterization and localization of the naturally occurring cross-links in vaccinia virus DNA. J Mol Biol 88: 785–796
Golini F, Kates JR (1985) A soluble transcription system derived from purified vaccinia virions. J Virol 53: 205–213
Guo P, Moss B (1990) Interaction and mutual stabilization of the two subunits of vaccinia virus mRNA capping enzyme co-expressed in Escherichia coli. Proc Natl Acad Sci USA 87: in press
Hanngi M, Bannwarth W, Stunnenberg HG (1986) Conserved TAAAT motif in vaccinia virus late promoters: overlapping TATA box and site of transcription initiation. EMBO J 5: 1071–1076
Harper JM, Parsonage MT, Pelham HR, Darby G (1978) Heat inactivation of vaccinia virus particle-associated functions: properties of heated particles in vivo and in vitro. J Virol 26: 646–659
Henikoff S, Cohen EH (1984) Sequences responsible for transcription termination on a gene segment in *Saccharomyces cerevisiae*. Mol Cell Biol 4: 1515–1520
Holowczak JA (1972) Uncoating of poxviruses. 1. Detection and characterization of subviral particles in the uncoating process. Virology 50: 216–232
Hooda-Dhingra U, Thompson CL, Condit RC (1989) Detailed phenotypic characterization of five temperature-sensitive mutants in the 22- and 147-kilodalton subunits of vaccinia virus DNA-dependent RNA polymerase. J Virol 63: 714–729
Hruby DE, Ball LA (1981) Control of expression of the vaccinia virus thymidine kinase gene. J Virol 40: 456–464
Hruby DE, Lynn DL, Kates JR (1979) Vaccinia virus replication requires active participation of the host cell transcriptional apparatus. Proc Natl Acad Sci USA 76: 1887–1890
Ichihashi Y, Oie M, Tsuruhara T (1984) Location of DNA-binding proteins and disulfide-linked proteins in vaccinia virus structural elements. J Virol 50: 929–938
Ink BS, Pickup DJ (1990) Vaccinia virus directs the synthesis of early mRNAs containing 5′ poly(A) sequences, Proc Natl Acad Sci USA 87: 1536–1540
Joklik WK (1964a) The intracellular uncoating of poxvirus DNA. I. The fate of radioactively-labeled rabbitpox virus. J Mol Biol 8: 263–276
Joklik WK (1964b) The intracellular uncoating of poxvirus DNA. II. The molecular basis of the uncoating process. J Mol Biol 8: 277–288
Jones EV, Puckett C, Moss B (1987) DNA-dependent RNA polymerase subunits encoded within the vaccinia virus genome. J Virol 61: 1765–1771
Kao S, Ressner E, Kates J, Bauer WR (1981) Purification and characterization of a superhelix binding protein from vaccinia virus. Virology 111: 500–508
Kassavetis GA, Zentner PG, Geiduschek EP (1986) Transcription at bacteriophage T4 variant late promoters. An application of a newly devised promoter-mapping method involving RNA chain retraction. J Biol Chem 261: 14256–14265
Kates J, Beeson J (1970a) Ribonucleic acid synthesis in vaccinia virus. II. Synthesis of polyriboadenylic acid. J Mol Biol 50: 19–23
Kates J, Beeson J (1970b) Ribonucleic acid synthesis in vaccinia virus. I. The mechanism of synthesis and release of RNA in vaccinia cores. J Mol Biol 50: 1–18
Kates JR, McAuslan B (1967a) Messenger RNA synthesis by a "coated" viral genome. Proc Natl Acad Sci USA 57: 314–320
Kates JR, McAuslan BR (1967b) Poxvirus DNA-dependent RNA polymerase. Proc Natl Acad Sci USA 58: 134–141
Kaverin NV, Varich NL, Surgay VV, Chernos VI (1975) A quantitative estimation of poxvirus genome fraction transcribed as early and late mRNA. Virology 65: 112–119
Keck JG, Baldick CJ Jr, Moss B (1990) Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late transactivator genes. Cell 60: in press

Keith J, Gershowitz A, Moss B (1980) Dinucleotide sequences at the 5′ ends of vaccinia virus mRNAs synthesized in vitro. J Virol 36: 601–605
Kleiman JH, Moss B (1975a) Purification of a protein kinase and two phosphate acceptor proteins from vaccinia virions. J Biol Chem 250: 2420–2429
Kleiman JH, Moss B (1975b) Characterization of a protein kinase and two phosphate acceptor proteins from vaccinia virions. J Biol Chem 250: 2430–2437
Kotwal GJ, Hugin AW, Moss B (1989) Mapping and insertional mutagenesis of a vaccinia virus gene encoding a 13,800-Da secreted protein. Virology. 171: 579–587
Kunzi MS, Traktman P (1989) Genetic evidence for involvement of vaccinia virus DNA-dependent ATPase I in intermediate and late gene expression. J Virol 63: 3999–4010
LaColla P, Weissbach A (1975) Vaccinia virus infection of HeLa cells. I. Synthesis of vaccinia DNA in host cell nuclei. J Virol 15: 305–315
Lakritz N, Fogelsong PD, Reddy M, Baum S, Hurwitz J, Bauer WR (1985) A vaccinia virus DNase preparation which cross-links superhelical DNA. J Virol 53: 935–943
Lee-Chen GJ, Niles EG (1988) Transcription and translation mapping of the 13 genes in the vaccinia virus *Hin*dII D fragment. Virology 163: 52–63
Lee-Chen GJ, Bourgeois N, Davidson K, Condit RC, Niles EG (1988) Structure of the transription initiation and termination sequences of seven early genes in the vaccinia virus *Hin*dIII D fragment. Virology. 163: 64–79
Mackett M, Smith GL, Moss B (1982) Vaccinia virus: a selectable eukaryotic cloning and expression vector. Proc Natl Acad Sci USA 79: 7415–7419
Mahr A, Roberts BE (1984a) Organization of six early transcripts synthesized from a vaccinia virus *Eco*RI fragment. J Virol 49: 479–509
Mahr A, Roberts BE (1984b) Arrangement of late RNAs transcribed from a 7.1 kilobase *Eco*RI vaccinia virus DNA fragment. J Virol 49: 510–520
Mars M, Beaud G (1987) Characterization of vaccinia virus early promoters and evaluation of their information content. J Mol Biol 198: 619–631
Martin CT, Muller DK, Coleman JE (1988) Processivity in early stages of transcription by T7 RNA polymerase. Biochemistry 27: 3966–3974
Martin SA, Moss B (1975) Modification of RNA by mRNA guanylyltransferase and mRNA (guanine-7-)methyl-transferase from vaccinia virions. J Biol Chem 250: 9330–9335
Martin SA, Moss B (1976) mRNA guanylyltransferase and mRNA (guanine-7-)methyltransferase from vaccinia virions. Donors and acceptor substrate activities. J Biol Chem 251: 7313–7321.
Martin SA, Paoletti E, Moss B (1975) Purification of mRNA guanylyltransferase and mRNA (guanine -7-)methyltransferase from vaccinia virus. J Biol Chem 250: 9322–9329
McAuslan BR (1963a) The induction and repression of thymidine kinase in the poxvirus-infected HeLa cell. Virology 21: 383–389
McAuslan BR (1963b) Control of induced thymidine kinase activity in the poxvirus infected cell. Virology. 20: 162–168
Merchlinsky M, Garon C, Moss B (1988) Molecular cloning and sequence of the concatemer junction from vaccinia virus replicative DNA: viral nuclease cleavage sites in cruciform structures. J Mol Biol 199: 399–413
Miner JN, Hruby DE (1989) DNA sequences that regulate expression of a vaccinia virus late gene (L65) and interact with a DNA-binding protein from infected cells. J Virol 63: 2726–2736
Miner JN, Weinrich SL, Hruby DE (1988) Molecular dissection of cis-acting regulatory elements from 5′-proximal regions of a vaccinia virus late gene cluster. J Virol 62: 297–304
Minnegan H, Moyer RW (1985) Intracellular localization of rabbit poxvirus nucleic acid within infected cells as determined by in situ hybridization. J Virol 55: 634–643
Morgan JR, Cohen LK, Roberts BE (1984) Identification of the DNA sequences encoding the large subunit of the mRNA capping enzyme of vaccinia virus. J Virol 52: 206–214
Morrison DK, Moyer RW (1986) Detection of a subunit of cellular Pol II within highly purified preparations of RNA polymerase isolated from poxvirus virions. Cell 44: 587–596
Morrison DK, Carter JK, Moyer RW (1985) Isolation and characterization of monoclonal antibodies directed against two subunits of rabbit poxvirus-associated DNA-directed RNA polymerase. J Virol 55: 670–680
Moss B, Salzman NP (1968) Sequential protein synthesis following vaccinia virus infection. J Virol 2: 1016–1027
Moss B, Rosenblum EN, Garon CF (1973) Glycoprotein synthesis in cells infected with vaccinia virus. III. Purification and biosynthesis of the virion glycoprotein. Virology 55: 143–156

Moss B, Rosenblum EN, Gershowitz A (1975) Characterization of a polyriboadenylate polymerase from vaccinia virions. J Biol Chem 250: 4722–4729
Moss B, Gershowitz A, Wei CM, Boone R (1976) Formation of the guanylylated and methylated 5′ terminus of vaccinia virus mRNA. Virology 72: 341–351
Munyon WH, Kit S (1966) Induction of cytoplasmic ribonucleic acid synthesis in vaccinia-infected LM cells during inhibition of protein synthesis. Virology 29: 303–306
Munyon WE, Paoletti E, Grace JT Jr. (1967) RNA polymerase activity in purified infectious vaccinia virus. Proc Natl Acad Sci USA 58: 2280–2288
Muthukrishnan S, Moss B, Cooper JA, Maxwell ES (1978) Influence of 5′ terminal cap structure on the initiation of translation of vaccinia virus mRNA. J Biol Chem 253: 1710–1715
Nevins JR, Joklik WK (1977a) Isolation and partial characterization of the poly(A) polymerases from HeLa cells infected with vaccinia virus. J Biol Chem 252: 6939–6947
Nevins JR, Joklik WK (1977b) Isolation and properties of the vaccinia virus-DNA-dependent RNA polymerase. J Biol Chem 252: 6930–6938
Niles EG, Lee-Chen G-J, Shuman S, Moss B, Broyles SS (1989) Vaccinia virus gene *D12L* encodes the small subunit of the viral mRNA capping enzyme. Virology 172: 513–522
Oda K, Joklik WK (1967) Hybridization and sedimentation studies on "early" and "late" vaccinia messenger RNA. J Mol Biol 27: 395–419
Opperman H, Koch G (1976) On the regulation of protein syntheis in vaccinia virus infected cells. J Gen Virol 32: 261–273
Pacha RF, Condit RC (1985) Characterization of a temperature-sensitive mutant of vaccinia virus reveals a novel function that prevents virus-induced breakdown of RNA. J Virol 56: 395–403
Paoletti E (1977a) High molecular weight virion-associated RNA of vaccinia. A possible precursor to 8 to 12S mRNA. J Biol Chem 252: 872–877
Paoletti E (1977b) In vitro synthesis of a high molecular weight virion-associated RNA by vaccinia. J Biol Chem 252: 866–871
Paoletti E, Grady LJ (1977) Transcriptional complexity of vaccinia virus in vivo and in vitro. J Virol 23: 608–615
Paoletti E, Lipinskas BR (198a) The role of ATP in the biogenesis of vaccinia virus mRNA in vitro. Virology 87: 317–325
Paoletti E, Lipinskas BR (1978b) Soluble endoribonuclease activity from vaccinia virus-specific cleavage of virion-associated high-molecular-weight RNA. J Virol 26: 822–824
Paoletti E, Cooper N, Moss B (1974a) Regulation of synthesis of two immunologically distinct nucleic acid-dependent nucleoside triphosphate phosphohydrolases in vaccinia virus-infected HeLa cells. J Virol 14: 578–586
Paoletti E, Cooper N, Moss B (1974b) Two nucleic acid-dependent nucleoside triphosphate phosphohydrolases from vaccinia virus. Nucleotide substrate and polynucleotide cofactor specificities. J Biol Chem 249: 3281–3286
Paoletti E, Rosemond-Hornbeak H, Moss B (1974c) Two nucleic acid-dependent nucleoside triphosphate phosphohydrolases from vaccinia virus: Purification and characterization. J Biol Chem 249: 3273–3280
Paoletti E, Lipinskas B, Panicali D (1980) Capped and polyadenylated low-molecular-weight RNA synthesized by vaccinia virus in vitro. J Virol 33: 208–219
Patel DD, Pickup DJ (1987) Messenger RNAs of a strongly-expressed late gene of cowpox virus contains a 5′-terminal poly(A) leader. EMBO J 6: 3787–3794
Patel DD, Pickup DJ (1989) The second-largest subunit of the poxvirus RNA polymerase is similar to the corresponding subunits of procaryotic and eucaryotic RNA polymerases. J Virol 63: 1076–1086
Pelham HRB (1977) Use of coupled transcription and translation to study mRNA production by vaccinia cores. Nature 269: 532–534
Pelham HRB, Sykes JMM, Hunt T (1978) Characteristics of a coupled cell-free transcription and translation system directed by vaccinia cores. Eur J Biochem 82: 199–209
Pennington TH (1974) Vaccinia virus polypeptide synthesis: sequential appearance and stability of pre- and post-replicative polypeptides. J Gen Virol 25: 433–444
Pennington TH, Follett EA (1974) Vaccinia virus replication in enucleated BSC-1 cells: particle production and synthesis of viral DNA and proteins. J Virol 13: 488–493
Plucienniczak A, Schroeder E, Zettelmeissl G, Streeck RE (1985) Nucleotide sequence of a cluster of early and late genes in a conserved segment of the vaccinia virus genome. Nucleic Acids Res 13: 985–998

Pogo BGT, Dales S (1969a) Two deoxyribonuclease activities within purified vaccinia virus. Proc Natl Acad Sci USA 63: 820–827
Pogo BGT, Dales S (1969b) Regulation of the synthesis of nucleotide phosphohydrolase and neutral deoxyribonuclease. Proc Natl Acad Sci USA 63: 1297–1303
Pogo BGT, O'Shea MT (1977) Further characterization of deoxyribonucleases from vaccinia virus. Virology 77: 55–66
Prescott DM, Kates J, Kirkpatrick JB (1971) Replication of vaccinia virus DNA in enucleated L-cells. J Mol Biol 59: 505–508
Proudfoot NJ, Brownlee GG (1976) 3′ non-coding region sequences in eucaryotic messenger RNA. Nature 263: 211–214
Puckett C, Moss B (1983) Selective transcription of vaccinia virus genes in template dependent soluble extracts of infected cells. Cell 35: 441–448
Reddy MK, Bauer WR (1989) Activation of the vaccinia virus nicking-joining enzyme by trypsinization. J Biol Chem 264: 443–449
Rodriguez JF, Kahn JS, Esteban M (1986) Molecular cloning, encoding sequence, and expression of vaccinia virus nucleic acid-dependent nucleoside triphosphatase gene. Proc Natl Acad Sci USA 83: 9566–9570
Rohrmann G, Moss B (1985) Transcription of vaccinia virus early genes by a template-dependent soluble extract of purified virions. J Virol 56: 349–355
Rohrmann G, Yuen L, Moss B (1986) Transcription of vaccinia virus early genes by enzymes isolated from vaccinia virions terminates downstream of a regulatory sequence. Cell 46: 1029–1035
Rosel J, Moss B (1985) Transcriptional and translational mapping and nucleotide sequence analysis of a vaccinia virus gene encoding the precursor of the major core polypeptide 4b. J Virol 56: 830–838
Rosel JL, Earl PL, Weir JP, Moss B (1986) Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the *Hin*dIII H genome fragment. J Virol 60: 436–439
Rosemond-Hornbeak H, Moss B (1974) Single-stranded deoxyribonucleic acid-specific nuclease from vaccinia virus. Endonucleolytic and exonucleolytic activities. J Biol Chem 249: 3292–3296
Rosemond-Hornbeak H, Paoletti E, Moss B (1974) Single-stranded deoxyribonucleic acid-specific nuclease from vaccinia virus. Purification and characterization. J Biol Chem 249: 3287–3291
Roth MJ, Hurwitz J (1984) RNA capping by the vaccinia virus guanylyltransferase. Structure of enzyme-guanylate intermediate. J Biol Chem 259: 13488–13494
Sarov I, Joklik WK (1972) Characterization of intermediates in the uncoating of vaccinia virus DNA. Virology 50: 593–602
Sato K, Ito R, Baek K-H, Aggarwal K (1988) A specific DNA sequence controls termination of transcription in the gastrin gene. Mol Cell Biol 6: 1032–1043
Schwer B, Stunnenberg HG (1988) Vaccinia virus late transcripts generated in vitro have a poly(A) head. EMBO J. 7: 1183–1190
Schwer B, Visca P, Vos JC, Stunnenberg HG (1987) Discontinuous transcription or RNA processing of vaccinia virus late messengers results in a 5′ poly(A) leader. Cell. 50: 163–169
Sebring ED, Salzman NP (1967) Metabolic properties of early and late vaccinia messenger ribonucleic acid. J Virol 1: 550–575
Shaffer R, Traktman P (1987) Vaccinia virus encapsidates a novel topoisomerase with the properties of a eucaryotic type I enzyme. J Biol Chem 262: 9309–9315
Shepard B, Panicali D, Huang C (1989) Transient expression system to measure the efficiency of vaccinia promoter regions. Plasmid. 18: 16–23
Shuman E, Spencer E, Furneaux H, Hurwitz J (1980) The role of ATP in in vitro vaccinia virus RNA synthesis. Effects of AMP-PNP and ATPγS. J Biol Chem 255: 5396–5403
Shuman S (1989) Functional domains of vaccinia virus mRNA capping enzyme. Analysis by limited tryptic digestion. J Biol Chem 264: 9690–9695
Shuman S, Hurwitz J (1981) Mechanism of mRNA capping by vaccinia virus guanylyltransferase: characterization of an enzyme-guanylate intermediate. Proc Natl Acad Sci USA 78: 187–191
Shuman S, Moss B (1987) Identification of a vaccinia virus gene encoding a type I DNA topoisomerase. Proc Natl Acad Sci USA 84: 7478–7482
Shuman S, Moss B (1988a) Factor-dependent transcription termination by vaccinia virus RNA polymerase: evidence that the cis-acting termination signal is in nascent RNA. J Biol Chem 263: 6220–6225
Shuman S, Moss B (1988b) Vaccinia virus poly(A) polymerase: specificity for nucleotides and nucleotide analogs. J Biol Chem 263: 8405–8412

Shuman S, Moss B (1989) Bromouridine triphosphate inhibits transcription termination and mRNA release by vaccinia virions. J Biol Chem (in press)
Shuman S, Surks M, Furneaux H, Hurwitz J (1980) Purification and characterization of a GTP-pyrophosphate exchange activity from vaccinia virions. Association of the GTP-pyrophosphate exchange activity with vaccinia mRNA guanylyltransferase-RNA (guanine-7-)methyltransferase complex capping enzyme. J Biol Chem 255: 11588–11598
Shuman S, Broyles SS, Moss B (1987) Purification and characterization of a transcription termination factor from vaccinia virions. J Biol Chem 262: 12372–12380
Shuman S, Golder M, Moss B (1988) Characterization of vaccinia virus DNA topoisomerase I expressed in *Escherichia coli*. J Biol Chem 263: 16401–16407
Shuman S, Golder M, Moss B (1989) Insertional mutagenesis of the vaccinia virus gene encoding a type 1 DNA topoisomerase: evidence that the gene is essential for virus growth. Virology 170: 302–306
Silver M, McFadden G, Wilton S, Dales S (1979) Biogenesis of poxviruses: role for the DNA dependent RNA polymerase II of the host during expression of late function. Proc Natl Acad Sci USA 76: 4122–4125
Soloski MJ, Holowczak JA (1981) Characterization of supercoiled nucleoprotein complexes released from detergent-treated vaccinia virions. J Virol 37: 770–783
Spencer E, Loring D, Hurwitz J, Monroy G (1978) Enzymatic conversion of 5′-phosphate terminated RNA to 5′-di-, and triphosphate-terminated RNA. Proc Natl Acad Sci USA 75: 4793–4797
Spencer E, Shuman S, Hurwitz J (1980) Purification and properties of vaccinia virus DNA-dependent RNA polymerase. J Biol Chem 255: 5388–5395
Tamin A, Villarreal EC, Weinrich SL, Hruby DE (1988) Nucleotide sequence and molecular genetic analysis of the vaccinia virus *Hin*dIII N/M region encoding the genes responsible for resistance to α-amanitin. Virology 165: 141–150
Tutas DJ, Paoletti E (1977) Purification and characterization of core-associated polynucleotide 5′-triphosphatase from vaccinia virus. J Biol Chem 252: 3092–3098
Van Meir E, Wittek R (1988) Fine structure of the vaccinia virus gene encoding the precursor of the major core protein 4a. Arch Virol. 102: 19–27
Varich NL, Sychova IV, Kaverin NV, Antonova TP, Chernos VI (1979) Transcription of both DNA strands of vaccinia virus genome in vivo. Virology. 96: 412–430
Vassef A (1987) Conserved sequences near the early transcription start sites of vaccinia virus. Nucleic Acids Res. 15: 1427–1443
Vassef A, Ben-Hamida F, Dru A, Beaud G (1982) Translational control of early protein synthesis at the late stage of vaccinia virus infection. Virology. 118: 45–53
Vassef A, Mars M, Dru A, Pluciennicak A, Streek RE, Beaud G (1985) Isolation of cis-acting vaccinia virus DNA fragments promoting expression of herpes simplex virus thymidine kinase by recombinant vaccinia viruses. J Virol 55: 1643–1672
Venkatesan S, Moss B (1981) In vitro transcription of the inverted terminal repetition of the vaccinia virus genome: correspondence of initiation and cap sites. J Virol 37: 738–747
Venkatesan S, Moss B (1982) Eukaryotic mRNA capping enzyme-guanylate covalent intermediate. Proc Natl Acad Sci USA 79: 340–344
Venkatesan S, Gershowitz A, Moss B (1980) Modification of the 5′- end of mRNA: association of RNA triphosphatase with the RNA guanylyltransferase-RNA (guanine-7-)methyltransferase complex from vaccinia virus. J Biol Chem 255: 903–908
Venkatesan S, Baroudy BM, Moss B (1981) Distinctive nucleotide sequences adjacent to multiple initiation and termination sites of an early vaccinia virus gene. Cell. 125: 805–813
Veomett GE, Kates JR (1973) ATP requirement for extrusion of RNA from vaccinia cores and release of RNA from nuclei in vitro. ICN-UCLA Symposium on Molecular Biology. Academic, New York
Villarreal EC, Hruby DE (1986) Mapping the genomic location of the gene encoding α-amanitin resistance in vaccinia virus mutants. J Virol 57: 65–70
Villarreal EC, Roseman NA, Hruby DE (1984) Isolation of vaccinia virus mutants capable of replicating independently of the host cell nucleus. J Virol 51: 359–366
Vos JC, Stunnenberg HG (1988) Depression of a novel class of vaccinia virus genes upon DNA replication. EMBO J. 7: 3487–3492
Wei CM, Moss B (1975) Methylated nucleotides block 5′-terminus of vaccinia virus mRNA. Proc Natl Acad Sci USA 72: 318–322
Weir JP, Moss B (1983) Nucleotide sequence of the vaccinia virus thymidine kinase gene and the nature of spontaneous frameshift mutations. J Virol 46: 530–537

Weir JP, Moss B (1984) Regulation of expression and nucleotide sequence of a late vaccinia virus gene. J Virol 51: 662–669
Weir JP, Moss B (1985) Use of a bacterial expression vector to identify the gene encoding a major core protein of vaccinia virus. J Virol 56: 534–540
Weir JP, Moss B (1987a) Determination of the promoter region of an early vaccinia virus gene encoding thymidine kinase. Virology 158: 206–210
Weir JP, Moss B (1987b) Determination of the transcriptional regulatory region of a vaccinia virus late gene. J Virol 61: 75–80
Wilson EM, Edbauer C, Hruby DE (1988) Characterization of a binding factor that interacts with the sequences upstream of the vaccinia virus thymidine kinase gene. Virus genes. 2: 31–48
Wilton S, Dales S (1986) Influence of RNA polymerase II upon vaccinia virus-related translation examined by means of α-amanitin. Virus Res 5: 323–341
Wing D, Weissbach A (1984) Vaccinia virus RNA polymerase associated with nuclei of infected HeLa cells. J Virol 49: 26–34
Wittek R, Moss B (1980) Tandem repeats within the inverted terminal repetition of vaccinia virus DNA. Cell 21: 277–284
Wittek R, Menna A, Muller K, Schumperli D, Bosley PG, Wyler R (1978) Inverted terminal repeats in rabbit poxvirus and vaccinia virus DNA. J Virol 28: 171–181
Wittek R, Cooper J, Barbosa E, Moss B (1980) Expression of the vaccinia virus genome—analysis and mapping of mRNAs encoded eithin the inverted terminal repetition. Cell 21: 487–493
Woodson B (1967) Vaccinia mRNA synthesis under conditions which prevent uncoating. Biochem Biophys Res Commun 27: 169–175
Woodson B, Joklik WK (1965) The inhibition of vaccinia virus multiplication by isatin-B-thiosemicarbazone. Proc Natl Acad Sci USA 54: 946–953
Wright CF, Moss B (1987) In vitro synthesis of vaccinia virus late mRNA containing a 5′ poly(A) leader sequence. Proc Natl Acad Sci USA 84: 8883–8887
Wright CF, Moss B (1989) Identification of factors specific for transcription of the late class of vaccinia virus genes. J Virol 63: 4224–4233
Yang WP, Bauer WR (1988) Purification and characterization of vaccinia virus structural protein VP8. Virology 167: 578–584
Yuen L, Moss B (1986) Multiple 3′ ends of mRNA encoding vaccinia virus growth factor occur within a series of repeated sequences downstream of T clusters. J Virol 60: 320–323
Yuen L, Moss B (1987) Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. Proc Natl Acad Sci USA 84: 6417–6421
Yuen L, Davison AJ, Moss B (1987) Early promoter-binding factor from vaccinia virions. Proc Natl Acad Sci USA 84: 6069–6073

The Role of Telomeres in Poxvirus DNA Replication

A. M. DeLange[1] and G. McFadden[2]

1 Introduction

Poxviruses comprise a family of eukaryotic viruses which replicate exclusively in the cytoplasm of host vertebrate or invertebrate cells. The life cycle of this virus family (reviewed in Dales and Pogo 1982; Moss 1990) is initiated upon entry of the virions into the host cell. The core of the virion harbors not only the large double-stranded linear DNA genome, but also a great variety of polypeptides that are utilized during early viral transcription and for other events necessary to initiate the infective cycle. The multisubunit virus-encoded RNA polymerase, RNA capping enzyme, and poly(A) polymerase are amongst the enzymes that

[1] Department of Human Genetics, University of Manitoba, Winnipeg, Manitoba R3E OW3, Canada
[2] Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

Current Topics in Microbiology and Immunology, Vol. 163

facilitate early gene expression in the virion core immediately upon entry into the host cell. Accurate early gene expression has also been induced in vitro. Following uncoating of the core, DNA replication takes place in specialized virus-induced structures which have been referred to as "factories", "virosomes", or "micro-nuclei". Our current knowledge of the replication mechanism(s) utilized by this family of viruses is incomplete. Even though poxvirus genome structure is fairly well understood, replicative intermediate structures are ill-defined, as are the enzymes that mediate poxvirus DNA replication (reviewed in McFadden and Dales 1982; Holowczak 1982; Moss 1990). The poxvirus genome consists of a single linear double-stranded $1-2 \times 10^8$ dalton DNA molecule, which is terminally cross-linked and often contains characteristic sets of tandem repeats in an apparently noncoding region adjacent to the terminal cross-link. Approximately 100–200 tightly packed intronless genes encode all or most proteins required for viral gene expression and DNA replication. Replication of this large DNA molecule is believed to be initiated and terminated at or near the cross-linked termini (Esteban et al. 1977; Pogo et al. 1981, 1984). The observation that nicks are introduced at or near the ends of parental DNA molecules soon after infection and then become sealed as cross-links in fully replicated mature daughter molecules (Esteban and Holowczak 1977; Pogo 1977, 1980; Pogo and O'shea 1978) lends additional credence to the view that the extreme termini perform a crucial function during initiation and termination of poxvirus DNA replication. This review will summarize our current knowledge on the structure and function of the telomeres of poxvirus genomes. Telomeres have been functionally defined as that region of DNA at the molecular end of a linear eukaryotic chromosome that is required for replication and stability of that chromosome (Blackburn and Szostak 1984). Here we arbitrarily define the poxvirus telomere as the noncoding DNA sequences at the end of the linear viral DNA molecule. Two elements of the poxviral telomere were first recognized in members of the *Orthopoxvirus* genus on the basis of structure and DNA sequence: (a) the terminal crosslink, which consists of an AT-rich incompletely base-paired hairpin structure; and (b) an adjacent region which usually comprises sets of relatively simple tandem repeats but may consist of more complex tandem elements (e.g., in the *Leporipoxvirus* genus). The structural and functional aspects of these elements are reviewed in the following sections. In addition, a third element, the telomere resolution target (TRT) region has been defined on the basis of its function during the segregation of daughter hairpin-terminated molecules from replicative intermediates.

2 Structure and Function of Eukaryotic Telomeres

The complete replication and stable maintenance of the ends of linear DNA molecules require special adaptations to account for the need of all known DNA polymerases to utilize a preexisting 3′-hydroxyl primer (Watson 1972;

CAVALIER-SMITH 1974; BATEMAN 1975) and to prevent the natural tendency of intracellular broken DNA ends to fuse or recombine (MCCLINTOCK 1941; 1942). These problems have been solved in a variety of ways. For example, linear genomes of phages T4 and T7 have sidestepped this problem by concatemerization (BROKER 1973; WATSON 1972). Alternatively, bacteriophage $\theta 29$ and the mammalian adenoviruses utilize a terminal covalently attached protein as primer for DNA synthesis (reviewed in KELLY et al. 1988). Another way of coping with these problems has been adopted and apparently conserved at the ends of higher order eukaryotic chromosomes. Terminal satellite-like repeats, initially identified in lower eukaryotes such as protozoa and fungi (reviewed in BLACKBURN and SZOSTAK 1984; WEINER 1988), have now also been found at the extreme ends of chromosomes in higher plants and mammals (RICHARDS and AUSUBEL 1988; MOYZIS et al. 1988). Telomere replication is thought to proceed in a template-independent terminal transferase type reaction (GREIDER and BLACKBURN 1985; 1989; CECH 1988). Another class of telomeres adopts a more simple structure, namely, a covalently closed hairpin. Hairpin termini have been observed in *Tetrahymena* rDNA (BLACKBURN and GALL 1978), *Paramecium* mitochondrial DNA (PRITCHARD and CUMMINGS 1981), linear plasmids of yeast (KIKUCHI et al. 1985), linear plastids of barley (ELLIS and DAY 1986), parvoviruses (BERNS et al. 1985), African swine fever virus (GONZALEZ et al. 1986), and poxviruses (BAROUDY et al. 1982a; DELANGE et al. 1986). Even the linear plasmid of the prokaryote *Borrelia burgdorferi*, the causative agent of Lyme disease, may prove to contain terminal hairpins (BARBOUR and GARON 1987).

3 Structure of Poxvirus Telomeres

3.1 Poxvirus Telomeres Are Cross-Linked: A Historical View

The existence of cross-links in the linear double-stranded DNA genome of poxviruses was first suggested by the observation that after denaturation of virion-derived poxvirus DNA a significant fraction of the DNA reanneals rapidly in a concentration-independent fashion. This "snapback" DNA of fowlpox (SZYBALSKI et al. 1963), cowpox and vaccinia (JUNGWIRTH and DAWID 1967) was initially detected by sedimentation in neutral gradients. Subsequently, BERNS and SILVERMAN (1970) demonstrated that the complementary strands of vaccinia virus DNA failed to separate in alkaline sucrose gradients.

Electron microscopic analysis of denatured and partially denatured vaccinia DNA located the "cross-links" at the two extreme ends of the linear DNA molecule (GESHELIN and BERNS 1974). Various methods have been used to identify terminal cross-links in a variety of poxvirus genera. Among these are: (a) electron microscopic observation of denatured DNA, (b) unique sedimentation behavior of intact, cross-linked molecules in alkaline sucrose gradients, and

(c) anomalous behavior of viral restriction fragments containing the terminal cross-links, when analyzed by gel electrophoresis under alkaline conditions (McCarron et al. 1978). The most widely used method, however, involves the rapid reannealing property of restriction fragments containing the terminal cross-links and their visualization in neutral agarose gels (DeFilippes 1976; Jaureguiberry 1977). Presently, terminal cross-links have been identified in every vertebrate poxvirus tested, including orthopoxviruses vaccinia (Geshelin and Berns 1974), rabbitpox (Wittek et al. 1977), cowpox, monkeypox, variola, ectromelia, taterapox, raccoonpox, and camelpox (Esposito and Knight 1985); parapoxviruses stomatitis papulosa virus (Menna et al. 1979), Milkers node virus (Thomas et al. 1980), and orf (Mercer et al. 1987); the avipoxvirus fowlpox (Szybalsky et al. 1963; Gafford et al. 1978); capripoxviruses (Black et al. 1986); leporipoxviruses Shope fibroma virus (Jacquemont et al. 1972; Wills et al. 1983); myxoma (McFadden, unpublished work); and unclassified poxviruses molluscum contagiosum (Parr et al. 1977) and Yaba monkey tumor poxvirus (Kilpatrick and Rouhandeh 1985, 1987). This comprehensive list leaves little doubt that terminal cross-links are a universal feature of poxvirus telomeres.

The precise molecular identification of the terminal cross-link was first achieved by direct sequencing of the DNA terminus from vaccinia virus strain *WR* (Baroudy et al. 1982a). This study established that the two complementary strands of the vaccinia linear genome are connected into a continuous polynucleotide chain. The resulting AT-rich terminal hairpin structure was shown to contain

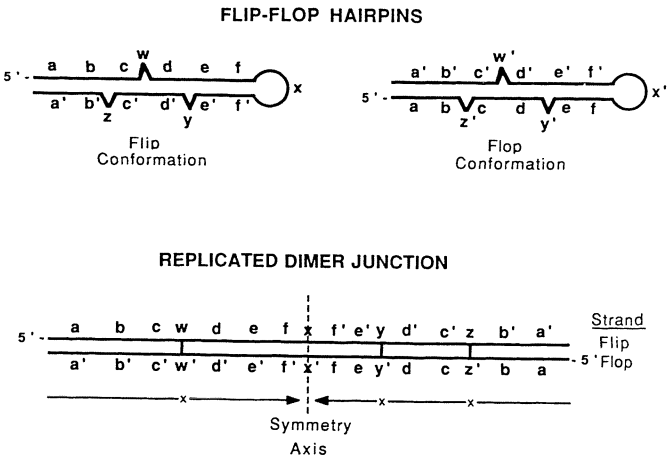


Fig. 1. Relationship of poxviral flip/flop hairpin termini to the dimer junction configuration. The relative sequence orientations of flip/flop terminal hairpins are represented for a hypothetical viral genome with three extrahelical bases (x,y,z) and multiple non-base-paired "turnaround" nucleotides (x). Note that the replicated dimer junction consists of one strand of the flip sequence and one of the complementary flop strand. Thus, the bases which are extrahelical in the hairpin configuration are nonpalindromic (denoted by the *crosses* in each arm of the dimer junction) in the inverted repeat arrangement of the dimer

extrahelical bases and exist in two conformations (flip and flop) which are inverted and complementary (shown schematically in Fig. 1). Subsequently, DeLange et al. (1986) confirmed this structure in vaccinia strain *IHD-W* and in the leporipoxvirus Shope fibroma virus (SFV). Interestingly, African swine fever virus (ASFV), a virus which was initially classified as an iridovirus, has a similar, AT-rich, incompletely base-paired hairpin (Gonzalez et al. 1986). Although the precise sequence of the hairpin is not conserved, the AT-richness, presence of extrahelical bases, and flip-flop conformations were observed in each case.

3.2 Conformation of Poxvirus Telomeres During DNA Replication In Vivo

The mature poxvirus genome that is packaged in virions contains a hairpin structure at each end. During DNA replication this hairpin becomes transiently converted into an inverted repeat conformation which has its axis of symmetry at the original hairpin end. This apparently replicative intermediate form was first identified in rabbitpox virus (Moyer and Graves 1981) and has since been detected in vaccinia virus (Baroudy et al. 1982b; Moss et al. 1983) and SFV (DeLange and McFadden 1986). Based on the relative amounts of the dimeric inverted repeat and hairpin termini, Moyer and Graves (1981) suggested that the inverted repeat conformation of the viral telomere joins genomic units into head-to-head and tail-to-tail concatemeric arrays. When replicating DNA is sedimented in a neutral sucrose gradient, the inverted repeat conformation of the viral telomere is found in DNA that sediments faster than unit-length molecules, similarly suggesting that they are part of concatemeric arrays (Moss et al. 1983). In subsequent experiments, Merchlinsky and Moss (1986, 1988) have referred to this conformation as "concatemer junction". In this review we will use the terms "telomere fusion" or "dimer junction" which do not infer the status of any one particular genomic milieu. We will use "telomere fusion" in a broad sense to designate the dimeric inverted repeat conformation and reserve "dimer fusion" for the quasi-palindromic region around the axis of symmetry (see Fig. 1). Several models have been proposed to account for the presence of the dimer junction conformation in replicating DNA. These models invoke either replication through the hairpin (i.e., synthesis of a daughter flip sequence from a flop template or vice versa) or the isomerization of two preexisting complementary hairpins. Two versions of the replication type model have been proposed. First, the self-priming model is an extension of the terminal palindrome model of Cavalier-Smith (1974), as modified for hairpin termini by Bateman (1975). The model proposes the introduction of a near-terminal nick, with the use of the resulting 3′ end as a primer for the synthesis of a terminal palindrome which folds back upon itself, thus acting as a primer for further DNA replication of the viral genome. The telomere fusion configuration would result when the replication fork reached the opposite end of the genome and replicated through an intact hairpin (Moyer and Graves 1981; Baroudy et al. 1982b; Moss et al. 1983). Second, the "de novo

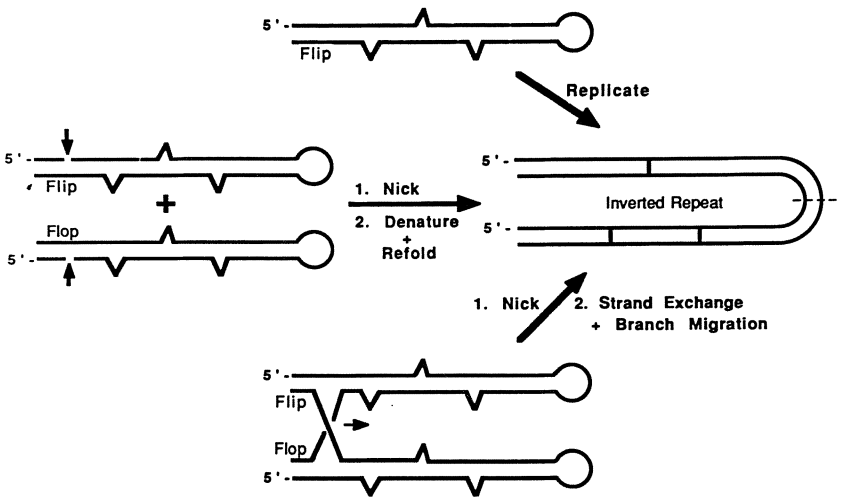


Fig. 2. Possible mechanisms for the conversion of hairpin termini to the dimer junction configuration. A dimer junction can be generated either by replication or hairpin annealing. A replication fork, initiated by a self-priming or de novo primed mechanism, will progress around a terminal hairpin to create an inverted repeat replicative intermediate structure. Alternatively, a flip and flop hairpin could potentially align and undergo an exchange and annealing event mediated by nicking, denaturation, and refolding, or by nicking, strand invasion, and branch migration towards the hairpin ends. Note that the combination of nicking, strand exchange, and branch migration, proposed here to generate dimer junctions, is similarly utilized in models of the reverse reaction from dimer junctions back to hairpin termini (see Fig. 5)

start" model proposes replication through hairpins from an internal RNA primer (ESTEBAN et al. 1977; BAROUDY et al. 1982b; MOSS et al. 1983). The isomerization models, on the other hand, do not require DNA synthesis (BAROUDY et al. 1982b). Instead, isomerization is made possible by the placement of an identical nick on adjoining flip and flop hairpins followed either by melting and reannealing or strand-exchange and branch migration (Fig. 2). Isomerization therefore will fuse preexisting hairpins into either circular or concatemeric DNA molecules. The major drawback of the model is that, to generate a correct dimer junction, it only allows joining of two hairpins that are inverted and complementary to each other, i.e., flip and flop. Isomerization of identical hairpins would generate an incompletely base-paired dimer junction.

3.3 Cloning of Poxvirus Telomeres

It is a well known fact that linear DNA molecules cannot be propagated in bacterial strains, such as *Escherichia coli*, that are commonly used for molecular cloning purposes. Several approaches designed to overcome this problem have allowed cloning of all or part of the poxvirus hairpin terminus. In each case, part

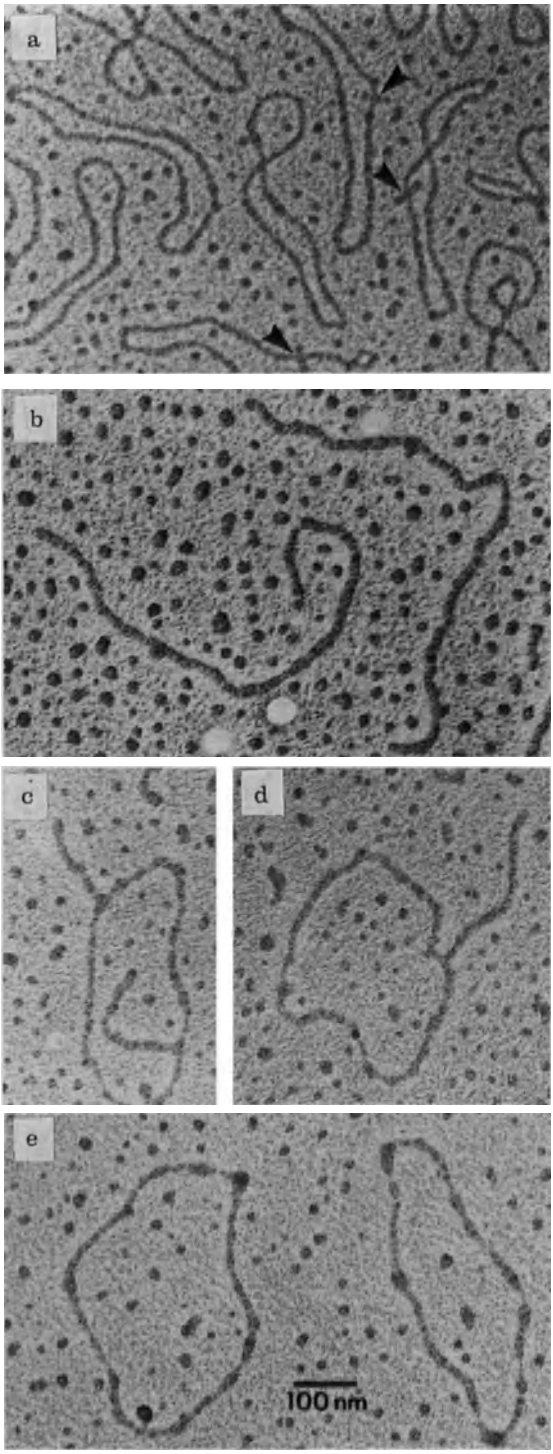


Fig. 3a–e. Electron micrographs of circular plasmids with telomere fusion inserts and the resolved linear minichromosomes from transfected cells. **a** Circular plasmids containing a 0.6 kb palindromic insert of the Shope fibroma virus telomere are visualized and the extruded cruciforms indicated by the arrows. Note that in any single molecule one cruciform arm corresponds to a flip sequence and the other to a flop. The base of the cruciform is a freely mobile Holliday cross-over junction. **b–e** Minichromosomes derived from transfection with either lineform or cruciform-containing plasmids into poxvirus-infected cells are shown at neutral pH (*b*) or under increasingly stringent denaturing conditions (*c–e*) (Reproduced from DeLange et al. (1986), with permission)

or all of the telomere fusion conformation was cloned into circular vectors. The first reported case involved cleavage of the hairpin of vaccinia strain *WR* with S1 nuclease and in vitro extension of the hairpin loop from a site of cleavage within the hairpin. This approach allowed synthesis and cloning of part of the telomere fusion element in an *E. coli* plasmid (PICKUP et al. 1983). DELANGE et al. (1984, 1986) utilized the ability of yeast to stably maintain long inverted repeats within circular plasmids. In this case, gel-purified hairpin-containing restriction fragments were ligated at both ends of a linearized yeast cloning vector and introduced into a recipient yeast strain. Circular plasmid clones, containing a complete copy of the telomere fusion elements of vaccinia virus *IHD-W* and of SFV were obtained in this fashion. To obtain larger amounts of the poxvirus insert, small restriction fragments containing the telomere fusion configuration of SFV and vaccinia were subcloned from yeast into pUC vectors propagated in highly recombination-deficient *E. coli* (DELANGE et al. 1986). The palindromic inserts were confirmed both by DNA sequencing and by electron microscopic observation of extruded cruciforms (see Fig. 3a). Two other approaches have been used to clone telomere fusion fragments of vaccinia. First, the fusion elements were generated in vitro by annealing of complementary strands from denatured hairpins. These were then cloned into an *E. coli* plasmid (WINTERS et al. 1985). Second, telomere fusion fragments were excised from replicating vaccinia DNA which was isolated from infected cells when their abundance was maximal and directly cloned into *E. coli* (MERCHLINSKY and MOSS 1986). In each case, the sequence of the cloned telomere fusion fragment was determined and found to be as expected for the proposed dimer junction configuration (Fig. 1). Interestingly, the vaccinia junction sequences, which contain 14 nonpalindromic base pairs, are stable in a *rec*A host, whereas the SFV sequences contain only 8 nonpalindromic base pairs but require a *rec*A *rec*BC host to be stably maintained.

4 Telomere-Associated Sequences

4.1 Repeated Sequences Near Ends of Linear Chromosomes

Telomere-associated sequences have been defined as those regions near the ends of chromosomes that contain repeated DNA sequences. Such repeats, which may extend many kilobases from the molecular end of chromosomal DNA, have been located in yeast, rye, *drosophila*, and *physarum* (reviewed in BLACKBURN and SZOSTAK 1984). In addition, the linear mitochondrial DNA molecule of *Tetrahymena* contains telomeric tandem repetitions 31–53 bp in length (MORIN and CECH 1986; 1988). The number of repeat units at any telomere varies, probably as a consequence of homologous recombination between repeats at different chromosome ends. The functional significance of these terminal repeats is still an

open question. It has been suggested that these elements mediate many of the interactions that occur both among telomeres and between telomeres and the nuclear envelope (YOUNG et al. 1983). Such associations may facilitate the apparent high frequency of recombination between repeat units on different chromosomes. Finally, at least in the case of *Physarum*, repeat units have been shown to be capable of forming complex hairpin structures which may both stabilize the ends and facilitate controlled DNA replication of the termini (BERGOLD et al. 1983; EMERY and WEINER 1981; JOHNSON 1980).

4.2 Poxvirus Repeats

Poxvirus telomeres contain reiterated DNA sequences, which are apparently noncoding (WITTEK et al. 1980; PICKUP et al. 1982; MACAULAY et al. 1987) and resemble in some respects telomere-associated sequences in higher order eukaryotic chromosomes. WITTEK and MOSS (1980) first identified tandem repeats near the ends of the vaccinia genome by the molar abundance of a 70 bp restriction fragment. Subsequent sequencing of the vaccinia terminal region revealed two blocks of repeats separated by a unique 325 bp sequence. The block of repeats distal to this 325 bp element contained thirteen 70 bp repeats, while the proximal region contained eighteen 70 bp repeats, two 125 bp repeats, and eight 54 bp repeats. The 125 bp and 70 bp elements appear to have evolved from the 54 bp repeat (BAROUDY et al. 1982a, b; BAROUDY and MOSS 1982). The tandem repeats of the related orthopoxvirus cowpox virus (CPV) are similarly arranged as two sets of repeated sequences separated by a unique 312 bp sequence, which is 97% homologous to the analogous 325 bp sequence of vaccinia virus (PICKUP et al. 1982). The DNA termini of CPV contain four types of repeat units which are related to each other, two of which are highly homologous to regions within the 70 bp repeat unit of vaccinia virus. That this repeat unit is highly conserved among orthopoxviruses was demonstrated by hybridization of the 70 bp repeat unit to terminal DNA fragments of 37 out of 38 tested orthopoxvirus isolates (ESPOSITO and KNIGHT 1985), including monkeypox, monkeypox white variants, variola, several vaccinia strains, ectromelia, taterapox, cowpox, and camelpox. Only raccoonpox (RCN) did not hybridize to the 70 bp repeat. DNA sequence analysis of the termini of this latter virus revealed two sets of tandem repeats separated by a 254 bp unique sequence, similar to the arrangement found in vaccinia and CPV. Instead of the homogeneous 70 bp repeat of vaccinia, RCN contains six related types of sequences that are reiterated more or less at random. The RCN repeats possess a core sequence in common with the repeats of vaccinia and CPV. The high degree of similarity between the ends of RCN DNA and the ends of the other orthopoxvirus DNAs suggests that these sequences are intimately associated with virus multiplication.

Besides the *Orthopoxvirus* genus, terminal tandem repeats have also been reported in the parapoxvirus orf (ROBINSON et al. 1987). On the other hand, only a moderate number of imperfect repeats can be found near the ends of the

leporipoxvirus SFV. This virus possesses five tandemly arranged related but nonidentical repeat units (UPTON et al. 1987). The observation that each of these repeats is an imperfect palindrome is so far unique to this poxvirus genus.

As has been observed for some higher order chromosomal telomeres, the length of the terminal restriction fragments of vaccinia virus is heterogeneous. This length heterogeneity, which is due to variation in the number of repeat units, may be caused by unequal crossing over between 70 bp repeat units or between sets of 70 bp repeats (MOSS et al. 1981; BAROUDY and MOSS 1982); however, length heterogeneity need not be universal in poxviruses, since it is not detected in the leporipoxviruses and parapoxviruses. The frequency of "illegitimate" unequal crossing over may well be under viral genetic control, but as yet little is known about such control mechanisms.

5 In Vivo Replication of Cloned Poxvirus Telomeres

5.1 Transfection Assay

Autonomous replication of viral or plasmid DNA in the nucleus of mammalian cells has a stringent requirement for the presence of replication origin sequences on the replicating plasmid DNA (reviewed by KUCHERLAPATI and SKOULTCHI 1984). In contrast, the cytoplasmically replicating poxviruses facilitate replication of transfected plasmid DNA in a sequence-nonspecific fashion (DELANGE and MCFADDEN 1986). The transfection assay used to monitor this replication utilized coprecipitation of plasmid DNA with calcium phosphate (GRAHAM and VAN DER EB 1973) to facilitate the entry of DNA into poxvirus-infected cells and made use of restriction enzymes such as *DpnI* which cleaves methylated "input" DNA but fails to cleave newly replicated unmethylated DNA (PEDEN et al. 1980). The origin-independent replication of plasmid DNA in poxvirus-infected cells has the following characteristics:

1 No viral or any other fortuitous replication origin sequence is required. As long as the template DNA is circular, efficient replication will be catalyzed (DELANGE and MCFADDEN 1986).
2 There is no evidence of cis-acting viral DNA sequences that enhance plasmid replication (MERCHLINSKY and MOSS 1988).
3 The variable fraction of the input transfected DNA that actually enters the replicative pool replicates autonomously into high molecular weight head-to-tail concatemers (DELANGE and MCFADDEN 1986).
4 A high frequency of homologous recombination between cotransfected plasmids always accompanies DNA replication (DELANGE et al. 1986; EVANS et al. 1988).

5 Autonomous plasmid replication does not occur in uninfected cells but is dependent on trans-acting viral replication factors (DeLange and McFadden 1986; Merchlinsky and Moss 1988).
6 Even though it is known that plasmid replication takes place in the cytoplasm of infected cells, the precise intracytoplasmic location is still undefined.

The failure to observe substantial levels of recombinants between the viral genome and the autonomous plasmid concatemers under conditions where recombination between transfecting plasmids is readily detected suggests that a site other than the viral "factories" is utilized. Finally, this replication system is now used extensively to study both cis- and trans-acting factors in viral DNA replication and recombination. The utility of such assay systems for dissecting viral replication schemes has been recently reviewed by Hay and Russell (1989). The following section describes the use of the transfection assay to study the mechanism of resolution of the replicative intermediate telomere fusion conformation to hairpin termini.

5.2 In Vivo Resolution of Cloned Poxvirus Telomeres

When circular plasmids containing telomere fusion fragments were transfected into poxvirus-infected cells, a high proportion of replicated plasmid DNA was found to be in a linear hairpin-terminated conformation (DeLange et al. 1986; Merchlinsky and Moss 1986). Interestingly, even though vaccinia virus and SFV are in two different genera and viral DNA probes do not cross-hybridize, the telomere fusion element from each virus is resolved into hairpin termini in cells infected with the heterologous virus. The generated linear "minichromosomes" possess intact hairpins at both ends and are covalently-closed through their length such that double-length single-stranded circles can be readily observed by electron microscopy under fully denaturing conditions (Fig. 3b-e). Using sets of staggered unidirectional deletions from either end and bidirectional central axis deletions from a unique *Afl*II site at the symmetry axis of the palindromic telomere fusion fragment of SFV, it was shown that all of the target sequences required for this resolution event lie within a 58–76 bp domain which is found in close proximity to, but does not include, the nonpalindromic nucleotides near the symmetry axis (Fig. 4). To function as substrate for telomere resolution, this target sequence must be present as two copies in the inverted repeat orientation (DeLange et al. 1986; DeLange and McFadden 1987). Parts of the sequence within the TRT domain of the leporipoxvirus SFV are fairly well conserved in vaccinia and other orthopoxviruses (DeLange et al. 1986; Esposito and Knight 1985; Parsons and Pickup 1987). A subset (region I) is even conserved in ASFV, an iridovirus which shares a number of genomic features, such as flip/flop hairpin termini, with members of the poxvirus family (Gonzalez et al. 1986). Functional analysis of the vaccinia telomere indicates that this region of homology is

Fig. 4. Boundaries of poxviral telomere resolution target domain. Top: schematic representation of the two inverted copies of the Shope fibroma virus (*SFV*) resolution domain in the dimeric conformation to illustrate the relative orientation of the eight nonpalindromic bases (*vertical lines*) with the symmetry axis (*dotted line*). The minimal domain, which includes regions I and IA, can catalyze accurate resolution at low efficiency. The auxiliary regions II and III are required for maximal efficiency. *Below*: comparable regions from three Orthopoxviruses: raccoonpox (*RCN*), cowpox (*CPV*) and vaccinia (*VAC*). The *boxed areas* mark regions of sequence conservation and the *dots* indicate mismatches. It is noteworthy that purines and pyrimidines are conserved rigidly in the boxed areas of regions II and III

similarly required for telomere resolution in vaccinia (DeLange et al. 1986; Merchlinsky and Moss 1986; 1988). To determine the spatial requirements and significance of symmetry between the two copies of the TRT domain, various nonviral DNA sequences were inserted at the axis of symmetry of a plasmid (pSAD2) which contains a perfect palindrome of the TRT domain of SFV. Such constructs demonstrate an absolute requirement for inverted symmetry at the central axis, but there appeared to be no sequence specificity in the region between the two copies of the TRT domain (McFadden et al. 1988). The most likely interpretation of this observation is that cis-acting TRT sequences function as targets for viral trans-acting factors and that some form of strand exchange, such as branch migration involving the central axis, is required for resolution.

The conserved TRT domain defined in SFV on the basis of deletion and insertion analysis consists of a "core" minimal resolution domain, region I/IA, and an auxiliary domain, regions II and III, plus possibly an intervening palindromic sequence PAL (DeLange et al. 1986). Deletion of any part of the core domain completely eliminates resolution, whereas deletion of regions II, III or PAL merely reduces the efficiency of resolution. The PAL in the SFV TRT domain is not present in the analogous domain of vaccinia and may contribute to the reduced efficiency of resolution of the vaccinia telomere fusion element in cells infected with SFV. A short palindrome at the distal end of region III of vaccinia is probably not required in the resolution event, since: (a) destruction of this palindrome, which harbors a *Xba*I site, has no apparent effect on resolution efficiency (Merchlinsky and Moss 1988) and (b) the distal part of region III, including the small palindrome, is not conserved in the Orthopoxvirus RCN

(PARSONS and PICKUP 1987). Except for the distal four nucleotides of region III, regions II and III of vaccinia, CPV and RCN differ by only one or two base pairs, in each case maintaining the order of purines and pyrimidines. This may be important since much of the conservation of regions II and III between vaccinia and SFV is based on a conserved order of purines and pyrimidines (DELANGE et al. 1986). Even though the significance of this observation is not yet understood, it appears that functional integrity of the TRT domain does not permit a significant deviation from the order of purines and pyrimidines. Finally, the 11 bp core element I is perfectly conserved in SFV, vaccinia, CPV, RCN, and even in ASFV (Fig. 4). Furthermore, the 5 bp sequence of region IA, which lies within the "core" domain, is also strictly conserved in SFV, vaccinia, RCN, and CPV. Recent site-directed mutagenesis experiments involving the vaccinia TRT indicate that both regions I and IA are particularly critical for accurate resolution, whereas the intervening four or five nucleotides appear to perform only a spacer function (MERCHLINSKY and MOSS, personal communication).

6 Genetics of Telomere Resolution

All available evidence indicates that most or all trans-acting factors required for poxvirus DNA replication and transcription are virus-encoded (MOSS 1990). This feature renders the poxvirus family particularly amenable to analysis by the powerful combination of genetic and molecular approaches. The genetic analysis of the prototype poxvirus vaccinia has been initiated through the isolation and characterization of drug-resistant and temperature-sensitive mutants (for review, see CONDIT and NILES, this volume). More recently, mutants have been isolated by reverse genetics or the directed mutagenesis approach (e.g., NILES and SETO 1988; BULLER et al. 1988). Characterization of temperature-sensitive and drug-resistant mutants has allowed identification of three complementation groups that are essential for poxvirus DNA synthesis. A detailed description of these genes and their gene products is presented elsewhere (TRAKTMAN, this volume).

The genetic approach has also been used to identify gene products that are required for the resolution of the replicative intermediate telomere fusion configuration into hairpin termini. Thus far, six noncomplementing temperature-sensitive mutants have been identified, each of which fails to resolve the great majority of telomere fusion elements (MERCHLINSKY and MOSS 1988; DELANGE, 1989). At the nonpermissive temperature these mutants generate a series of linear concatemers which are visualized by pulsed field gel electrophoresis (MERCHLINSKY and MOSS, 1989; DELANGE, 1989). In addition, much of the replicating viral DNA that is unable to enter the agarose gel is similarly concatemeric. Resolution-defective mutants *tsC7*, *tsC21*, *tsC22*, *tsC53*, *tsC63* (THOMPSON and CONDIT, 1986), and *ts9383* (DALES et al. 1978) were subdivided into three categories, (DELANGE, 1989). [The designation *tsC7*,... rather

than *ts7*,... has been adopted to avoid confusion with isolates from other mutant collections (SETO et al. 1987).] First, mutants in complementation groups *gp2* (*tsC21*), *gp10* (*tsC53*), and *gp17* (*tsC7*) are extremely defective in late protein synthesis. *gp10* and *gp17* are known to encode the 147 kDa and 22 kDa subunits of the virus-encoded RNA polymerase (THOMPSON et al. 1989; HOODA-DHINGRA et al. 1989), and *gp2* probably encodes the 21 kDa subunit of RNA polymerase (SETO et al. 1987). Second the *gp25* (*tsC22*) mutant displays a normal switch from early to late protein synthesis followed by an abrupt cessation of late protein synthesis. This phenotype, which has been referred to as "abortive late", is caused by the rapid degradation of late RNA (PACHA and CONDIT 1985). These two classes of mutants functionally link the presence of late proteins with the ability to resolve the telomere fusion element, strongly suggesting that one or more late proteins are required for efficient telomere resolution. Consistent with this notion, the antipoxviral drug isatin beta-thiosemicarbazone, which blocks late protein synthesis, also causes concatemer buildup (MERCHLINSKY and MOSS 1989). A third category of resolution-defective mutants, represented by *gp14* (*tsC63*) and *ts9383*, has an apparently normal switch from early to late protein synthesis and only a nominal decrease of some or all late proteins at later times. Whether these genes encode subunits of the hypothetical poxvirus telomere resolvase remains to be determined.

Thus, the overall conclusion is that while poxviral early proteins are clearly required for replication and amplification of telomeric DNA sequences, synthesis of one or more late proteins appears to be a prerequisite for efficient resolution into daughter terminal hairpins. Preliminary evidence also indicates that resolution can still occur in the presence of cytosine arabinoside, an inhibitor of DNA synthesis, suggesting that replication and resolution need not occur concomitantly (MERCHLINSKY and MOSS 1989).

7 In Vitro Studies

7.1 Extrusion of Cloned Poxvirus Telomeres into Cruciforms

The poxvirus telomere fusion insert within circular plasmids isolated from *E. coli* can exist in the lineform or cruciform. The extruded cruciform is found in negatively supercoiled plasmids isolated from bacteria and, as a consequence, can completely relax the plasmid if the inverted repeat is sufficiently long (see Fig. 3a). The kinetics of cruciformation have received recent attention because one of the models for telomere resolution postulates a cruciform as an intermediate (MCFADDEN and MORGAN 1982; SZOSTAK 1983). The cruciform intermediate is especially attractive because recombination "Holliday resolvases" such as T4 endonuclease VII (MIZUUCHI et al. 1982; KEMPER et al. 1984; LILLEY and KEMPER 1984) and T7 endonuclease I (PANAYOTATOS and WELLS 1981; DE MASSY et al.

1984; DICKIE et al. 1987b) effectively nick cruciforms at or very near their base and in the process resolve cruciform-containing molecules to linear hairpin-terminated products (MCFADDEN et al. 1988). The energetics of cruciformation of poxviral telomere fusion inserts have been examined by two-dimensional agarose gel electrophoresis (DICKIE et al. 1987a; REDDY 1988). This approach allows determination of parameters that govern lineform-to-cruciform transition. It has been shown that cruciformation of the native SFV telomere fusion requires a high energy of formation ($G_f = 44$ kcal/mol) and an apparently low activation energy (DICKIE et al. 1987a). In these in vitro studies, the high energy of formation is provided by the torsional energy of supercoiling, G_s. Once the initial energy of formation is provided by topoisomers of sufficient negative supercoiling, the low activation energy ensures that cruciform extension is rapid. By comparing cruciformation in a series of bidirectional central axis deletions of the telomeric insert it was shown that the presence of extrahelical bases in the terminal hairpin structures contribute substantially to the high G_f value. Perhaps more surprisingly, TRT-proximal sequences that flank the extruded cruciform appear to markedly influence the G_f value. The kinetics of cruciformation of a vaccinia telomere fusion insert have been determined by examining head-to-head dimers containing two copies of a vaccinia virus telomere fusion fragment. In each case, only one set of stem-loop structures per molecule was observed, suggesting that, as with the SFV telomere, the initial formation of a small cruciform rather than cruciform extension is the rate limiting step in cruciformation (MERCHLINSKY et al. 1988). Taken together, these data indicate that no insurmountable energetic or thermodynamic barriers exist which would preclude cruciformation of these telomeric fusion sequences in vivo. Whether cruciforms are transiently formed as obligatory intermediates in infected cells remains to be determined.

7.2 In Vitro Resolution of Cloned Poxvirus Telomeres

Once the telomere fusion elements were cloned and shown to function as bona fide targets for resolution into genuine hairpin termini, attempts were made to utilize these as substrates for enzyme activities in vitro. To begin to reconstitute resolution in vitro, two approaches have been adopted. First, cruciform-extruded telomeric inserts have been treated with T4 endonuclease VII or T7 endonuclease I to directly generate linear hairpin-terminated plasmids (DICKIE et al. 1987b; 1988; MERCHLINSKY et al. 1988). Even though these linearized molecules contain nicks, a DNA ligase activity can seal these and hence generate an intact, covalently closed, hairpin-terminated DNA molecule (MCFADDEN et al. 1988). These two recombination endonucleases have also been used to map the locations of the mobile cruciform base in vitro. Second, a search for a poxvirus enzymatic activity capable of resolving the telomere fusion configuration to terminal hairpins has been initiated. LAKRITZ et al. (1985) have identified a nuclease activity from vaccinia virions which introduces nicks and cross-links in negatively supercoiled target DNA plasmids. The activity comigrates with a

50 kDa polypeptide which was initially identified as a single-strand-specific endonuclease with a pH optimum of 7.8. Whether or not these two activities are catalyzed by the same protein is not yet established, inasmuch as the nicking/cross-linking activity of the cross-linking enzyme has a pH optimum of 6.5. This activity was shown to preferentially cleave at or near the axis of symmetry of the telomere fusion insert (REDDY 1988; REDDY and BAUER 1989; MERCHLINSKY et al. 1988). Under optimal conditions the enzyme cleaved preferentially within an S1-sensitive region in or just proximal to region IA, which is required for telomere resolution (MERCHLINSKY et al. 1988). The relatively low efficiency of nicking and cross-linking observed in this in vitro reaction indicates that if the 50 kDa nuclease is indeed involved in the telomere resolution event, one or more factor(s) is (are) probably still missing. That one such factor may be a protease is indirectly suggested by the finding that trypsin significantly stimulates cross-linking activity of the enzyme (REDDY 1988; REDDY and BAUER 1989). Finally, it was found that the nuclease probably generates 5′-hydroxyl and 3′-phosphate ends, reminiscent of eukaryotic topoisomerase I-cleaved DNA (MERCHLINSKY et al. 1988).

8 Models for Poxvirus Telomere Resolution

Two principle types of models have been advanced to rationalize the resolution of hairpin termini from an inverted repeat replicative intermediate (Fig. 5). Neither model involves the synthesis of new phosphodiester bonds but merely serves to isomerize phosphodiesters existing in the palindromic intermediate into the segregated hairpin arrangement. The "nicking and strand exchange" model is a version of the terminal palindrome model of CAVALIER-SMITH (1974) as modified for hairpin termini (BATEMAN 1975). In this model resolution is initiated by the introduction of staggered nicks. The BATEMEN version originally proposed unwinding and subsequent reannealing to generate hairpin termini, but the absolute requirement for symmetry of DNA between the two inverted copies of the TRT domain suggests that branch migration rather than unwinding must be incorporated in the model (MCFADDEN et al. 1988). This modified scheme, illustrated in the bottom part of Fig. 5, involves nicking at or near the TRT followed by strand exchange and branch migration to generate the flip and flop daughter hairpins. The "cruciform-extrusion" model postulates that the first event involves helix unwinding, which can be initiated at the central axis or at some defined internal sequence (for example, within the TRT region; see MCFADDEN et al. 1988). The generated Holliday structure could then be resolved to hairpin termini by an enzyme activity analogous to the T4 endonuclease VII or T7 endonuclease I. It should be noted that the isomerization reactions of both models involve nicking, closing, and branch migration activities. To maintain the intact flip and flop hairpin structures in either case it is essential that the staggered nicks occur in a symmetrical fashion outside the central region of imperfect symmetry.

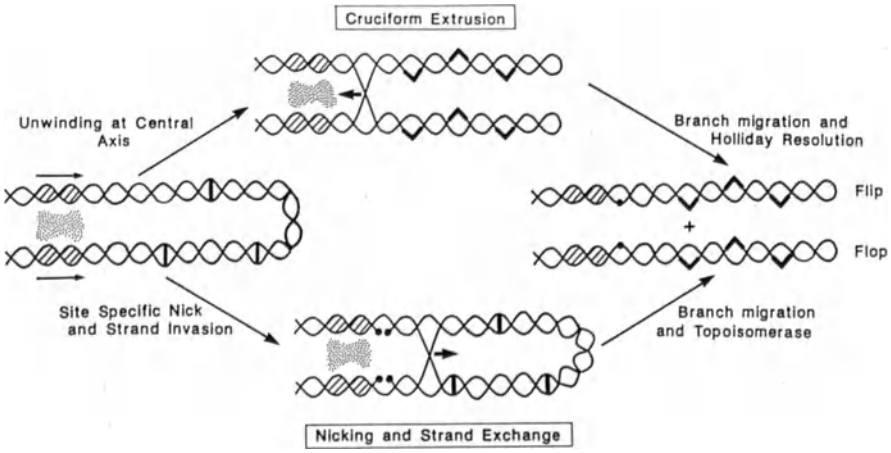


Fig. 5. Models to explain poxviral telomere resolution. The substrate replicative intermediate form is shown at *left* with the telomere resolution target (TRT, *shaded*) aligned by a hypothetical trans-acting factor. The cruciform extrusion model postulates central axis unwinding (for example by helicase activity) and refolding into a transient cruciform which functions as a substrate for Holliday resolution. The nicking and strand exchange model is modified from that proposed by Bateman (see text) to account for central axis strand exchange, and requires topoisomerase activity to segregate daughter molecules. The cross-over may involve either the two nicked or the two unnicked strands. Note that both models involve nicking/resealing at a site internal to all the extrahelical bases (highlighted in diagram) and is arbitrarily positioned adjacent to the TRT site

They must therefore occur at least 64 bp apart in the case of SFV and 104 bp in the case of vaccinia. The location of at least some of the nicks introduced in vitro by the 50 kDa nuclease from vaccinia virions is consistent with this requirement. Note that in all respects telomeric resolution appears to be completely analogous to a site-specific recombination event, with the TRT presumably functioning as the initial blinding site for the viral transacting factors which facilitate the actual strand exchange that precedes the final segregation into daughter hairpins.

9 Summary and Prospectives

The poxvirus telomere consists of at least three DNA domains, the AT-rich incompletely base-paired hairpin terminus, the nearby region of repeats, and the intervening TRT domain. The only sequence to which a definite function has been ascribed to date is the TRT domain, which constitutes the target region necessary for telomere resolution. The strict conservation of AT-richness and incomplete base-pairing of the hairpin termini strongly imply their essential function in the life cycle of poxviruses. Similarly, the identical location of the

region of repeats, irrespective of their complexity, implies that these elements fulfill some essential function(s). Yet, neither the hairpin nor the region of repeats is obligatorily required in the process that converts the replicative intermediate configuration into hairpin termini. Possible functions that can be ascribed to either or both of these DNA elements are:

(a) initiation of DNA replication;
(b) packaging of the genome into the virion core;
(c) an attachment site to promote telomere-matrix or telomere-telomere association;
(d) isomerization of adjoining hairpin termini to generate the replicative intermediate telomere fusion configuration;
(e) transient maintenance of the dimer junction configuration prior to resolution; and
(f) recombination between genomic termini, which may be an essential step during poxvirus DNA replication.

The challenge for the future will be to develop new and innovative approaches to define the function(s) of these telomere regions. The development of the transfection assay has been one such approach which, in a relatively short time, has contributed greatly to our current level of understanding of telomere resolution. To further adapt this assay to study other potential functions, such as initiation of DNA replication, it is necessary to gain a better understanding of the mechanism of the apparently origin-independent replication of transfected plasmid DNA. Such studies may well answer the question whether poxviruses in fact use defined origins of replication.

The efficient replication and resolution of plasmid DNA in poxvirus-infected cells has allowed determination of the domain boundaries of a telomeric region (TRT) that is required in cis for the resolution of the telomere fusion element to hairpin termini. This region was shown to contain a minimal core region, which is absolutely required for resolution, and an auxiliary region which promotes optimal resolution efficiency. The availability of cloned wild type and mutant substrates will, in all likelihood, lead to an efficient in vitro resolution system. The development of such a system is now within reach and should, in combination with genetic studies, allow identification of the various components necessary to generate hairpin termini from replicative intermediate telomere fusion elements. Finally, a better understanding of molecular mechanisms utilized for poxviral telomere replication and resolution will help to resolve the issue of whether or not common pathways or analogous mechanisms are also utilized for higher order eukaryotic chromosomes.

Acknowledgments. We wish to thank the Alberta Heritage Foundation for Medical Research and the Medical Research Council of Canada for financial support. We are grateful to R.T. Hay, M. Merchlinsky, and B. Moss for communicating information prior to publication and to R. Condit and M. Reddy for helpful discussions.

References

Barbour AG, Garon CF (1987) Linear plasmids of the bacterium *Borrelia burgdorferi* have covalently closed ends. Science 237: 409–411

Baroudy BM, Moss B (1982) Sequence homologies of diverse lengths tandem repetitions near the ends of vaccinia virus genome suggest unequal crossing over. Nucleic Acids Res 10: 5673–5679

Baroudy BM, Venkatesan S, Moss B (1982a) Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. Cell 28: 315–324

Baroudy BM, Venkatesan S, Moss B (1982b) Structure and replication of vaccinia virus telomeres. Cold Spring Harbor Symp Quant Biol 47: 723–729

Bateman AJ (1975) Simplification of palindromic telomere theory. Nature 253: 379–380

Bergold PJ, Campbell GR, Littau VC, Johnson EM (1983) Sequence and hairpin structure of an inverted repeat series at termini of the *Physarum* extrachromosomal rDNA molecule. Cell 32: 1287–1299

Berns KI, Silverman C (1970) Natural occurrence of cross-linked vaccinia virus deoxyribonucleic acid. J Virol 5: 299–304

Berns KI, Muzcyczka N, Hauswirth WW (1985) Replication of parvoviruses. In: Fields BN (ed) Virology. Raven, New York, pp 415–532

Black DN, Hammond JM, Kitching RP (1986) Genomic relationship between capripoxviruses. Virus Res 5: 277–292

Blackburn EH, Gall JG (1978) A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in *Tetrahymena*. J Mol Biol 120: 33–53

Blackburn EH, Szostak JW (1984) The molecular structure of centromeres and telomeres. Ann Rev Biochem 53: 163–194

Broker TR (1973) An electron microscopic analysis of pathways for bacteriophage T4 DNA recombination J Mol Biol 81: 1–16

Buller RML, Chakrabarti S, Cooper JA, Twardzik DR, Moss B (1988) Deletion of the vaccinia virus growth factor gene reduces virus virulence. J Virol 62: 866–874

Cavalier-Smith T (1974) Palindromic base sequences and replication of eukaryotic chromosomes. Nature 250: 467–470

Cech TR (1988) G-strings at chromosome ends. Nature 332: 777–778

Dales S, Pogo BGT (1982) The biology of poxviruses. Springer Berlin Heidelberg New York

Dales S, Milanovitch V, Pogo BGT, Weintraub SB, Huima T, Wilton S, McFadden G (1978) Biogenesis of vaccinia: isolation of conditional lethal mutants and electron microscopic characterization of their phenotypically expressed defects. Virology 84: 403–428

DeFilippes FM (1976) Restriction enzyme digests of rapidly renaturing fragments of vaccinia virus DNA. J Virol 70: 227–238

DeLange AM (1989) Identification of temperature-sensitive mutants of vaccinia virus that are defective in conversion of concatemeric replicative intermediates to the mature linear DNA genome. J Virol 63: 2437–2444

DeLange AM, McFadden G (1986) Sequence nonspecific replication of transfected plasmids in poxvirus-infected cells. Proc Natl Acad Sci USA 83: 614–618

DeLange AM, McFadden G (1987) Efficient resolution of replicated poxvirus telomeres to native hairpin structures requires two inverted symmetrical copies of a core target DNA sequence. J Virol 61: 1957–1963

DeLange AM, Futcher B, Morgan R, McFadden G (1984) Cloning of the vaccinia virus telomere in a yeast plasmid vector. Gene 27: 13–21

DeLange AM, Reddy M, Scraba D, Upton C, McFadden G (1986) Replication and resolution of cloned poxvirus telomeres in vivo generates linear minichromosomes with intact viral hairpin termini. J Virol 59: 249–259

deMassy B, Studier FW, Dorgai L, Appelbaum L, Weisberg RA (1984) Enzymes and sites of genetic recombination: studies with gene-3 endonuclease of phage T7 and with site-affinity mutants of phage lambda. Cold Spring Harbor Symp Quant Biol 49: 715–726

Dickie P, Morgan AR, McFadden G (1987a) Cruciform extrusion in plasmids bearing the replicative intermediate configuration of a poxvirus telomere. J Mol Biol 196: 541–558

Dickie P, McFadden G, Morgan AR (1987b) The site-specific cleavage of synthetic Holliday junction analogs and related branched DNA structures by bacteriophage T7 endonuclease I. J Biol Chem 262: 14826–14836

Dickie P, Morgan AR, McFadden G (1988) Conformational association of the Holliday junction associated with a cruciform during branch migration in supercoiled plasmid DNA. J Mol Biol 201: 19–30

Ellis THN, Day A (1986) A hairpin plastid genome in barley. EMBO J 5: 2769–2776

Emery HS, Weiner AM (1981) An irregular satellite sequence is found at the termini of the linear extrachromosomal rDNA in *Dictyostelium discoideum*. Cell 26: 411–419

Esposito JJ, Knight JC (1985) Orthopoxvirus DNA: a comparison of restriction profiles and maps. Virology 143: 230–251

Esteban M, Holowczak J (1977) Replication of vaccinia DNA in mouse L cells. I. in vivo DNA synthesis. Virology 78: 57–75

Esteban M, Flores L, Holowczak JA (1977) Model for vaccinia virus DNA replication. Virology 83: 467–473

Evans DH, Stuart D, McFadden G (1988) High levels of genetic recombination among cotransfected plasmid DNAs in poxvirus-infected mammalian cells. J Virol 62: 367–375

Gafford LG, Mitchell EB, Randall CC (1978) A comparison of sedimentation behaviour of three poxvirus DNAs. Virology 89: 229–239

Geshelin P, Berns KI (1974) Characterization and localization of the naturally occurring cross-links in vaccinia virus DNA. J Mol Biol 88: 785–796

Gonzalez A, Talavera A, Almendral JM, Viñuela E (1986) Hairpin loop structure of African swine fever virus DNA. Nucleic Acids Res 14: 6835–6844

Graham FL, Van der Eb AJ (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52: 456–467

Greider CW, Blackburn EH (1985) Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. Cell 43: 405–413

Greider CW, Blackburn EH (1989) A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. Nature 337: 331–336

Hay RT, Russell WC (1989) Recognition mechanisms in the synthesis of animal virus DNA. Biochem J 258: 3–16

Holowczak JA (1982) Poxvirus DNA. Curr Top Microbiol Immunol 97: 27–79

Hooda-Dhingra U, Thompson CL, Condit RC (1989) Detailed phenotypic characterization of five temperature-sensitive mutants in the 22- and 147-kilodalton subunits of vaccinia virus DNA-dependent RNA polymerase. J Virol 63: 714–729

Jacquemont B, Grange J, Gazzolo L, Richard MH (1972) Composition and size of Shope fibroma virus deoxyribonucleic acid. J Virol 9: 836–841

Jaureguiberry G (1977) Cleavage of vaccinia virus DNA by restriction endonucleases *Bal*I, *Eco*RI, *Bam*HI. Isolation of the natural cross-links. FEBS Lett 83: 111–117

Johnson EM (1980) A family of inverted repeat sequences and specific single-strand gaps at the termini of the *Physarum* rDNA palindrome. Cell 22: 875–886

Jungwirth C, Dawid IB (1967) Vaccinia DNA: separation of viral from host cell DNA. Arch Virol 20: 464–468

Kelly TJ, Wold MS, Li J (1988) Initiation of viral DNA replication. Adv Virus Res 34: 1–42

Kemper B, Jensch F, v Depka-Prondzynski M, Fritz HJ, Borgmeyer U, Mizuuchi K (1984) Resolution of Holliday structures by endonuclease VII as observed in interactions with cruciform DNA. Cold Spring Harbor Symp Quant Biol 49: 815–825

Kikuchi Y, Hirai K, Gunge N, Hishinuma F (1985) Hairpin plasmid—a novel linear DNA of perfect hairpin structure. EMBO J 4: 1881–1886

Kilpatrick DR, Rouhandeh H (1985) Cloning and physical mapping of Yaba monkey tumor virus DNA. Virology 143: 399–406

Kilpatrick DR, Rouhandeh H (1987) The analysis of Yaba monkey tumor virus DNA. Virus Res 7: 151–157

Kucherlapati R, Skoultchi AI (1984) Introduction of purified genes into mammalian cells. CRC Crit Rev Biochem 16: 349–379

Lakritz N, Foglesong PD, Reddy M, Baum S, Hurwitz J, Bauer WR (1985) A vaccinia virus DNase preparation which cross-links superhelical DNA. J Virol 53: 935–943

Lilley DM, Kemper B (1984) Cruciform-resolvase interactions in supercoiled DNA. Cell 36: 413–422

Macaulay C, Upton C, McFadden G (1987) Tumorigenic poxviruses: transcriptional mapping of the terminal inverted repeats of Shope fibroma virus. Virology 158: 381–393

McCarron RJ, Cabrera CV, Esteban M, McAllister W, Holowczak J (1978) Structure of vaccinia DNA: analysis of the viral genome by restriction endonucleases. Virology 86: 88–101
McClintock B (1941) The stability of broken ends of chromosomes in *Zea mays*. Genetics 26: 234–282
McClintock B (1942) The fusion of broken ends of chromosomes following nuclear fusion. Proc Natl Acad Sci USA 28: 458–463
McFadden G, Dales S (1982) Organization and replication of poxvirus DNA. In: Kaplan AS (ed) Organization and replication of viral DNA. CRC, Boca Raton, pp 173–190
McFadden G, Morgan AR (1982) DNA cruciform structures: implications for telomere replication in eukaryotes and instability of long palindromic DNA sequences in prokaryotes. J Theor Biol 97: 343–349
McFadden G, Stuart D, Upton C, Dickie P, Morgan AR (1988) Replication and resolution of poxvirus telomeres. In: Kelly T, Stillman B (eds) Cancer Cells 6: Eukaryotic DNA Replication. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp 77–85
Menna A, Wittek R, Bachmann PA, Mayr A, Wyler R (1979) Physical characterisation of a stomatitis populosa virus genome: a cleavage map for the restriction endonucleases *Hin*dIII and *Eco*RI. Arch Virol 59: 145–156
Mercer AA, Fraser K, Barns G, Robinson AJ (1987) The structure and cloning of orf virus DNA. Virology 157: 1–12
Merchlinsky M, Moss B (1986) Resolution of linear minichromosomes with hairpin ends from circular plasmids containing vaccinia virus concatemer junctions. Cell 45: 879–884
Merchlinsky M, Moss B (1988) Sequence-independent replication and sequence-specific resolution of plasmids containing the vaccinia virus concatemer junction: requirements for early and late trans-acting factors. In: Kelly T, Stillman B (eds) Cancer Cells 6: Eukaryotic DNA Replication. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp 87–93
Merchlinsky M, Garon CF, Moss B (1988) Molecular cloning and sequence of the concatemer junction from vaccinia virus replicative DNA. Viral nuclease cleavage sites in cruciform structures. J Mol Biol 199: 399–413
Merchlinsky M, Moss B (1989) Resolution of vaccinia virus DNA concatemer junctions requires late-gene expression. J Virol 63: 1595–1603
Mizuuchi K, Kemper B, Hays J, Weisberg RA (1982) T4 endonuclease VII cleaves Holliday structures. Cell 29: 357–365
Morin GB, Cech TR (1986) The telomeres of the linear mitochondrial DNA of *Tetrahymena thermophila* consist of 53 bp tandem repeats. Cell 46: 873–883
Morin GB, Cech TR (1988) Mitochondrial telomeres: surprising diversity of repeated telomeric DNA sequences among six species of *Tetrahymena*. Cell 52: 367–374
Moss B (1990) Replication of poxviruses. In: Fields BN (ed) Virology. 2nd Ed. Raven, New York, pp 2074–2111
Moss B, Winters E, Cooper N (1981) Instability and reiteration of DNA sequences within the vaccinia virus genome. Proc Natl Acad Sci USA 78: 1614–1618
Moss B, Winters E, Jones EV (1983) Replication of vaccinia virus. In: Cozzarelli NR (ed) Mechanisms of DNA replication and recombination. Liss, New York, pp 449–461
Moyer RW, Graves RL (1981) The mechanism of cytoplasmic orthopox replication. Cell 27: 391–401
Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MD, Meyne J, Ratliff RL, Wu JR (1988) A highly conserved repetitive DNA sequence, $(TTAGGG)_n$, present at the telomeres of human chromosomes. Proc Natl Acad Sci USA 85: 6622–6626
Niles EG, Seto J (1988) Vaccinia virus gene *D8* encodes a virion transmembrane protein. J Virol 62: 3772–3778
Pacha RF, Condit RC (1985) Characterization of a temperature-sensitive mutant of vaccinia virus reveals a novel function that prevents virus-induced breakdown of RNA J. Virol 56: 395–403
Panayotatos N, Wells RD (1981) Cruciform structures in supercoiled DNA. Nature 289: 466–470
Parr RP, Burnett JW, Garon CF (1977) Structural characterization of the molluscum contagiosum virus genome. Virology 81: 247–256
Parsons BL, Pickup DJ (1987) Trandemly repeated sequences are present at the end of the DNA of raccoonpox virus. Virology 161: 45–53
Peden KWC, Pipas JM, Pearson-White S, Nathans D (1980) Isolation of mutants of an animal virus in bacteria. Science 209: 1392–1396
Pickup DJ, Bastia D, Stone HO, Joklik WK (1982) Sequence of terminal regions of cowpox virus DNA: arrangement of repeated and unique sequence elements. Proc Natl Acad Sci USA 79: 7112–7116

Pickup DJ, Bastia D, Joklik WK (1983) Cloning of the terminal loop of vaccinia virus DNA. Virology 124: 215–217

Pogo BGT (1977) Elimination of naturally occurring cross-links in vaccinia DNA after virus penetration. Proc Natl Acad Sci USA 74: 1739–1742

Pogo BGT (1980) Changes in parental vaccinia virus DNA after penetration into cells. Virology 101: 520–524

Pogo BGT, O'Shea MT (1978) The mode of replication of vaccinia virus DNA. Virology 86: 1–8

Pogo BGT, O'Shea M, Freimuth P (1981) Initiation and termination of vaccinia virus DNA replication. Virology 108: 241–248

Pogo BGT, Berkowitz EM, Dales S (1984) Investigation of vaccinia virus DNA replication employing a conditional lethal mutant defective in DNA. Virology 132: 436–444

Pritchard AE, Cummings DJ (1981) Replication of linear mitochondrial DNA from *Paramecium*: sequence and structure of the initiation-end crosslink. Proc Natl Acad Sci USA 78: 7341–7345

Reddy M (1988) Purification of the nicking-joining nuclease and its interaction with vaccinia viral replicative intermediates. Ph D thesis, Dept Microbiology, SUNY, Stony Brook, NY

Reddy M, Bauer WR (1989) Activation of the vaccinia virus nicking-joining enzyme by trypsinization. J Biol Chem 264: 443–449

Richards EJ, Ausubel FM (1988) Isolation of a higher eukaryotic telomere from *Arabidopsis thaliana*. Cell 53: 127–136

Robinson AJ, Barns G, Fraser K, Carpenter E, Mercer AA (1987) Conservation and variation in orf virus genomes. Virology 157: 13–23

Seto J, Celenza LM, Condit R, Niles EG (1987) Genetic map of the vaccinia virus *Hin*dIII D fragment. Virology 160: 110–119

Szostak JW (1983) Replication and resolution of telomeres in yeast. Cold Spring Harbor Symp Quant Biol 47: 1187–1194

Szybalski WR, Erikson RL, Gentry LG, Gafford LG, Randall CC (1963) Unusual properties of fowlpox virus DNA. Virology 19: 586–589

Thomas V, Flores L, Holowczak JA (1980) Biochemical and electron microscopic studies of the replication and composition of Milker's Node virus. J Virol 34: 244–255

Thompson CL, Condit RC (1986) Marker rescue mapping of vaccinia virus temperature-sensitive mutants using overlapping cosmid clones representing the entire virus genome. Virology 150: 10–20

Thompson CL, Hooda-Dhingra U, Condit RC (1989) Fine structure mapping of five temperature-sensitive mutants in the 22- and 147-kilodalton subunits of vaccinia virus DNA-dependent RNA polymerase. J Virol 63: 705–713

Upton C, DeLange AM, McFadden G (1987) Tumorigenic poxviruses: genomic organization and DNA sequence of the telomeric region of the Shope fibroma virus genome. Virology 160: 20–30

Watson JD (1972) Origin of concatemeric T7 DNA. Nature New Biol 239: 197–201

Weiner AM (1988) Eukaryotic nuclear telomeres: molecular fossils of the RNP world? Cell 52: 155–157

Wills A, DeLange AM, Gregson C, Macauley C, McFadden G (1983) Physical characterization and molecular cloning of the Shope fibroma virus genome. Virology 130: 403–414

Winters E, Baroudy BM, Moss B (1985) Molecular cloning of the terminal hairpin of vaccinia virus DNA as an imperfect palindrome in an *Escherichia coli* plasmid. Gene 37: 221–228

Wittek R, Moss B (1980) Tandem repeats within the inverted terminal repetition of vaccinia virus DNA. Cell 21: 277–284

Wittek R, Menna A, Schümperli D, Stoffel S, Müller HK, Wyler R (1977) *Hin*dIII and *Sst*I restriction sites mapped on rabbit poxvirus and vaccinia virus DNA. J Virol 23: 669–678

Wittek R, Cooper JA, Barbosa E, Moss B (1980) Expression of the vaccinia virus genome: analysis and mapping of mRNAs encoded within the inverted terminal repetition. Cell 21: 487–493

Young BS, Pession A, Traverse KL, French C, Pardue ML (1983) Telomere regions in *Drosophila* share complex DNA sequences with pericentric heterochromatin. Cell 34: 85–94

The Enzymology of Poxvirus DNA Replication

P. TRAKTMAN

1 Introduction

DNA replication is arguably the most fundamental of biological processes and serves as a pivotal element in the poxvirus life cycle. Replication generates progeny genomes and initiates the transition to late gene expression. Unraveling

Departments of Cell Biology and Anatomy and Microbiology, Cornell University Medical College, New York, NY 10021, USA

Current Topics in Microbiology and Immunology, Vol. 163

the mechanism of poxvirus replication and identifying the panoply of enzymatic activities which it requires has been a focus of much recent research. The intent of this chapter is to move beyond previous reviews (McFADDEN and DALES 1982; HOLOWCZAK 1983) and focus on viral proteins whose participation in DNA replication is presumed on the basis of genetic or biochemical data. Implicit in this discussion was the choice of a broad definition of DNA replication, one which centers on DNA synthesis but also encompasses regulation of the topology of genomic DNA and its assembly into a nucleoprotein complex.

1.1 Poxvirus DNA Replication: Experimental Parameters

Cytoplasmic localization and genetic autonomy are two features of poxvirus infection with profound implications for experimental analysis. The well-founded expectation that these viruses will encode all of the essential functions involved in the replication and structural organization of the chromosome has fostered increasingly sophisticated genetic analyses. Poxvirus genetics is discussed in detail elsewhere (see CONDIT and NILES, this volume); it is sufficient to reiterate that the isolation of drug-resistant and temperature-sensitive mutants has accelerated the identification of the enzymes and genes involved in viral replication. The availability of modern techniques of random and directed mutagenesis, coupled with the ability to reintroduce altered viral genes into the genome via marker rescue (NAKANO et al. 1982; FATHI et al. 1986), provides a fruitful opportunity for investigating structure-function relationships. Biochemical analyses have been aided by the encapsidation of some enzymes within the virion (e.g., topoisomerase) and by the localization of viral replication in a distinct cytoplasmic compartment removed from the events of host nucleic acid metabolism. Adding to the clarity with which the events and components of viral replication have been identified are the synchrony of poxvirus infection and the ability to prepare high titer viral stocks and perform high multiplicity infections. Due to the inhibition of host DNA replication, transcription, and translation which accompanies infection, the progression of the viral life cycle stands out boldly against a diminished background of host metabolism.

1.2 Vaccinia DNA Replication: Timing and Localization

The vaccinia life cycle can be roughly divided into the phases of early gene expression, DNA replication, and late gene expression. Entry into each of the later phases depends upon completion of the prior steps. Early gene expression, which is mediated by the encapsidated transcriptional machinery, occurs within membrane-bound viral cores. The viral mRNAs are translated on cytoplasmic polysomes, and the pool of early proteins provides additional transcriptional machinery and the array of proteins required for DNA replication (to be described below). Synthesis of these proteins (which can be prevented by infection

in the presence of cycloheximide) also leads to the full induction of viral cytopathic effects and is required for the "secondary uncoating" of the viral cores (SAROV and JOKLIK 1973). This uncoating releases the viral genome into the cytoplasm, an obligate step for the phase of DNA replication.

At reasonably high multiplicities of infection (15), the onset of DNA replication occurs before 2 hours post infection (hpi). Viral DNA synthesis has historically been measured by monitoring the cytoplasmic incorporation of exogenously added [^{3}H] thymidine into acid-precipitable material. As observed by this method, DNA synthesis initiates early and proceeds rapidly, and a strong peak of thymidine incorporation is seen between 2 and 4 hpi. After 5 hpi, little if any incorporation of thymidine is detected. Analysis of the density shift of progeny synthesized in the presence of BUdR has revealed that synthesis is semiconservative and that experimental inhibition of ongoing protein synthesis limits replication to one, and only one, round of synthesis (ESTEBAN and HOLOWCZAK 1978).

Models of replication based on these studies assumed that replication proceeded in a rapid wave and ceased by 5–6 hpi; however, accumulation of replicated DNA can also be monitored by performing quantitative filter hybridizations. Equal portions of cellular extracts are prepared throughout infection and immobilized on filters; the bound DNA is then allowed to hybridize with radiolabeled probes representing vaccinia sequences. Quantitation of the amount of probe bound (ENSINGER 1987; HOODA-DHINGRA et al. 1989; REMPEL et al. 1990) allows an assessment of the actual accumulation of viral sequences due to replication. These studies have revealed an extended pattern of DNA synthesis distinct from that which emerged from monitoring [^{3}H]thymidine incorporation. Increases in intracellular vaccinia DNA are first detected at approximately 3 hpi; sequences then accumulate rapidly and at a constant rate until approximately 10–12 hpi. Replication then appears to slow down and a plateau in the level of viral DNA is reached. Apparently, the phase of synthesis detected by thymidine incorporation represents only the earliest hours of DNA replication. The lack of incorporation of exogenous thymidine after 5 hpi might reflect the depression of thymidine kinase activity at later times of infection (see below for a discussion of the inhibitory feedback on thymidine kinase by the products of its own reaction) and/or extensive changes in intracellular nucleotide pools (see below for a discussion of the viral ribonucleotide reductase). One can then conclude that, during high multiplicity infections, replication initiates at approximately 2 hpi and proceeds for at least 10 h.

The observation that inhibition of DNA synthesis at late times post infection (6 h) does not adversely affect viral yield (SALZMAN et al. 1963) suggests that the final hours of DNA replication may not contribute to the production of mature progeny virus. Indeed, investigators have found that, although the total level of vaccinia sequences which accumulate is roughly equivalent to half of the nuclear DNA content, only one-third of these sequences are encapsidated (JOKLIK and BECKER 1964). The onset of late gene expression, which depends upon genomic

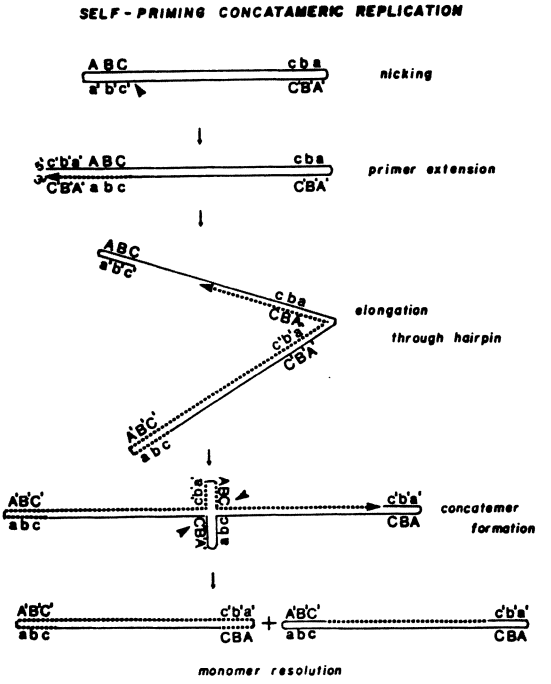
replication, begins shortly after DNA synthesis commences and continues for the duration of the infection. As might be expected, the detectable progression to each sequential phase is delayed when infections are performed at lower multiplicities.

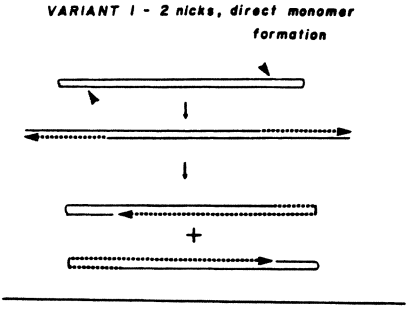
1.3 Proposed Model for Poxvirus DNA Replication

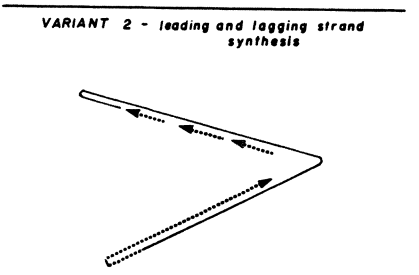
Despite the progress which has been made in studying the structure of the viral genome and in identifying viral functions involved in replication, many aspects of the mechanism of poxvirus replication remain unclear. Nevertheless, a working model has been put forward (McFADDEN and DALES 1982) which can accommodate recent findings and will stimulate future studies. The essential features of this model are illustrated in Fig. 1. The prototypic poxvirus genomes are large DNA duplexes (185 kb for vaccinia) with terminal cross-links. With the exception of variola, similar DNA sequences are found at the two ends of poxvirus genomes in the form of long inverted terminal repeats of 5–10 kb. Directly flanking the terminal hairpins are numerous tandem repeats of short AT-rich sequences (WITTEK and MOSS 1980 and described in greater detail by DELANGE and McFADDEN, in this volume). These direct repeats are implicated in inter- and intragenomic recombinational events. The hairpins (104 bp in vaccinia), despite their extensive self-complementarity, contain extrahelical bases and are characterized as single-stranded. They occur in two isomeric forms (termed flip and flop) which are inverted and complementary (BAROUDY et al. 1982 and described in greater detail by DELANGE and McFADDEN, in this volume).

The first event in viral replication is presumed to be a nick in one of the viral DNA strands near the terminus. This nick has been detected by the increased topological mobility and altered sedimentation properties that it confers on viral genomes (POGO 1977). This nick creates an available 3′OH primer terminus for DNA polymerase; elongation then displaces the complementary strand and yields a longer-than-genome-length duplex with one open and one hairpin

Fig. 1. A schematic outline of the self-priming concatameric model of poxvirus replication. The linear genome with hairpin termini is indicated; inverted repeat sequences nearest the termini are labeled *a*, *b*, *c*, or the complementary *A*, *B*, *C*. In mature termini, the hairpin regions are not fully complementary, since one strand carries extrahelical bases shown here by the addition of a prime (′). Replication is indicated as commencing with a single nick near the terminal region, which allows strand displacement and elongation of the newly created 3′ end. The self-complementarity of the elongated strand allows it to fold back on itself and prime further elongation. This elongation can progress through the hairpin terminus, creating a tail-to-tail genomic dimer. Although not shown here, the synthesis of longer concatemers is presumed and has been detected. The concatemer junctions contain inverted repeats which, as shown here, may undergo cruciform extrusion. Resolution of the concatemers is discussed more fully in the chapter by DELANGE and McFADDEN but is depicted here as involving scission at the base of the cruciform. Resolved monomers contain the appropriate termini in which incompletely base-paired sequences occur in the flip or flop conformation. Two variants of the model are shown in the *lower panel*. In some molecules, two nicks might occur, allowing primer extension of both strands. Monomers would be formed directly in this case. In the second variant, the possibility that concatemer formation might involve lagging and leading strand synthesis is shown


SELF-PRIMING CONCATAMERIC REPLICATION
nicking
primer extension
elongation
through hairpin
concatemer
formation
monomer resolution


VARIANT 1 - 2 nicks, direct monomer
formation


VARIANT 2 - leading and lagging strand
synthesis

terminus. The elongated strand now contains an inverted repeat and this self-complementarity allows the 3′ end to fold back on itself and prime replication which can continue around the hairpin terminus to form a dimeric tail-to-tail concatemer. This process can continue through several cycles, and indeed concatemeric intermediates of at least four genomes in length have been detected (MOYER and GRAVES 1981; DELANGE 1989). [^{3}H]thymidine pulse-labeling of cultures infected with temperature-sensitive replication mutants immediately upon shift to the permissive temperature has confirmed that radiolabeled precursors are first incorporated into sequences derived from the ends of the genome (POGO et al. 1984). Sequences derived from both termini were labeled, suggesting that nick-promoted initiation might occur from either terminus or simultaneously from both termini. Since concatemers are seen, it seems that at least in some cases initiation begins at only one terminus. The sequence or structural specificity of the site of terminal nicking is unknown at present. Any definition of a poxviral origin of replication must accommodate the important observation that all circular DNA plasmids introduced into infected cells by transfection replicate (DELANGE and MCFADDEN 1986).

The simplest model for subsequent genomic synthesis invokes only unidirectional, leading strand synthesis; however, leading and discontinuous lagging strand syntheses, which converge at the hairpin, cannot be definitively ruled out. Although early reports presented evidence for the synthesis of short DNA strands which were "chased" into larger forms (ESTEBAN and HOLOWCZAK 1977b), these data have not been confirmed with more recent techniques. Moreover, the detection of large amounts of single-stranded genomic DNA during replication (ESTEBAN and HOLOWCZAK 1977b; POGO et al. 1981) argues for unidirectional, strand-displacement synthesis.

That concatemeric forms are true replication intermediates has been confirmed by several investigators (DELANGE et al. 1986; DELANGE 1989; MERCHLINSKY and MOSS 1986; MERCHLINSKY et al. 1988; MERCHLINSKY and MOSS 1989) and is discussed elsewhere in this volume. Briefly, species representing concatemeric junctions have been isolated from cytoplasmic DNA prepared from infected cells (MERCHLINSKY et al. 1988). Plasmids containing the concatemeric junction fragment are resolved in poxvirus-infected cells into linear minichromosomes containing authentic hairpin termini (DELANGE et al. 1986; MERCHLINSKY and MOSS 1986). In addition, some vaccinia mutants defective in the expression of late viral gene products accumulate concatemeric intermediates at the expense of monomeric forms (DELANGE 1989; MERCHLINSKY and MOSS 1989). As discussed in detail by DELANGE and MCFADDEN in this volume, various models for concatemer resolution invoking staggered nicking and ligation or cruciform extrusion and Holliday-junction resolution are under consideration. The general validity of the proposed replication model has been underscored by the analysis of concatemeric intermediates and the determination that they contain head-head and tail-tail arrays of genomes as predicted (MOYER and GRAVES 1981); however, recent studies of concatemers which accumulate in resolution-deficient mutants (DELANGE 1989; MERCHLINSKY and MOSS 1989) indicate that under these circumstances many junctions indicative of head-to-tail

tandem arrangements are also seen. These forms probably arise as a consequence of high levels of recombination mediated by the numerous terminal tandem repeats.

1.4 Implications for the Repertoire of Replication Functions

Drawing on the model described above and on the growing understanding of a variety of replication systems (CAMPBELL 1986), the functions likely to be required for poxvirus replication can be outlined. Initiation, which shares little with that commonly seen at internal origins, will probably be effected by an endonuclease whose specificity may require accessory proteins. Elongation will certainly involve the already identified DNA polymerase, and it is likely that processivity will be conferred by accessory proteins. Moreover, a helicase is invariably required to open the DNA duplex ahead of the advancing polymerase. Stabilization of the displaced strand can be presumed to rely on stoichiometric quantities of a single-strand DNA binding protein. In the unlikely event that both leading and lagging strand synthesis occur, primase, RNAse H, and DNA ligase activities will be needed to synthesize and remove primers and to link Okazaki fragments. Precursors will be provided by ribonucleotide reductase (and thymidine kinase); presumably, a kinase will be required to convert the dNDPs generated by ribonucleotide reductase to the dNTPs utilized by the replication machinery. The torsional stress generated ahead of the replication fork is likely to be relieved by the action of a type I topoisomerase. Resolution of the concatemeric intermediates into unit-length genomes is thought to involve cleavage and fold-back of each strand near the concatemeric junction, followed by ligation to reform the covalently closed hairpins. This might be accomplished by an enzyme capable of concerted nicking and joining or by distinct nuclease and ligase activities. Significant levels of homologous recombination appear to occur during replication; the enzymes involved in this process might be included in the compilation of replication proteins. Finally, incorporation of the genome into progeny virions involves significant condensation and wrapping of the genome with several proteins into a nucleoprotein complex whose fine structure remains poorly understood.

2 Replication Functions Identified by Genetic and Biochemical Studies

2.1 Essential Viral-Encoded Functions with Roles in Replication

Genetic analyses of poxviruses have been of central importance in defining essential replicative functions. The collections of temperature-sensitive mutants generated by CONDIT and ENSINGER have been sorted into numerous complementation

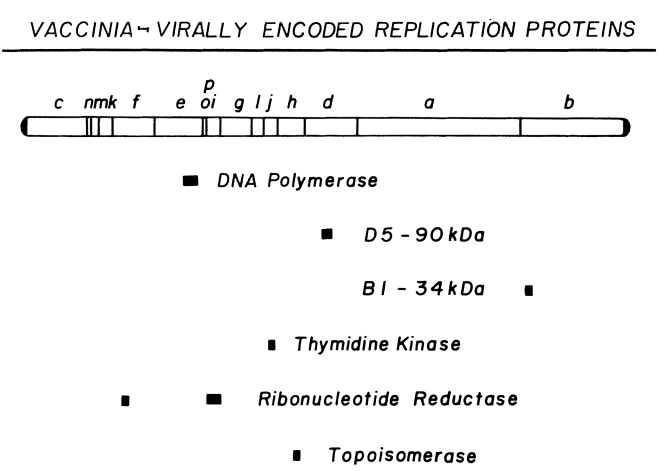


Fig. 2. The 185 kb vaccinia genome is drawn with the 16 fragments resulting from *Hin*dIII restriction displayed (*a–p* in diminishing size order). Genomic positions of the genes encoding the DNA polymerase, 90 kDa D5 protein, 34 kDa B1 protein, thymidine kinase, ribonucleotide reductase, and topoisomerase are indicated. Thymidylate kinase and DNA ligase, whose positions are not shown, map within the rightmost portion of the *Hin*dIII A fragment

groups and assayed for early gene expression, DNA synthesis, and late gene expression (CONDIT and MOTYCZKA 1981; CONDIT et al. 1983; ENSINGER 1982; ENSINGER and ROVINSKY 1983; THOMPSON and CONDIT 1986). Three complementation groups capable of expressing early genes but not of synthesizing viral DNA at the nonpermissive temperature have been defined. These mutants carry lesions in genes presumed to encode essential replicative functions. The location of the genes affected in these three groups is shown in Fig. 2. Each is discussed in greater detail below.

2.1.1 DNA Polymerase

Reports of increased DNA polymerase activity in the cytoplasm following poxvirus infection were made more compelling by the observation that the induced enzyme was immunologically distinct from the host enzyme (MAGEE and MILLER 1967; BERNS et al. 1969; CITARELLA et al. 1972). Isolation of viral mutants resistant to classic inhibitors of DNA polymerase function, such as phosphonoacetic acid (PAA) (MOSS and COOPER 1982; SRIDHAR and CONDIT 1983) and aphidicolin (DE FILIPPES 1984), strengthened expectations that the virus would encode a DNA polymerase. A temperature-sensitive mutant belonging to the same complementation group as a PAA^r allele was found to possess a thermolabile polymerase activity (SRIDHAR and CONDIT 1983). The gene encoding the enzyme was identified by genetic rescue of polymerase mutants and by comparison of the purified enzyme (CHALLBERG and ENGLUND 1977a) with the in vitro translation products of mRNA hybrid-selected with candidate DNA

fragments (JONES and MOSS 1984; TRAKTMAN et al. 1984). Encoded within the right half of the *Hin*dIII E fragment, the 3.4 kb polymerase mRNA is transcribed from right to left with respect to the viral genome. The complete sequence of the 1,006 amino acid open reading frame has been obtained (Fig. 3 and legend; EARL et al. 1986; BINNS et al. 1987; TRAKTMAN et al. 1989), as has the corresponding sequence of fowlpox DNA polymerase (BINNS et al. 1987). The cytoplasmic enzyme has been purified to apparent homogeneity and characterized biochemically (CHALLBERG 1979a, b). The enzyme is a monomer of 110 kDa which possesses polymerase activity and intrinsic 3′–5′ exonuclease function. The polymerase function, which requires a primed template, is not highly processive and cannot displace strands or pass through barriers of secondary structure. The Km for each nucleotide is approximately 15 u*M*. The exonuclease activity, which is presumed to provide a proofreading function, is inhibited in the presence of dNTPs or competitor single-strand DNA.

Comparison of the DNA sequence with those of a variety of other polymerases reveals regions of significant homology (EARL et al. 1986; BERNAD et al. 1987; LARDER et al. 1987; PIZZAGALLI et al. 1988; WONG et al. 1988). The six polymerase domains which are shared among a number of alpha-like polymerases are also conserved in the vaccinia enzyme (see Fig. 3). To date, two temperature-sensitive lesions, *Cts24* and *NG26* (CONDIT et al. 1983; SRIDHAR and CONDIT 1983), and two independent but identical PAA^r lesions (EARL et al. 1986; SRIDHAR and CONDIT 1983) have been mapped to single nucleotides within the polymerase sequence (EARL et al. 1986; TRAKTMAN et al. 1989b and Fig. 3). The temperature-sensitive mutants show an essentially complete absence of DNA synthesis at the nonpermissive temperature, and viral yields are less than 1% of those obtained at the permissive temperature. Thus, the polymerase is essential for viral DNA replication and the impact of its inactivation is not relieved by a cellular component. Aphidicolin^r mutants have also been isolated (DE FILIPPES 1984; TADDIE and TRAKTMAN, manuscript in preparation); the more recent collection of mutants map within a central portion of the gene containing conserved domains 2 and 3 (see Fig. 3). Herpes simplex virus DNA polymerase mutants bearing resistance to nucleotide analogs map predominantly to region 2, suggesting that the vaccinia mutants may well affect nucleotide binding. This is consistent with studies indicating that aphidicolin functions as a competitive inhibitor of dCTP and a noncompetitive inhibitor of dATP, dGTP, and TTP (PEDRALI-NOY and SPADARI 1980). Notably, one aphidicolin^r mutant isolated by TADDIE and TRAKTMAN appears to have a generally increased frequency of spontaneous mutation; the acquisition of a mutator phenotype strengthens the argument that these lesions affect nucleotide binding and selection.

Preparation of an anti-DNA polymerase antiserum (CROZEL and TRAKTMAN, unpublished data) has allowed the monitoring of polymerase synthesis following infection. Contrary to early reports that the DNA polymerase was a delayed early function, the 110 kDa polymerase can be detected within the first hour of infection. Moreover, the polymerase mRNA is abundant in RNA prepared from cells infected in the presence of cycloheximide (TRAKTMAN et al. 1984). Synthesis

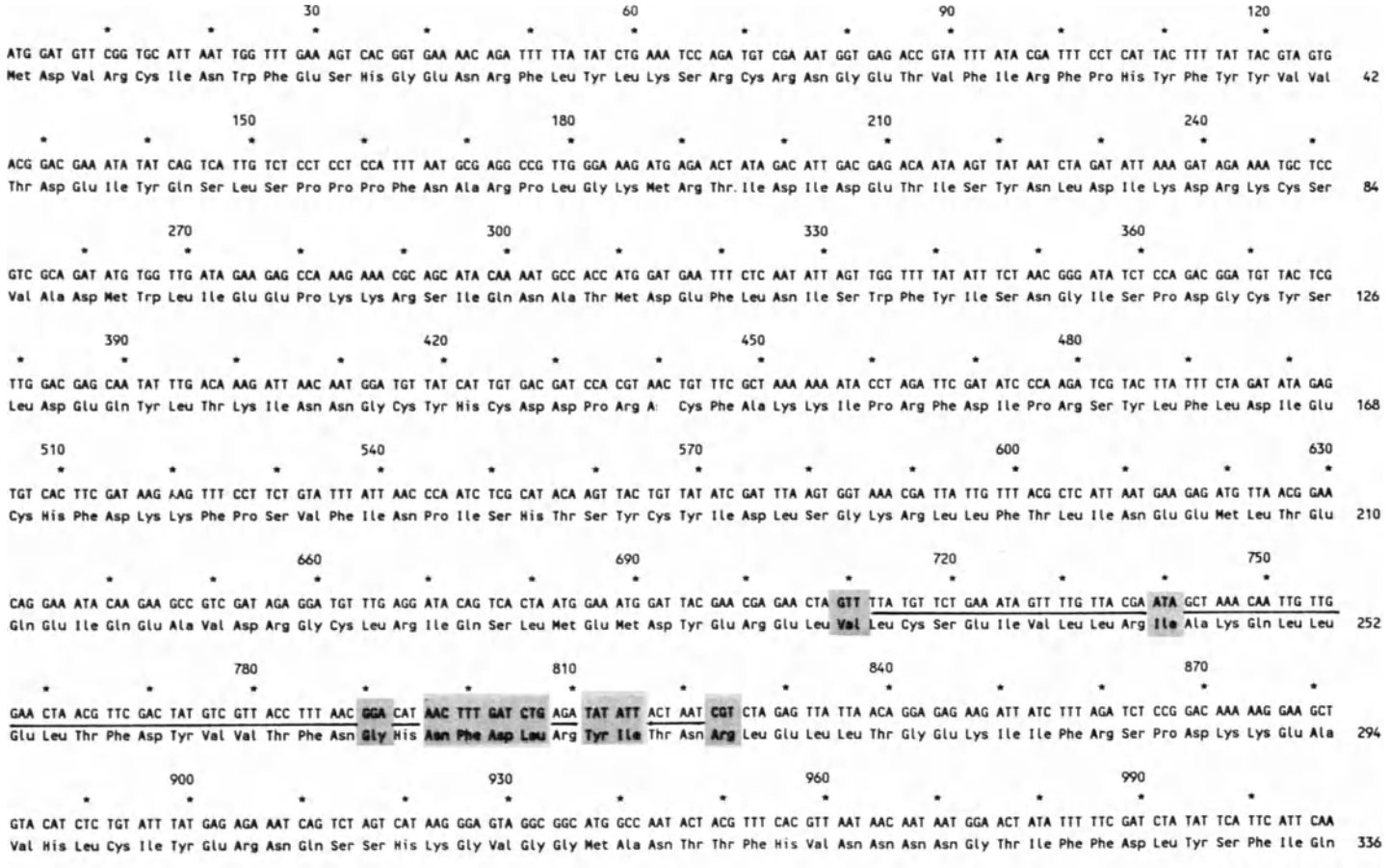


Fig. 3. (*Continued*)

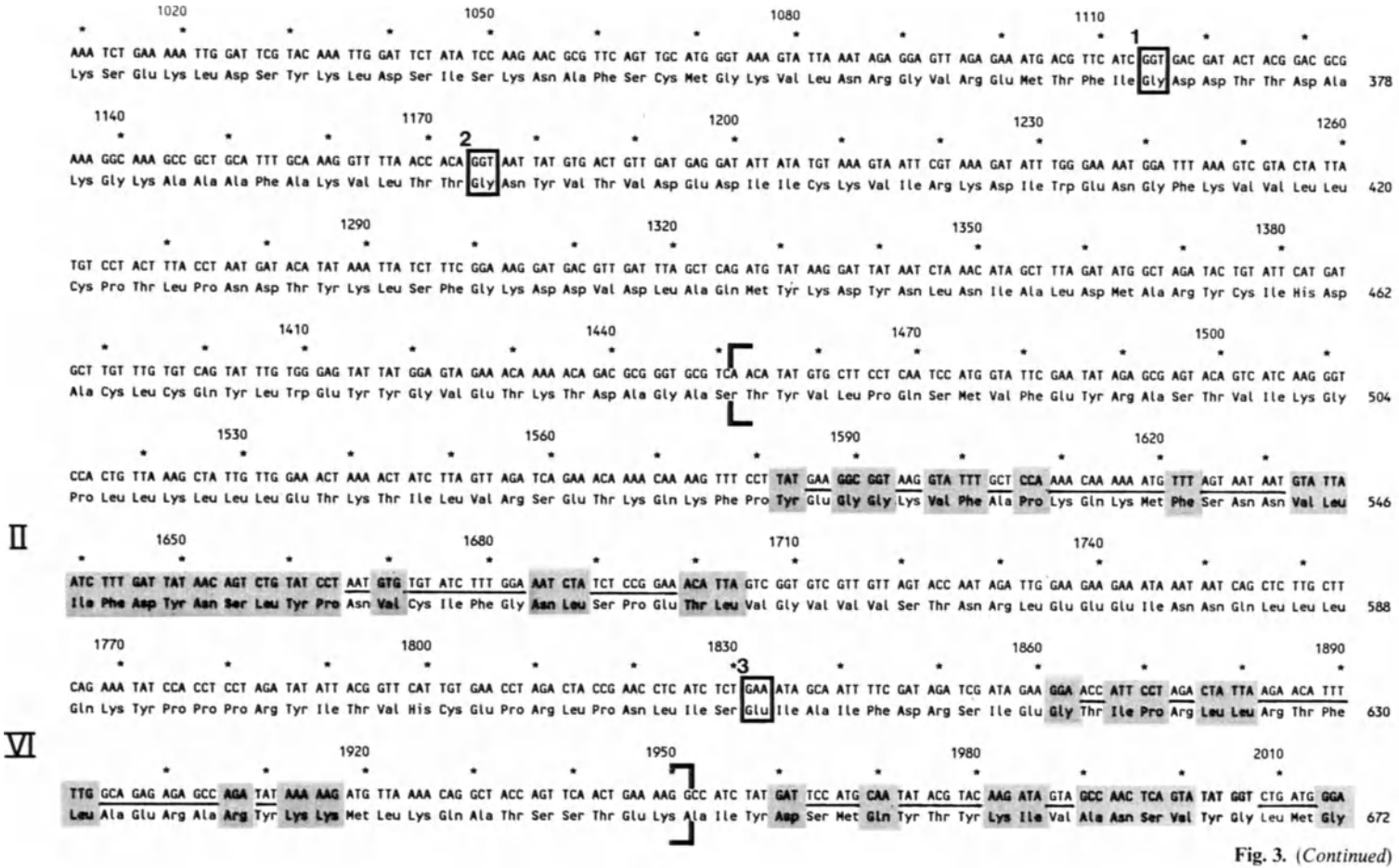


Fig. 3. (*Continued*)

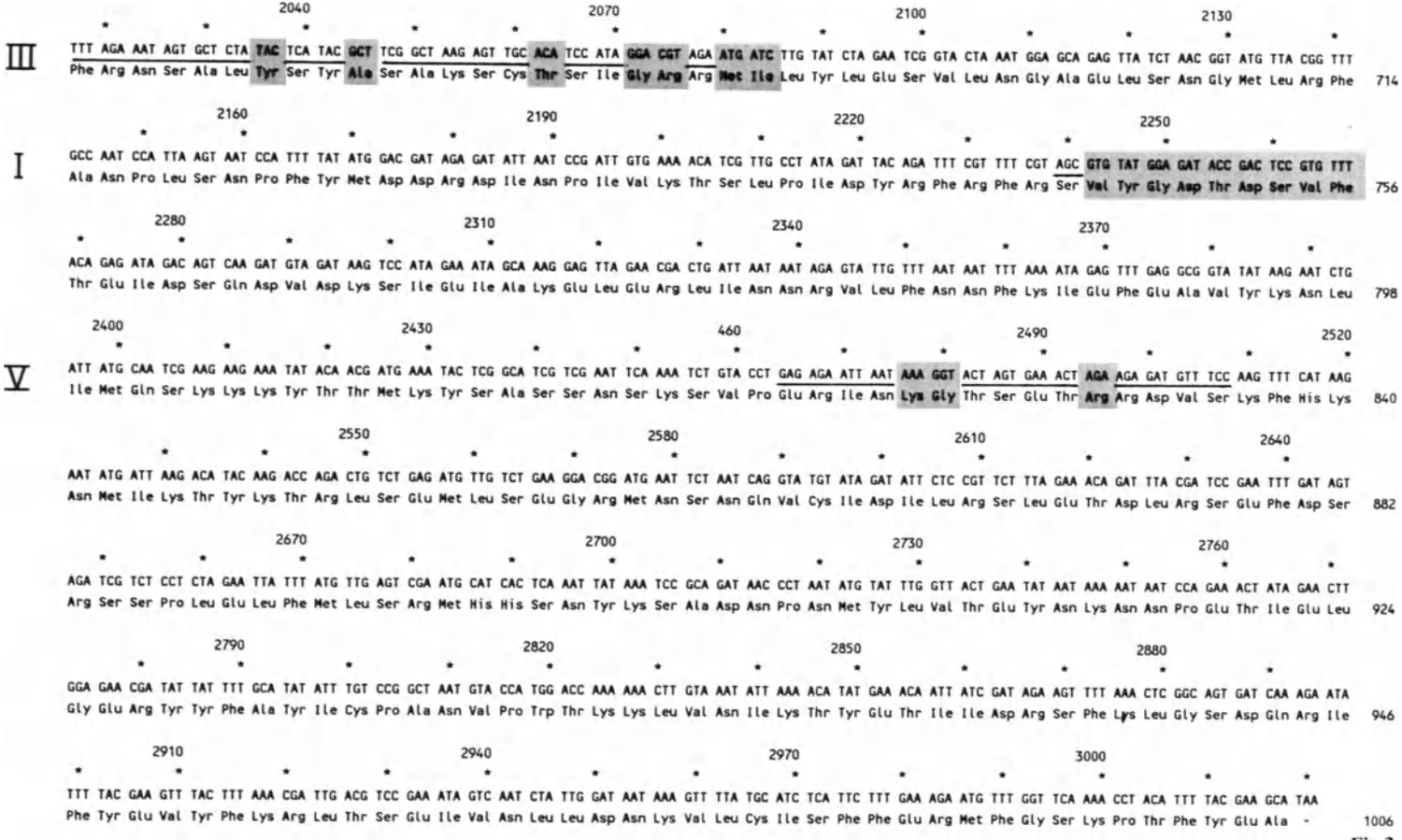


Fig 3

peaks at approximately 2.5 hpi and subsequently declines. Surprisingly, this shutoff of polymerase synthesis is seen even when temperature-sensitive DNA$^-$ mutants are infected at nonpermissive temperature. Even in the absence of DNA replication or late gene expression, when synthesis of many early proteins has been shown to persist (CONDIT and MOTYCZKA 1981), DNA polymerase expression remains transient (MCDONALD and TRAKTMAN, unpublished work). Since this pattern has also been seen with the D5 90 kDa protein (see below), it is tempting to speculate that expression of replication proteins is intrinsically regulated in a manner independent of the onset of replication and late gene expression. Perhaps a necessary virion component decays with time or a negative regulator accumulates post infection. Adding to the complexity of polymerase regulation are the observations that the polymerase gene contains a consensus termination signal (T_5NT) (YUEN and MOSS 1987) downstream of the RNA start site and the initiating ATG and that three potential late start sites (TAAAT) (HANGGI et al. 1986; ROSEL et al. 1986) are present within the upstream region of the polymerase. Indeed, late transcription from the DNA polymerase gene has been seen (KÜNZI and TRAKTMAN 1989; MCDONALD and TRAKTMAN, unpublished work). By extrapolation from other systems, complex regulation of polymerase synthesis at the levels of transcription and translation is likely. It has been reported that the translation of the HSV DNA polymerase mRNA may be affected by the long 5′ leader which possesses potential secondary structural features and short upstream orfs (DORSKY and CRUMPACKER 1988). Similarly, some cytomegalovirus mRNAs, including that encoding the DNA polymerase, contain brief upstream orfs whose interruption can enhance translation (KOUZARIDES et al. 1987; GEBALLE and MOCARSKI 1988). The DNA polymerase of bacteriophage T4, by virtue of binding to its own mRNA, functions as an autogenous translational repressor (ANDRAKE et al. 1988). Complex regulation of viral DNA polymerases appears to be ubiquitous, and it will be of interest to determine the mode by which the expression of the poxvirus enzyme is modulated.

Fig. 3. The complete sequence of the open reading frame encoding the vaccinia virus DNA polymerase is shown. The 1006 amino-acid sequence has been updated to correct a recently discovered error in the original sequence (AHN and MOSS, personal communication, McDonald and Traktman, unpublished work). Correction of an erroneous base insertion in the N-terminal coding region now places the initiating ATG codon further upstream. The open reading frame initiates within the TAAATG motif located one nucleotide downstream of the early transcriptional start site. The corrected sequence thus encodes an additional 25 N-terminal amino acids and alters the next 13 amino acids as a result of a shift in the reading frame. The remainder of the sequence is as previously reported and corrected (EARL et al. 1986; BINNS et al. 1987; TRAKTMAN et al. 1989).

The six regions of homology which are conserved within alpha-like DNA polymerases (WONG et al. 1988) are numbered and their extent indicated with a horizontal line. The shaded residues are the most highly conserved. Boxes 1, 2 and 3 indicate the positions of the PAA^r mutation in NG26, the ts mutation in NG26 and the ts mutation in ts42, respectively. The brackets show the boundaries within which some aphidicolin^r mutations have been localized.

2.1.2 D5: 90 kDa

A second complementation group of temperature-sensitive (ts) mutants with primary defects in DNA replication has been mapped to the *Hin*dIII D fragment and specifically the *D5* orf (EVANS and TRAKTMAN 1987; ROSEMAN and HRUBY 1987). This group contains mutants *Cts17*, *Cts24* (CONDIT et al. 1983), and *Ets69* (ENSINGER and ROVINSKY 1983) of the *WR* strain and mutant *ts6389* of the *IHD* strain of vaccinia (MCFADDEN and DALES 1980). The *D5* orf encodes a protein of 785 amino acids with a predicted molecular weight (MW) of 90,332 (NILES et al. 1986; ROSEMAN and HRUBY 1987); the 3 kb mRNA is transcribed from left to right with respect to the viral genome. A readthrough transcript originating with the upstream *D4* orf and continuing through the *D5* orf is also synthesized. The gene is transcribed early after infection and transcripts are easily detected in RNA preparations isolated after infection in the presence of cycloheximide. In addition to being preceded by early promoter elements, the initiating ATG is part of a TAAATG motif associated with late transcription (ROSEMAN and HRUBY 1987). mRNA hybrid-selected to DNA derived from the *D5* gene can be translated in vitro into a protein of approximately 90 kDa which comigrates with the intracellular product immunoprecipitated with an antiserum generated to the *D5* gene product (EVANS and TRAKTMAN 1987). Thus, there is no evidence for intracellular processing of the protein. The *D5* gene product has previously been called "82 kDa" (EVANS and TRAKTMAN 1987) in the literature because its electrophoretic migration is slightly faster than that of the large subunit of the guanylyltransferase (orf *D1*, predicted to be 96,708 kDa, NILES et al. 1986) which had been referred to as 86 kDa (MORGAN et al. 1984). Synthesis of the protein peaks by 2.5 hpi and has essentially ceased by 4.5 hpi (EVANS and TRAKTMAN 1987), making the presence of the late transcriptional signal an unsolved puzzle. As mentioned above in the discussion of the DNA polymerase, this regulated expression of the 90 kDa protein persists even when DNA replication and late gene expression are blocked by performing infections with ts DNA$^-$ mutants at the nonpermissive temperature. Pulse-chase experiments indicate that intracellular 90 kDa protein is quite stable (EVANS and TRAKTMAN 1987), although the proteins synthesized during infections with *Cts17* and *Cts24* at the nonpermissive temperature are far more labile and show half-lives of as little as 30 min.

The lesions in the three *WR* mutants have been mapped to specific nucleotide changes (ROSEMAN and HRUBY 1987; EVANS and TRAKTMAN, unpublished work). *Cts27* and *Cts24* are closely linked (54 nucleotides apart) and lie within the N-terminal portion of the orf (amino acids 143 and 161). Interestingly, both of these N-terminal changes are predicted to abolish potential phosphorylation sites (as predicted by the IBI-Pustell software for nucleic acid analysis). The lesion in *ts6389* is also N-terminal. The lesion in *Ets69* is in the C-terminal portion of the protein (amino acid 682). Comparison of the *D5* orf with available protein databases does not reveal any significant homologies.

D5 mutants show a significant deficit in DNA replication as assayed by quantitation of accumulated viral DNA (dot blot hybridization) or incorporation

of [^{3}H]thymidine. Incubation of infected cultures at the permissive temperature for 2.5 h prior to shifting to nonpermissive temperatures does not relieve the block in [^{3}H]thymidine incorporation. Moreover, shifting the cultures to the non-permissive temperature during DNA replication leads to an immediate cessation in [^{3}H]thymidine incorporation (EVANS and TRAKTMAN, unpublished work). The *D5* gene product appears to be essential for continuing DNA synthesis, and the "fast-stop" phenotype of the mutants suggests that the D5 90 kDa protein may well act directly at the replication fork. Future biochemical studies of D5 function will be facilitated by the availability of a recombinant vaccinia virus which directs the inducible overexpression of the 90 kDa protein (EVANS and TRAKTMAN, submitted).

In addition to their replication defect, *Cts17*, *Cts24*, and *ts6389* display unusual behavior during marker rescue analyses. Efficient mapping of other mutants by marker rescue has been demonstrated with fragments as small as a few hundred base pairs (TRAKTMAN et al. 1989b). These three mutants, however, could not be rescued by fragments smaller than 2 kb, and rescue was quite sensitive to the site of plasmid linearization. This block to rescue could be relieved by continuing the incubation at the permissive temperature for 2–4 h post transfection (EVANS and TRAKTMAN, unpublished work). This requirement for the presence of "functional" 90 kDa following transfection suggests that the protein may play some role in stabilizing, activating, or facilitating the actual recombination of DNA. An understanding of these observations will require future studies, although degradation of the transfected DNA at the nonpermissive temperature has not been observed and is therefore an unlikely explanation. Lastly, some aspect of thymidine kinase (tk) regulation appears to be altered during infection with *Cts17* and *Cts24* at the nonpermissive temperature. Typically, the level of tk activity detected in crude extracts reaches a plateau at approximately 4 hpi. This plateau, which is ascribed to feedback inhibition by the intracellular products of tk enzymatic activity, is not seen in extracts derived from non-permissive infections with *Cts17* and *Cts24* (ROSEMAN and HRUBY 1987). This observation remains unexplained.

2.1.3 B1: 34 kDa

Two additional mutants yielding replication defects, *Cts2* and *Cts25* (CONDIT et al. 1983), form the third complementation group. Originally mapped to 25 kb within the rightmost quarter of the genome by marker rescue with cosmid clones (THOMPSON and CONDIT 1986), these mutants have now been mapped to the *B1* orf (REMPEL et al. 1990). The gene is expressed early after infection; the 1 kb mRNA is transcribed in a rightward direction relative to the viral genome. The in vitro translation product of the mRNA hybrid-selected by the gene is a 30 kDa polypeptide. Transcriptional initiation and termination signals are typical of early transcripts; the orf consists of 900 nucleotides predicted to encode a 300 amino acid protein of 34,271 Da (TRAKTMAN et al. 1989). Sequence for this region

has also been obtained in the laboratory of G. SMITH (personal communication). The lesions in *ts2* and *ts25* have been mapped to amino acids 79 and 227, respectively (TRAKTMAN et al. 1989a).

Most interestingly, the predicted amino acid sequence shows significant homology to a family of serine-threonine protein kinases. Sequences homologous to the ATP-binding domain and the phosphorylation receptor domain of these kinases are well-conserved in the *B1* sequence. Biochemical characterization of the putative kinase remains preliminary, although the availability of an anti-34 kDa antibody will facilitate these studies. The role of this protein in viral replication is not known, nor is the detailed nature of the replication defect in *Cts2* and *Cts25* during infection at the nonpermissive temperature. Interestingly, the arrest of the mutants is severe at low or high multiplicities in mouse L cells (nonpermissive DNA synthesis is $< 5\%$ that seen in permissive infection, viral yields are 0.1%–1% of those obtained in permissive infection), whereas the defect in monkey BSC40 cells is less severe (REMPEL et al. 1990). For example, infection of these cells with *ts25* at an moi of 15 results in significant levels of DNA synthesis (50%–65% relative to permissive infection) and of infectious progeny (24 h yield of 10%–20% relative to permissive infection). The cause of this variation in phenotype is unknown.

2.2 Vaccinia-Encoded Enzymes Involved in Precursor Metabolism

2.2.1 Thymidine Kinase

The *tk* was one of the first viral genes to be identified genetically and to be localized on the vaccinia genome (HRUBY and BALL 1982; HRUBY et al. 1983; WEIR et al. 1982; WEIR and MOSS 1983). The ability to isolate tk^- viral mutants by applying 5-bromodeoxyuridine (BUdR) selection during growth on tk^- cell lines established that the enzyme was not essential for growth in tissue culture. This finding opened the way for molecular dissection of the regulation of the *tk* locus and for its later use as an insertion site for foreign genes. Genes encoding tk have also been mapped and sequenced in fowlpox (BOYLE et al. 1987) and Shope fibroma virus (UPTON and MCFADDEN 1986).

The vaccinia tk is synthesized as a 19 kDa monomer; the tetrameric form is enzymatically active in phosphorylating thymidine to dTMP. Translation of the viral mRNA in reticulocyte extracts or in injected *Xenopus* oocytes yields active enzyme, suggesting that the enzyme requires no unusual modifications or cofactors. The gene for tk is located within the leftmost region of the *Hin*dIII J fragment and contains the typical regulatory signals for early transcription. The steady-state level of tk mRNA peaks at approximately 3–4 hpi, after which transcripts diminish and become undetectable by 8 hpi (HRUBY and BALL 1981).

These data exemplify the observation that early vaccinia mRNAs are short-lived, a consequence of transient synthesis and apparent lability. Enzyme activity can be detected almost immediately in infected cell extracts and increases during the first 4 hpi. Enzyme activity plateaus abruptly at this point and remains unchanged for the duration of the infection. No net increase in intracellular enzyme activity occurs after 4 hpi even though translatable tk mRNA is still present in vivo. This static level of tk activity was thought to reflect either repressed translation of tk mRNA or inhibition of enzymatic activity at late times after infection. In support of the latter possibility was the finding that the vaccinia tk is inhibited significantly by dTDP and dTTP (HRUBY 1985). Cytoplasmic dTDP and dTTP generated by tk itself might produce the plateau seen in the level of enzyme activity measured in unfractionated cellular extracts. Although the viral ribonucleotide reductase would also generate these products, the profile of tk activity was found to be unchanged in the presence of hydroxyurea, a potent inhibitor of ribonucleotide reductase. The regulation of tk enzymatic activity is apparently independent of the late stages of viral infection. Normal down-regulation occurs in most viral mutants blocked in DNA synthesis and in nonpermissive cell lines which permit neither replication nor late gene expression (FRANKE et al. 1985).

Preparation of an anti-serum directed against tk has allowed quantitation of protein levels which do not rely on measurements of enzymatic activity. Immunoblot and immunoprecipitation analyses indicate that the level of tk protein rises steeply during early infection, slows, and then remains level for the duration of infection (HRUBY, personal communication). These data suggest that synthesis of the enzyme continues as long as the mRNA persists and that the protein is quite stable. The early plateau seen in intracellular enzyme activity probably reflects the balance between continued synthesis of low levels of tk and feedback inhibition of its activity. In vivo inhibition of tk at approximately 4 hpi partially explains the observation that incorporation of exogenously added thymidine into nascent DNA ceases by approximately 5 hpi, although filter hybridization analyses indicate that DNA accumulation continues for an additional 4–7 h.

Although the viral tk is not essential in tissue culture, its inactivation has a dramatic effect on the virus' ability to spread in animals. Comparison of the LD_{50} of tk^- virus and wild type virus in mice reveals that the pathogenicity of tk^- virus after intracerebral inoculation is reduced by at least four orders of magnitude (BULLER et al. 1985; see also chapter by TURNER and MOYER, this volume).

2.2.2 Thymidylate Kinase

During the preparation of this chapter, evidence for a vaccinia-encoded orf with strong homology to the *S. cerevisiae* thymidylate kinase gene was obtained (SMITH et al. 1989b). The gene maps within the *Hin*dIII A fragment and is

designated as the 13th orf of the *Sal*I F fragment (*Sal*F *13R*). The gene is transcribed early after infection and the mature protein is predicted to have a MW of 23,319. This gene, whose putative product would act directly after tk in the purine biosynthetic pathway, is not essential for viral growth in tissue culture.

2.2.3 Ribonucleotide Reductase

Vaccinia virus encodes another enzymatic component directed towards the synthesis of precursors necessary for DNA replication. The viral ribonucleotide reductase (rr) is a two subunit enzyme with structural and functional similarities to analogous enzymes from mammalian and procaryotic systems (SLABAUGH et al. 1984; SLABAUGH and MATHEWS 1984). Ribonucleotide reductase catalyzes the conversion of rNDP to dNDP. Like the mammalian enzyme, vaccinia rr is allosterically inhibited by dATP and dTTP. The 34 kDa M2 subunit is encoded by the *Hin*dIII F fragment; analysis of the deduced amino acid sequence reveals 80% homology to the cellular enzyme (SLABAUGH et al. 1988). This catalytic subunit contains the essential active site tyrosine. The large M1 subunit of the rr, predicted to contain the regulatory nucleotide binding sites, has been mapped to the *Hin*dIII *I3* gene (TENGELSEN et al. 1988). The derived amino acid sequence shares a high level of homology with the mouse M1 subunit sequence. Selection of viral mutants resistant to hydroxyurea yielded isolates which had amplified the M2 gene (SLABAUGH and MATHEWS 1986; SLABAUGH et al. 1988). Mutants contained 2–15 tandemly arrayed copies of this gene; overexpression of the 34 kDa subunit alone led to a significant increase in intracellular rr activity, implying that the M2 subunit is normally limiting. Overproduction of enzyme activity conferred resistance to hydroxyurea (HU), although the excess enzyme showed no intrinsic resistance to the drug.

Expression of the enzyme after wild type (wt) infection begins immediately; cytoplasmic extracts show a peak of activity at 4–5 hpi with a subsequent plateau (SLABAUGH and MATHEWS 1986). In an earlier report, enzyme activity was found to decline during the late phase of infection (SLABAUGH and MATHEWS 1984). The M1 and M2 mRNAs are synthesized early; their levels peak by 3 hpi and then gradually decline, remaining detectable until 9 hpi. Immunological profiles of the synthesis and accumulation of the M1 and M2 proteins mirror the profile of mRNA levels and are consistent with the observed pattern of intracellular enzyme activity. Levels of M2 mRNA and protein are consistently higher than those of the corresponding M1 species. Pulse-chase analyses indicate that both subunits are quite stable (ROSEMAN and SLABAUGH, personal communication).

In a variety of other systems, rr is implicated in regulating the onset of DNA synthesis. Thus, it is of interest to comment on DNA synthesis in the HU^r mutants which overproduce enzyme activity (SLABAUGH and MATHEWS 1986). These mutants incorporate more [^{3}H]thymidine into DNA than does wt virus, and the peak of maximal incorporation occurs at an earlier time after infection. These

results suggest that the rate of vaccinia replication may be determined, in part, by the generation of sufficient precursor pools. Although it might have been a reasonable prediction that the pattern of DNA synthesis in HU^r mutants would be equivalent in the presence or absence of HU, that is not the case. Following infection with HU^r isolates in the presence of HU, the levels of thymidine incorporation are far lower than under control conditions and appear to increase linearly for up to 12 h. Why thymidine incorporation persists for so long under these conditions has not been explained, although the smaller precursor pools present as a result of HU inhibition may relieve the feedback inhibition of tk.

Like tk, vaccinia rr is not essential under the conditions of tissue culture (HRUBY and SLABAUGH, personal communication); however, viral DNA synthesis is completely inhibited in the presence of HU, an inhibitor of both cellular and viral enzymes. These data suggest that functional rr, albeit not necessarily viral, is required for efficient replication. Previous observations that mammalian rr is localized within the cytoplasm would be consistent with the substitution of the cellular enzyme for the viral counterpart; nevertheless, the strict cell cycle regulation of the cellular enzyme makes its availability to the infecting virus problematic. With regard to the essentiality of the vaccinia enzyme in animal infections, the pathogenicity of viruses containing inactivated M1 genes has been tested intracerebrally in mice. Attenuation was moderate, causing an increase in LD_{50} of two orders of magnitude. Viruses with inactivated rr M1 and tk genes were more severely compromised than were viruses with either single gene deficiency (HRUBY and BULLER, personal communication).

2.3 Topoisomerase

A type I topoisomerase activity has been purified from vaccinia virions (BAUER et al. 1977; FOGLESONG and BAUER 1984; SHAFFER and TRAKTMAN 1987; SHUMAN and MOSS 1987). It is a single subunit enzyme of 32 kDa (SHAFFER and TRAKTMAN 1987). Like eucaryotic type I enzymes in general, the enzyme will relax negatively or positively supercoiled DNA without an energy cofactor. Relaxation involves the formation of a transient intermediate in which the enzyme cleaves one strand of the DNA and covalently binds to the 3′ phosphate of the broken strand (SHAFFER and TRAKTMAN 1987). DNA relaxation is stimulated by, but does not require, a divalent cation. Unlike other type I eucaryotic enzymes, camptothecin does not inhibit the enzyme (SHUMAN et al. 1988; COUNTRYMAN and TRAKTMAN, unpublished work); however, classical inhibitors of type II enzymes such as novobiocin, coumermycin, and the epipodophyllotoxins are potent inhibitors of the vaccinia enzyme (FOGLESONG and BAUER 1984; SHAFFER and TRAKTMAN 1987; COUNTRYMAN and TRAKTMAN, unpublished work). This paradox has not been resolved.

The gene for the topoisomerase was mapped by comparing DNA sequence deduced from N-terminal protein sequence to the available database of genomic vaccinia sequences (SHUMAN et al. 1987). The enzyme is encoded by the *H7* orf. Sequence comparison reveals that the vaccinia enzyme shares several regions of homology with the yeast, rat, and human type I topoisomerases. This conservation of structural features is especially interesting considering that the vaccinia enzyme is approximately one-third the MW of the other enzymes. Corresponding topoisomerase genes have been found in Shope fibroma virus (UPTON and MCFADDEN, personal communication) and fowlpox (BINNS and BOURSNELL, personal communication).

The orf begins with the consensus late transcriptional signal, TAAATG (HANGGI et al. 1986; ROSEL et al. 1986). Immunoprecipitation of metabolically labeled extracts reveals de novo synthesis of the topoisomerase with kinetics characteristic of late gene products (COUNTRYMAN and TRAKTMAN, unpublished work). Intracellular topoisomerase activity is first observed at 3 h following infection of cytoplasts (PODDAR and BAUER 1986). Attempts to create a virus lacking functional topoisomerase by interrupting the gene with a foreign, selectable marker have been unsuccessful (Shuman et al. 1989), making it almost certain that the enzyme is essential for viral replication. Encapsidation of the enzyme implies a role in early transcription, a suggestion consistent with studies in other systems. Likewise, the observations that topoisomerase is required to relieve the torsional stress generated during DNA synthesis of SV40, adenovirus, and bacteriophage T4 make it most likely that the enzyme will also play an important role in facilitating DNA replication. The enzyme has been overproduced in *E. coli* (SHUMAN et al. 1988; COUNTRYMAN and TRAKTMAN, unpublished work), and the recombinant enzyme displays indistinguishable biochemical properties from the authentic topoisomerase.

2.4 Nicking-Joining Nuclease

As described above, initiation of viral DNA synthesis is thought to require the formation of a specific nick near the hairpin terminus of the viral genome. Resolution of unit length viral genomes from concatameric replication intermediates must also involve scission and religation of the viral DNA at sites within or near the hairpin termini. These features of the replication cycle have focused attention on several nuclease activities induced after infection or encapsidated within vaccinia virions (ROSEMOND-HORNBEAK and MOSS 1974; ROSEMOND-HORNBEAK et al. 1974; POGO and DALES 1969; POGO and O'SHEA 1977; LAKRITZ et al. 1985; MERCHLINSKY et al. 1988; REDDY and BAUER 1989). Early reports presented evidence for a neutral-alkaline (pH 7.8 optimum) endonuclease and an acidic (pH optimum 4.5) exonuclease within viral cores. The endonuclease was found to leave 5′OH and 3′P termini and the exonuclease was found to degrade

DNA from the 5′ terminus (POGO and O'SHEA 1977). Treatment of viral cores with heat or trypsin preferentially enhanced the activity of the pH 7.8 endonuclease. Both activities appeared to reside in polypeptides of 50 kDa. Recent evidence (LAKRITZ et al. 1985; REDDY and BAUER 1989) suggests that the multiple activities probably reside in one homo- or heterodimeric enzyme with a native MW of 105,000 and subunits of 50,000.

This enzyme performs sequential operations on supercoiled plasmid DNA—nicking, subsequent linearization, and, lastly, cross-linking of products—to produce hairpin termini (LAKRITZ et al. 1985; REDDY and BAUER 1989). These activities occur independently of an energy cofactor. The purified intermediate products are not substrates for the later reactions, implying that these are concerted reactions which may involve covalent enzyme-DNA intermediates. Analogies can be made to the mechanism of action of topoisomerase I enzymes, which nick and reseal DNA in the absence of an energy cofactor by forming a covalent enzyme-DNA intermediate. The observed 3′P and 5′OH termini found after endonucleolytic cleavage with the 50 kDa nuclease reinforce this mechanistic similarity with eucaryotic topoisomerases. Only reactions performed at elevated temperatures or in the presence of denaturants have generated significant levels of linearized or cross-linked products, suggesting that progression of the reaction beyond nicking is facilitated by melting of the DNA duplex. Moreover, trypsinization of the 50 kDa enzyme, with the resultant production of a 45 kDa product, significantly increases cross-linking activity and brings the optima of the enzyme closer to physiological salt and pH conditions.

Sequence specificity for DNA linearization has been demonstrated for the nuclease. Incubation with plasmids containing the nearly palindromic vaccinia concatameric junction fragment (in an extruded cruciform) leads to cleavage at sites near the central axis of the palindrome (the apex of the extruded cruciform) (MERCHLINSKY et al. 1988). To fulfill the role of the resolving enzyme, cleavage should be at the base of the extruded cruciform, suggesting that participation of this enzyme in resolution might require modulation with other proteins capable of providing additional site specificity. Although our understanding of the enzyme is incomplete, it remains a good candidate for one of the enzymes involved in initiating and/or terminating DNA replication. Modulation of nicking activity vs cross-linking activity might allow encapsidated nuclease to initiate replication and processed, intracellular enzyme to resolve concatameric intermediates.

2.5 DNA Ligase

Although the most widely accepted model for vaccinia DNA replication does not invoke the formation and subsequent ligation of Okazaki fragments, the need for a DNA ligase in vaccinia infection has been proposed. First, intra- and

intergenomic homologous recombination proceed at a high rate following vaccinia infection, as suggested experimentally by genetic exchange between viral genomes and/or transfected DNA plasmids (BALL 1987; EVANS et al. 1988, SPYROPOULOS et al. 1988). A DNA ligase might play a role in sealing the nicks resulting from recombinational exchanges. Moreover, the resolution of concatemers into monomeric genomes is thought to involve scission of the concatemers at the terminal repeat region (invoking either the staggered nick model or the cruciform extrusion/resolution model) followed by religation of the daughter hairpins. There were two early reports concerning a putative vaccinia ligase. SAMBROOK and SHATKIN (1969) demonstrated more than a tenfold increase in ATP-dependent DNA ligase activity in cytoplasmic extracts following infection with vaccinia virus. This rise in activity was dependent on protein synthesis but not on viral DNA synthesis, and no activity was found in viral cores. An increase in intracellular DNA ligase activity following vaccinia infection was also reported by SPADARI (1976). Although a more modest rise in enzyme activity was found, the kinetics of ligase appearance were consistent with its characterization as an early viral protein. Using the criteria of chromatographic behavior, cofactor requirements, and MW, however, the author could not distinguish between cellular and viral enzymes.

More recent evidence for the presence of a viral DNA ligase was obtained by detecting a novel enzyme-[^{32}P]AMP covalent intermediate (of approximately 65 kDa) in extracts of infected cells (ANDERSON and TRAKTMAN, unpublished work). Definitive proof of a virally-encoded enzyme appeared in the literature during the preparation of this chapter (SMITH et al. 1989a; KERR and SMITH 1989). The *Sal*F *15R* orf (mapping in the extreme right of the *Hin*dIII A genomic fragment) was shown to encode a 552 amino acid protein with significant homology to yeast DNA ligases. An antiserum prepared to the product of this gene immunoprecipitated a 61 kDa protein from infected cells as well as the ligase-[^{32}P]AMP covalent intermediate that could be generated in infected cell extracts. This important finding opens the way for the determination of the role of the viral ligase in replication, repair, and recombination.

2.6 Virion-Associated DNA-Binding Proteins

2.6.1 VP11: An 11 kDa Virion Polypeptide

One of the most abundant polypeptides within the vaccinia core is an 11 kDa polypeptide (ROSEMOND-HORNBEAK and MOSS 1973; POGO et al. 1975; KAO et al. 1981; KAO and BAUER 1987). This protein accounts for approximately 11% of the viral core by mass. Synthesis of this highly basic protein during infection is dependent upon DNA synthesis, making it a member of the late class of viral gene products. The majority of the protein becomes phosphorylated at two different serine residues, and the phosphorylated forms are preferentially encapsidated

into virions. The protein has been shown to aggregate in vitro and to possess DNA binding activity. Maximal DNA binding is obtained with positively or negatively supercoiled substrates. Comparisons to histone H1 have been made, elicited by the basic nature of the protein and its ability to saturate DNA substrates with high levels of bound protein. The current model of VP11 action is as a DNA binding protein involved in condensation of the viral genome into the viral equivalent of a chromatin conformation. Observations of an 11 kDa protein in virosomes containing replicating DNA have been inconsistent, and any involvement in facilitating or regulating transcription, replication, concatemer resolution, or DNA encapsidation remains speculative. The gene encoding this 11 kDa protein has not yet been identified, although a gene encoding a late 11 kDa protein has been characterized (WITTEK et al. 1984). Several 11 kDa species have been detected in virions (PERSON-FERNANDEZ and BEAUD 1986; BROCK and TRAKTMAN, unpublished work), and 11 kDa proteins have also been implicated in cytoskeletal interactions (HILLER and WEBER 1982) and the inhibition of host protein synthesis (PERSON-FERNANDEZ and BEAUD 1986).

2.6.2 VP8: A 25 kDa Virion Polypeptide

Another abundant virion protein with affinity for DNA has been described: Accounting for some 7% of the protein mass of the vaccinia particle, 7×10^3 copies of VP8 are estimated to be present per virion (YANG and BAUER 1988a). The monomeric VP8 protein has equivalent affinities for circular, single-, and double-stranded linear DNA at low salt concentrations (25 m*M*), but reveals a clear preference for circular DNA substrates when assayed at 100 m*M* salt. No sequence specificity for DNA binding was observed. Participation of VP8, along with VP11, in the condensation and organization of the viral nucleoprotein complex is suggested. SAROV and JOKLIK (1973) have shown that during infection, as viral DNA is lost from intracellular cores during secondary uncoating, VP8 loses its association with the cores as well. This suggests that the association of VP8 and the viral genome persists only through the early phase of viral gene expression. Any role for VP8 during the phases of replication and/or late gene expression has yet to be demonstrated. VP8 is made de novo only after viral DNA replication has begun and presumably is then complexed with progeny genomes during encapsidation. The gene encoding VP8 has been localized to the *Hin*dIII L fragment (YANG and BAUER 1988b). The primary translation product has a MW of 28000, which is then processed proteolytically into the mature 25000 MW protein. Processing occurs between amino acid 32 (gly) and 33 (ala). Pulse-chase experiments have shown that the precursor persists for approximately 2 h before being processed into the 25 kDa form. Like the proteolytic maturation of other virion proteins (4b, for example), VP8 processing is inhibited by rifampicin. The inhibition of protein maturation by rifampicin blocks virion assembly, suggesting that processing of VP8 may regulate its ability to form nucleoprotein complexes which participate in or induce genome encapsidation.

2.7 Virosome-Associated DNA-Binding Proteins

Vaccinia DNA replication occurs in dense cytoplasmic aggregates of viral DNA and proteins known as virosomes. Several investigators have developed techniques for the partial purification of virosomes by sucrose gradient centrifugation of cytoplasmic extracts (DAHL and KATES 1970; NOWAKOWSKI et al. 1987a, b; POLISKY and KATES 1972, 1975). Parallel treatment of uninfected cultures yields little if any material. Proteolytic and endonucleolytic digestion has confirmed that both DNA and protein are responsible for the formation of the dense aggregate. Newly synthesized viral proteins and DNA can be identified by their incorporation of radiolabeled precursors introduced into the culture medium at the time of infection. Studies of the major virosomal polypeptides by different investigators have not been wholly consistent. Nevertheless, it is possible to compare molecular weight, posttranslational modification and chromatographic behavior of the reported proteins and arrive at some consensus. Abundant virosomal proteins with affinity for DNA might represent such replication components as single-strand DNA binding proteins.

2.7.1 p34 kDa

Virosomes contain a number of newly labeled protein components, several of which are highly abundant and have been purified. One is a phosphoprotein of 34 kDa described variously as FP11 (NOWAKOWSKI et al. 1978 a, b) and polypeptide B (POLISKY and KATES 1972, 1975; SAROV and JOKLIK 1973). This protein comprises 17% of the [^{3}H]methionine incorporated into virosomes but 33% of the virosomally incorporated [^{3}H]lysine. Despite this high content of basic residues, the purified protein has an isolectric point close to 5.5. This discrepancy may arise from the apparent phosphorylation of p34 kDa on multiple threonine residues. Affinities for native calf thymus DNA cellulose (from which it can be eluted with 0.6 *M* Nacl; POLISKY and KATES 1975), and for single-stranded DNA cellulose (from which it can be eluted with 0.25 *M* NaCl; NOWAKOWSKI et al. 1978a, b) have been demonstrated. Radiolabeled p34 kDa appears shortly after infection, and its synthesis is not impaired by inhibitors of DNA replication. Affinity for DNA has also been confirmed by assembly of nonspecific protein-DNA aggregates in vitro (POLISKY and KATES 1976). No evidence has been found for encapsidation of p34 kDa in virions.

2.7.2 28 kDa

Like p34 kDa, 28 kDa has also been purified by single-stranded DNA-cellulose chromatography of isolated virosomes (NOWAKOWSKI et al. 1978a). The 28 kDa protein binds more tightly to the resin and is eluted as a broad peak with 0.45–0.8 *M* NaCl. It accounts for approximately 10% of the radioactivity incorporated into virosomal proteins.

2.7.3 40 kDa

A third virosomal protein, 40 kDa, has been identified (POLISKY and KATES 1972; SAROV and JOKLIK 1973; NOWAKOWSKI et al. 1978a). This polypeptide was eluted from single-stranded DNA-cellulose with 0.08 *M* NaCl. The synthesis of this polypeptide lags behind that of p34 kDa (POLISKY and KATES 1972) and can be blocked by cytosine arabinoside (SAROV and JOKLIK 1973), indicating that it is a late protein whose expression depends on prior DNA replication. In conflict with these observations was the unimpaired expression observed by POLISKY and KATES (1972) in the presence of 5-fluorouracil deoxyribonucleoside (FudR). Rapid sedimentation of this protein was not disrupted by treatment of virosomal complexes with DNAse providing evidence of its inclusion in a macromolecular complex (POLISKY and KATES 1972). The 40 kDa protein may thus have an intrinsic tendency of aggregate. In vitro reconstitution of nonspecific protein DNA aggregates has confirmed the affinity of the 40 kDa protein for DNA (POLISKY and KATES 1976). No evidence has been found for the inclusion of the 40 kDa in virions.

3 In Vitro Analyses of Poxvirus Replication

The fullest understanding of DNA replication has come from those systems where in vitro replication assays are available to complement genetic and biochemical approaches followed in vivo. Although quite preliminary, some in vitro analysis of poxvirus replication has been reported (ESTEBAN and HOLOWCZAK 1972; LAMBERT and MAGEE 1977). Concentrated cytoplasmic extracts of infected cells have been shown to be competent for DNA synthesis on endogenous templates using endogenous enzymes. Completion of one round of semiconservative synthesis has been reported; no evidence of subsequent initiations was seen. Extracts were stimulated by ATP, rNTPs, and dNTPs. Synthesis has been shown to be inhibited by PAA and HU, inhibitors of intracellular DNA replication (SHAFFER and TRAKTMAN, unpublished work).

Preliminary fractionation of the extracts was accomplished by centrifugation: soluble enzymes were recovered from the top of sucrose gradients and virosomal aggregates as pellets (LAMBERT and MAGEE 1977). The soluble enzymes, either as prepared or after chromatographic fractionation, were capable of augmenting the replication activity of the virosomal fraction. These studies provided a promising foundation for the eventual purification of an in vitro replication system; however, cytoplasmic extracts prepared from cells infected with replication mutants have not yet reflected the phenotype of the mutants (SHAFFER and TRAKTMAN, unpublished work). Thus, the system requires refinement prior to providing an in vitro complementation system for the biochemical dissection of gene products by analysis of conditionally lethal mutants.

4 Expectations for Future Research

The last 5 years have seen an enormous amount of progress in the dissection of poxvirus replication. Our understanding of the structure of the genomic termini and of the concatemeric replication intermediates has deepened significantly (for greater detail, see DELANGE and MCFADDEN, in this volume). The genes for tk, rr, thymidylate kinase, DNA polymerase, topoisomerase, DNA ligase, and two other essential replication components have been identified and their transcriptional pattern and DNA sequence determined (see above). Purification of some of these proteins has been facilitated by the use of pro- and eucaryotic expression vectors. Nonetheless, many of the presumed components of the replication machinery have yet to be detected or purified, and the functions of the essential *D5* (90 kDa) and *B1* (34 kDa) gene products remain unknown. These are the primary tasks for the next few years, amenable to a combined genetic, biochemical, and molecular biological approach. Reports of intracellular proteins with affinity for DNA need to be reexamined in the light of our current predictions for the array of replication functions. Polymerase accessory proteins, a single-strand binding protein, and a DNA helicase must be sought within infected cells. Effort must also be applied to the refinement of the in vitro replication system. In vitro replication studies of defined, exogenous templates would clarify our understanding of the events of the replication process. Iteration of mutant phenotypes in vitro would permit functional complementation with purified replication proteins. In concert, these studies will provide insight into the functional roles of the viral proteins engaged in the replication of the poxvirus genome.

Acknowledgments. I would like to thank the members of my laboratory who provided support and helpful criticism throughout the preparation of this chapter and to acknowledge the various colleagues who generously communicated information prior to publication. Some of the work described in this chapter was supported by grants from the NIH (A121758) and NSF (PYI DMB8451881) and by funds provided by Reliance Group Industries, the Merck Development Program for Woman Scientists, and a special group of donors from the Dorothy Rodbell Cohen Foundation. The author was a recipient of an Andrew W. Mellon Teacher–Scientist Award and is currently supported by an Irma T. Hirschl Career Scientist Award.

References

Andrake M, Guild N, Hsu T, Gold L, Tuerk C, Karam J (1988) DNA polymerase of bacteriophage T4 is an autogenous translational repressor. Proc Natl Acad Sci USA 85: 7942–7946

Ball LA (1987) High-frequency homologous recombination in vaccinia virus DNA. J Virol 61: 1788–1795

Baroudy BM, Venkatesan S, Moss B (1982) Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. Cell 28: 315–324

Bauer WR, Ressner EC, Kates J, Patzke JV (1977) A DNA nicking-closing enzyme encapsidated in vaccinia virus: partial purification and properties. Proc Natl Acad Sci USA 74: 1841–1845

Bernad A, Zaballos A, Salas M, Blanco L (1987) Structural and functional relationships between prokaryotic and eukaryotic DNA polymerases. EMBOJ 6: 4219–4225

Berns KI, Silverman C, Weissbach A (1969) Separation of a new deoxyribonucleic acid polymerase from vaccinia-infected HeLa cells. J Virol 4: 15–23

Binns MM, Stenzler L, Tomley FM, Campbell J, Boursnell MEG (1987) Identification by a random sequencing strategy of the fowlpoxvirus DNA polymerase gene, its nucleotide sequence and comparison with other viral DNA polymerases. Nucleic Acids Res 15: 6563–6573

Boyle DB, Coupar BE, Gibbs AJ, Seigman LJ, Both GW (1987) Fowlpox virus thymidine kinase: nucleotide sequence and relationships to other thymidine kinases. Virology 156: 355–365

Buller RM, Smith GL, Cremer K, Notkins AL, Moss B (1985) Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. Nature 317: 813–815

Campbell JL (1986) Eukaryotic DNA replication. Annu Rev Biochem 55: 733–771

Challberg MD, Englund PT (1979a) Purification and properties of the deoxyribonucleic acid polymerase induced by vaccinia virus. J Biol Chem 254: 7812–7819

Challberg MD, Englund PT (1979b) The effect of template secondary structure on vaccinia DNA polymerase. J Biol Chem 254: 7820–7826

Citarella RV, Muller R, Schlabach A, Weissbach A (1972) Studies on vaccinia virus-directed deoxyribonucleic acid polymerase. J Virol 10: 721–729

Condit RC, Motyczka A (1981) Isolation and preliminary characterization of temperature sensitive mutants of vaccinia virus. Virology 113: 224–241

Condit RC, Motyczka A, Spizz G (1983) Isolation, characterization and physical mapping of temperature sensitive mutants of vaccinia virus. Virology 128: 429–443

Dahl R, Kates JR (1970) Intracellular structures containing vaccinia DNA: isolation and characterization. Virology 42: 453–462

DeFilippes FM (1984) Effect of aphidicolinon vaccinia virus: isolation of an aphidicolin-resistant mutant. J Virol 52: 474–482

DeLange AM (1989) Identification of temperature-sensitive mutants of vaccinia virus that are defective in conversion of concatemeric replicative intermediates to the mature linear DNA genome. J Virol 63: 2437–2444

DeLange AM, McFadden G (1986) Sequence-nonspecific replication of transfected plasmid DNA in poxvirus-infected cells. Proc Natl Acad Sci USA 83: 614–618

DeLange AM, Reddy M, Scraba D, Upton C, McFadden G (1986) Replication and resolution of cloned poxvirus telomeres in vivo generates linear minichromosomes with intact viral hairpin termini. J Virol 59: 249–259

Dorsky DI, Crumpacker CS (1988) Expression of herpes simplex virus type 1 DNA polymerase gene by in vitro translation and effects of gene deletions on activity. J Virol 62: 3224–3232

Earl PL, Jones EV, Moss B (1986) Homology between DNA polymerase of poxviruses, herpesviruses and adenoviruses: nucleotide sequence of the vaccinia virus DNA polymerase gene. Proc Natl Acad Sci USA 83: 3659–3663

Ensinger M (1982) Isolation and genetic characterization of temperature-sensitive mutants of vaccinia virus WR. J Virol 43: 778–790

Ensinger MJ (1987) Phenotypic characterization of temperature-sensitive mutants of vaccinia virus with mutations in a 135,000-Mr subunit of the virion-associated DNA-dependent RNA polymerase. J Virol 61: 1842–1850

Ensinger M, Rovinsky M (1983) Marker rescue of temperature sensitive mutations of vaccinia virus WR: correlation of genetic and physical maps. J Virol 48: 419–428
Esteban M, Holowczak JA (1977a) Replication of vaccinia DNA in mouse L cells. II. In vitro DNA synthesis in cytoplasmic extracts. Virology 78: 76–86
Esteban M, Holowczak JA (1977b) Replication of vaccinia DNA in mouse L cells. III. Intracellular forms of viral DNA. Virology 82: 308–322
Esteban M, Holowczak JA (1978) Replication of vaccinia in mouse L cells. IV. Protein synthesis and viral DNA replication. Virology 86: 376–390
Evans E, Traktman P (1987) Molecular genetic analysis of a vaccinia virus gene with an essential role in DNA replication. J Virol 61: 3152–3162
Evans DH, Stuart D, McFadden G (1988) High levels of genetic recombination among contransfected plasmid DNAs in poxvirus-infected mammalian cells. J Virol 62: 367–375
Fathi Z, Sridhar P, Pacha RF, Condit RC (1986) Efficient targeted insertion of an unselected marker into the vaccinia virus genome. Virology 155: 97–105
Fogelsong PD, Bauer WR (1984) Effects of ATP and inhibitory factors on the activity of vaccinia virus type I topoisomerase. J Virol 49: 1–8
Franke CA, Roseman NA, Hruby DE (1985) Expression and regulation of the vaccinia virus thymidine kinase gene in non-permissive cells. Virus Res 3: 13–17
Geballe AP, Mocarski ES (1988) Translational control of cytomegalovirus gene expression is mediated by upstream AUG codons. J Virol 62: 3334–3340
Hanggi M, Bannwarth W, Stunnenberg HG (1986) Conserved TAAAT motif in vaccinia virus late promoters: overlapping TATA box and site of transcription initiation EMBOJ 5: 1071–1076
Hiller G, Weber K (1982) A phosphorylated basic vaccinia virion polypeptide of molecular weight 11,000 is exposed on the surface of mature particles and interacts with actin-containing cytoskeletal elements. J Virol 44: 647–657
Holowczak JA (1983) Poxvirus DNA replication. In: Becker Y (ed) Replication of viral and cellular genomes. Martinus Nijhoff Boston pp 205–236
Hooda-Dhingra U, Thompson CL, Condit RC (1989) Detailed phenotypic characterization of five temperature-sensitive mutants in the 22- and 147-kDa subunits of vaccinia virus DNA-dependent RNA polymerase. J Virol 63: 714–729
Hruby DE (1985) Inhibition of vaccinia virus thymidine kinase by the distal products of its own metabolic pathway. Virus Res 2: 151–156
Hruby DE, Ball LA (1981) Control of expression of the vaccinia virus thymidine kinase gene. J Virol 40: 456–464
Hruby De, Ball LA (1982) Mapping and identification of the vaccinia virus thymidine kinase gene. J Virol 43: 403–409
Hruby DE, Maki RA, Miller DB, Ball LA (1983) Fine structure analysis and nucleotide sequence of the vaccinia virus thymidine kinase gene. Proc Natl Acad Sci USA 80: 3411–3415
Joklik WK, Becker Y (1964) The replication and coating of vaccinia DNA. J Mol Biol 10: 452–474
Jones EV, Moss B (1984) Mapping of the vaccinia virus DNA polymerase gene by marker rescue and cell-free translation of selected RNA. J Virol 49: 72–77
Kao SY, Bauer WR (1987) Biosynthesis and phosphorylation of vaccinia virus structural protein VP11. Virology 159: 399–407
Kao S, Ressner E, Kates J, Bauer WR (1981) Purification and characterization of a superhelix binding protein from vaccinia virus. Virology 111: 500–508
Kerr SM, Smith GS (1989) Vaccinia virus encodes a polypeptide with DNA ligase activity. Nucleic Acids Res 17: 9039–9050
Kouzarides T, Bankier AT, Satchwell SC, Weston K, Tomlinson P, Barrell BG (1987) Sequence and transcription analysis of the human cytomegalovirus DNA polymerase gene. J Virol 61: 125–133
Künzi MS, Traktman P (1989) Genetic evidence for involvement of vaccinia virus DNA-dependent ATPase I in intermediate and late gene expression. J Virol 63: 3999–4010
Lakritz N, Foglesong D, Reddy M, Baum S, Hurwitz J, Bauer WR (1985) A vaccinia virus DNase preparation which cross-links superhelical DNA. J Virol 53: 935–943
Lambert DM, Magee WE (1977) Characterization of vaccinia virus DNA replication in vitro. Virology 79: 342–354
Larder BA, Kemp SD, Darby G (1987) Related functional domains in virus DNA polymerases. EMBOJ 6: 169–175
Magee WE, Miller OV (1967) Immunological evidence for the appearance of a new DNA polymerase in cells infected with vaccinia virus. Virology 31: 64–69

McFadden G, Dales S (1980) Biogenesis of poxviruses: preliminary characterization of conditional lethal mutants of vaccinia virus defective in DNA synthesis. Virology 103: 68–79
McFadden G, Dales S (1982) Organization and replication of poxvirus DNA. In: Kaplan AS (ed) Organization and replication of viral DNA. CRC Boca Raton pp 173–190
Merchlinsky M, Moss B (1986) Resolution of linear minichromosomes with hairpin ends from circular plasmids containing vaccinia virus concatemer junctions. Cell 45: 879–884
Merchlinsky M, Moss B (1989) Resolution of vaccinia virus DNA concatemer junctions requires late-gene expression. J Virol 63: 1595–1603
Merchlinsky M, Garon CF, Moss B (1988) Molecular cloning and sequence of the concatemer junction from vaccinia virus replicative DNA. J Mol Biol 199: 399–413
Morgan JR, Cohen LK, Roberts BE (1984). Identification of the DNA sequences encoding the large subunit of the mRNA-capping enzyme of vaccinia virus. J Virol 52: 206–214
Moss B, Cooper N (1982) Genetic evidence for vaccinia virus encoded DNA polymerase: isolation of phosphonoacetic acid resistant enzyme from the cytoplasm of cells infected with mutant virus. J Virol 673–678
Moyer RW, Graves RL (1981) The mechanism of cytoplasmic orthopoxvirus DNA replication. Cell 27: 391–401
Nakano E, Panicali D, Paoletti E (1982) Molecular genetics of vaccinia virus: demonstration of marker rescue. Proc Natl Acad Sci USA 79: 1593–1596
Niles EG, Condit RC, Caro P, Davidson K, Matusick L, Seto J (1986) Nucleotide sequence and genetic map of the 16 kb vaccinia virus *Hind*III D fragment. J Virol 153: 96–112
Nowakowski M, Kates J, Bauer W (1978a) Isolation of two DNA binding proteins from the intracellular replication complex of vaccinia virus. Virology 84: 260–267
Nowakowski M, Bauer W, Kates J (1978b) Characterization of a DNA-binding phosphoprotein from vaccinia virus replication complex. Virology 86: 217–225
Pedrali-Noy G, Spadari S (1980) Mechanism of inhibition of herpes simplex virus and vaccinia virus DNA polymerases by aphidicolin, a highly specific inhibitor of DNA replication. J Virol 36: 457–464
Person-Fernandez A, Beaud G (1986) Purification and characterization of a protein synthesis inhibitor associated with vaccinia virus. J Biol Chem 261: 8283–8289
Pizzagalli A, Valsasnini P, Plevani P, Lucchini G (1988) DNA polymerase I gene of *Saccharomyces cerevisiae*: nucleotide sequence mapping of a temperature-sensitive mutation, and protein homology with other DNA polymerases. Proc Natl Acad Sci USA 85: 3772–3776
Poddar SK, Bauer WR (1986) Type I topoisomerase activity after infection of enucleated, synchronized mouse L cells by vaccinia virus. J Virol 57: 433–437
Pogo BG (1977) Elimination of naturally occurring crosslinks in vaccinia virus DNA after viral penetration into cells. Proc Natl Acad Sci USA 74: 1739–1742
Pogo BGT, Dales S (1969) Two deoxyribonuclease activities within purified vaccinia virus. Proc Natl Acad Sci USA 63: 820–827
Pogo BGT, O'Shea MT (1977) Further charcterization of deoxyribonucleases from vaccinia virus. Virology 77: 56–66
Pogo BGT, Katz JR, Dales S (1975) Biogenesis of poxviruses: synthesis and phosphorylation of a basic protein associated with the DNA. Virology 64: 531–543
Pogo BGT, O'Shea M, Freimuth P (1981) Initiation and termination of vaccinia virus DNA replication. Virology 108: 241–248
Pogo BGT, Berkowitz EM, Dales S (1984) Investigation of vaccinia virus DNA replication employing a conditional lethal mutant defective in DNA. Virology 132: 436–444
Polisky B, Kates J (1972) Vaccinia virus intracellular DNA-protein complex: biochemical characteristics of associated protein. Virology 49: 168–179
Polisky B, Kates J (1975) Viral-specific polypeptides associated with newly replicated vaccinia DNA. Virology 66: 128–139
Polisky B, Kates J (1976) Interaction of vaccinia DNA-binding proteins with DNA in vitro. Virology 69: 143–147
Reddy MK, Bauer WR (1989) Activation of the vaccinia virus nicking-joining enzyme by trypsinization. J Biol Chem 264: 443–449
Rempel RE, Anderson MK, Traktman P (1990) Temperature-sensitive vaccinia virus mutants identify a gene with an essential role in viral replication. J Virol 64: 574–583
Rosel J, Earl PL, Weir J, Moss B (1986) Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the *Hind*III H genome fragment. J Virol 60: 436–449

Roseman NA, Hruby DE (1987) Nucleotide sequence and transcript organization of a region of the vaccinia virus genome which encodes a constitutively expressed gene required for DNA replication. J Virol 61: 1398–1406

Rosemond-Hornbeak H, Moss B (1973) Phosphoprotein components of vaccinia virus. J Virol 11: 961–970

Rosemond-Hornbeak H, Moss B (1974) Single-stranded deoxyribonucleic acid-specific nuclease from vaccinia virus. Endonucleolytic and exonucleolytic activities. J Biol Chem 249: 3292–3296

Rosemond-Hornbeak H, Paoletti E, Moss B (1974) Single-stranded deoxyribonucleic acid-specific nuclease from vaccinia virus. Purification and characterization. J Biol Chem 249: 3287–4391

Salzman NP, Shatkin AJ, Sebring ED (1963) Viral protein and DNA synthesis in vaccinia virus-infected HeLa cell cultures. Virology 19: 542–550

Sambrook J, Shatkin AJ (1969) Polynucleotide ligase activity in cells infected with simian virus 40, polyoma virus or vaccinia virus. J Virol 4: 719–726

Sarov I, Joklik WK (1973) Isolation and characterization of intermediates in vaccinia virus morphogenesis. Virology 52: 223–233

Shaffer RS, Traktman P (1987) Vaccinia encapsidates a novel topoisomerase with the properties of a eucaryotic type I enzyme. J Biol Chem 262: 9309–9315

Shuman S, Moss B (1987) Identification of a vaccinia virus gene encoding a type I DNA topoisomerase. Proc Natl Acad Sci USA 84: 7478–7482

Shuman S, Golder M, Moss B (1988) Characterization of vaccinia virus DNA topoisomerase I expressed in *Escherichia coli*. J Biol Chem 263: 16401–16407

Shuman S, Golder M, Moss B (1989) Insertional mutagenesis of the vaccinia virus gene encoding a type I topoisomerase: evidence that the gene is essential for virus growth. Virology 170: 302–306

Slabaugh MB, Mathews CK (1984) Vaccinia virus-induced ribonucleotide reductase can be distinguished from host cell activity. J Virol 52: 501–506

Slabaugh MB, Mathews CK (1986) Hydroxyurea-resistant vaccinia virus: overproduction of ribonucleotide reductase. J Virol 60: 506–514

Slabaugh MB, Johnson TL, Mathews CK (1984) Vaccinia virus induces ribonucleotide reductase in primate cells. J Virol 52: 507–514

Slabaugh MB, Roseman N, Mathews C (1988) Vaccinia-virus encoded ribonucleotide reductase: sequence conservation of the gene for the small subunit and its amplification in hydroxyurea resistant mutants. J Virol 62: 519–527

Smith GL, Chan YS, Kerr SM (1989a) Transcriptional mapping and nucleotide sequence of a vaccinia virus gene encoding a polypeptide with extensive homology to DNA ligases. Nucleic Acids Res 17: 9051–9062

Smith GL, de Carlos A, Chan YS (1989b) Vaccinia virus encodes a thymidylate kinase gene: sequence and transcriptional mapping. Nucleic Acids Res 17: 7581–7590

Spadari S (1976) Properties of DNA ligase from uninfected and virus-infected HeLa cells. Nucleic Acids Res 3: 2155–2167

Spyropoulos DD, Roberts BE, Panicali DL, Cohen LK (1988) Delineation of the viral products of recombination in vaccinia virus-infected cells. J Virol 62: 1046–1054

Sridhar P, Condit RC (1983) Selection for ts mutations in specific vaccinia virus genes: isolation of a phosphonoacetic acid-resistant, temperature sensitive virus mutant. J Virol 128: 444–457

Tengelsen LA, Slabaugh MB, Bibler JK, Hruby DE (1988) Nucleotide sequence and molecular genetic analysis of the large subunit of ribonucleotide reductase encoded by vaccinia virus. Virology 164: 121–131

Thompson CL, Condit RC (1986) Marker rescue mapping of vaccinia virus temperature sensitive mutants using overlapping cosmid clones representing the entire virus genome. Virology 150: 10–20

Traktman P, Sridhar P, Condit RC, Roberts BE (1984) Transcriptional mapping of the DNA polymerase gene of vaccinia virus. J Virol 49: 125–131

Traktman P, Anderson MK, Rempel RE (1989a) Vaccinia virus encodes an essential gene with strong homology to protein kinases. J Biol Chem 264: 21458–21461

Traktman P, Kelvin M, Pacheco S (1989b) Molecular genetic analysis of vaccinia virus DNA polymerase mutants. J Virol 63: 841–846

Upton C, McFadden G (1986) Identification and nucleotide sequence of the thymidine kinase gene of Shope fibroma virus. J Virol 60: 920–927

Weir J, Moss B (1983) Nucleotide sequence of the vaccinia virus thymidine kinase gene and the nature of spontaneous frameshift mutations. J Virol 46: 530–537

Weir JP, Bajszar G, Moss B (1982) Mapping of the vaccinia virus thymidine kinase gene by marker rescue and by cell free translation of selected mRNA. Proc Natl Acad Sci USA 79: 1210–1214

Wittek R, Moss B (1980) Tandem repeats within the inverted terminal repetition of vaccinia virus DNA. Cell 21: 277–284

Wittek R, Hanggi M, Hiller G (1984) Mapping of a gene coding for a major late structural polypeptide on the vaccinia virus genome. J Virol 49: 371–378

Wong SW, Wahl AF, Yuan PM, Arai N, Pearson BE, Arai K-I, Korn D, Hunkapiller MW, Wang TS-F (1988) Human DNA polymerase alpha gene expression is cell proliferation dependent and its primary structure is similar to both prokaryotic and eukaryotic replicative DNA polymerases. EMBOJ 7: 37–47

Yang W-P, Bauer WR (1988a) Purification and characterization of vaccinia virus structural protein VP8. Virology 167: 578–584

Yang W-P, Bauer WR (1988b) Biosynthesis and post-translational cleavage of vaccinia virus structural protein VP8. Virology 167: 585–590

Yuen L, Moss B (1987) Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. Proc Natl Acad Sci USA 84: 6417–6421

The Molecular Pathogenesis of Poxviruses

P. C. TURNER and R. W. MOYER

1 Introduction

Over the years, there have been a number of excellent reviews and articles which discuss aspects of orthopoxvirus virulence and pathogenesis (FENNER 1948a; FENNER 1948b; FENNER 1949a; FENNER 1949b; FENNER and CAIRNS 1959; MIMS

Department of Immunology and Medical Microbiology, College of Medicine, University of Florida, Box J-266, JHMHC, Gainesville, FL 32610, USA

1964; BLANDEN 1970; BLANDEN 1971a; BLANDEN 1971b; JACOBY et al. 1983; JACOBY and BHATT 1987; BHATT and JACOBY 1987a; BHATT and JACOBY 1987b; BHATT et al. 1988; FENNER et al. 1988a; FENNER et al. 1988b; JOKLIK et al. 1988). In particular, we would refer readers to the recent, excellent book by FENNER, WITTEK and DUMBELL (1989) upon which we have drawn extensively for this review and that provides over 40 years of personal perspectives on the subject.

We will focus on one aspect of this complex process, namely, the ability of the virus through deletion and rearrangement of genes to alter growth patterns and by so doing, offer the potential for host adaptation and variations in pathogenic properties. There are two separate, albeit related, aspects of poxvirus pathogenesis which must be considered: virus growth and the onset of clinical symptoms.

Generically, the process of virus growth in vivo consists of several discrete states. After the initial entry of the virus into the host through one of a number of routes specific for the individual virus, there is a stage of primary multiplication at or near the site of inoculation. Thereafter, there may be spread of the virus to secondary target sites. How the virus spreads and the nature of the secondary sites in many if not all cases is again dependent on the specific virus and must be considered within the context of tissue and cell tropism. One must also consider the immune response of the host, both humoral and cellular, as a major factor in whether resolution of the infection occurs. Products stemming from the immune response may also provide components which initiate or exacerbate clinical symptoms. Extensive viral growth is almost always linked to cell damage and destruction of tissue but does not necessarily result in clinical symptoms. Finally, some virus infections can become persistent and enter into an equilibrium with the host.

Natural infections of hosts by poxviruses are generally characterized by systemic infections with the involvement of a number of tissues and organs. Entry of the virus into a given host can be by several routes: the skin, respiratory, or oral routes. While it must be recognized that there are variations on the theme (FENNER 1948a; FENNER et al. 1988b; FENNER et al. 1989, pp. 85–142), the generally accepted working model of the systemic infection is that derived from ectromelia (mousepox) virus. Scarification leads to localized growth at the site of inoculation and thereafter involvement of the regional lymph nodes. An initial viremia then leads to infection of the liver and spleen, after which a second viremia results in further involvement of other organs including skin, intestines, and lungs.

It is reasonable to assume that without growth and concomitant cellular damage no clinical symptoms will result. In order to obtain the full spectrum of viral growth and maximum tissue and organ involvement, the virus must first travel from the site of initial inoculation to the appropriate target organs where extensive viral growth ensues. It is recognized that many diverse host factors may strongly influence a normal viral growth pattern, such as genotype, age, sex, microbial status, and environment (see JACOBY et al. 1982 for review), as can the state of the virus (genetic content, enveloped versus nonenveloped). We will only

consider the genetic constitution of the virus as it relates to the ability of the virus to grow in the expected organs and tissues as the infection runs its course and whether growth in cell culture can serve to predict growth patterns and virulence in the host organism.

With the development of the tools of molecular biology, many interesting features of these viruses emerged which have not been discussed within the context of the consequences of a natural infection. One such feature which is relevant to this discussion is the rapid deletion, transposition and rearrangement to which large segments of the terminal sequences of the viral DNA are subject. Most, but not all, of the specific genes which we will discuss are located within this region of the genome. This plasticity provides the potential for rapid evolution of these viruses and a means to adapt to changing circumstances in various hosts.

2 The General Structural Features of Poxvirus DNA

2.1 The Overall Organization of Viral Genes

The DNA of orthopoxviruses consists of a double-stranded duplex of approximately 190 kb (GESHELIN and BERNS 1974; GANGEMI and SHARP 1976; WITTEK et al. 1977; MCCARRON et al. 1978; MULLER et al. 1978; MACKETT and ARCHARD 1979; DEFILIPPES 1982; ESPOSITO and KNIGHT 1985). Within this 190 kb, the 20–30 kb regions of DNA nearest to the terminal extremes of the DNA are the most subject to variation from strain to strain in natural isolates. A comparison of the genomes of various orthopoxviruses reveals a central "core" of well conserved genes between different species, but there is extensive diversity at the terminal extremes of the DNA (ESPOSITO et al. 1978; MACKETT and ARCHARD 1979; ESPOSITO and KNIGHT 1985) (Fig. 1). Recent evidence suggests that at least part of the core region is conserved amongst all the poxviruses and not restricted solely to orthopoxviruses. For example, the region of fowlpox virus (*Avipoxvirus*) which cross hybridizes with the vaccinia *Hin*dIII J fragment that contains the vaccinia thymidine kinase (*TK*) gene has been examined (DRILLIEN et al. 1987; BINNS et al. 1988). Comparison with the vaccinia virus sequences reveals that, although the *TK* gene of fowlpox virus is located elsewhere within the fowlpox genome, the organization of open reading frames (ORFs) around the missing *TK* gene is similar to that in vaccinia, and they are closely related in terms of nucleotide and amino acid sequences. The same organization has been noted for the leporipoxviruses and capripoxviruses except that, unlike fowlpox virus, the *TK* gene remains as part of the conserved block (UPTON and MCFADDEN 1986b; GERSHON and BLACK 1989). A conserved core of genes can conceptually be viewed as a region encoding functions essential for the basic survival of this family of viruses. On the other hand, the terminal regions are quite variable, largely nonessential in

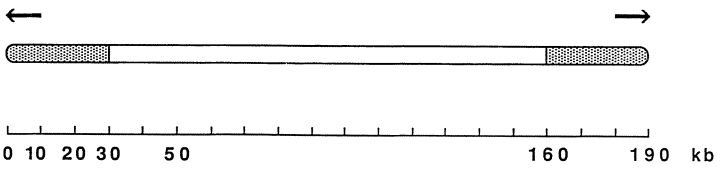


Fig. 1. A stylized poxvirus genome indicating terminal cross-links, inverted terminal repetitions (*arrows*), and near-terminal regions (*shaded*) which are subject to rearrangement by deletion and transposition. The central "core" region is strongly conserved between poxviruses and contains essential genes

tissue culture, and may contain the genes responsible for species specificity, tropism, and host range. The following sections will discuss this concept in detail and present data to generally support this hypothesis.

2.2 Structural Features of the Terminal Regions of Poxvirus DNA

The terminal regions of the poxvirus genome are replete with interesting structural features. One such feature is the presence of cross-linked termini structurally identical to telomeres (BERNS and SILVERMAN 1970; GESHELIN and BERNS 1974).

An examination of the "loops" which comprise the two telomeric termini at either extreme of the DNA revealed that they were not identical but instead consisted of "flip" and "flop" isomers which are inverted and complementary to one another (BAROUDY et al. 1982; BAROUDY et al. 1983). Immediately adjacent to the terminal loop sequences are a series of tandem repeats extending in vaccinia virus for about 3.5 kb. The repeat elements are small with the first (70 bp) being repeated 13 and then 18 times, with the two clusters of repeats separated by approximately 300 bases of unique sequence (WITTEK and MOSS 1980b; BAROUDY et al. 1982; BAROUDY et al. 1983). Thereafter follows a twice repeated 125 bp element and a 54 bp element repeated eight times. Finally, there are incomplete copies of both the 54 and 70 bp elements after which several copies of a 6 or 7 bp element end this region. The presence of multiple repeat elements, whose exact function is unknown, could provide one mechanism for the genetic instability (expansion and deletion) of DNA observed within this region through normal recombinatorial processes (WITTEK and MOSS 1980b). This repetitious region is not solely structural as sequence analysis has revealed that this region encodes mRNAs which are translated into polypeptides (WITTEK et al. 1980a; WITTEK et al. 1981; COOPER et al. 1981).

On a larger scale, hybridization (PEDRALI NOY and WEISSBACH 1977; GRADY and PAOLETTI 1977; WITTEK et al. 1977; WITTEK et al. 1978; MACKETT and ARCHARD 1979) and electron microscopic (GARON et al. 1978) experiments suggested that sequences at one end were repeated at the other in the form of

inverted repeats which in the case of vaccinia are about 10 kb. The only possible exception to this rule is variola virus (ARCHARD and MACKETT 1979; ESPOSITO and KNIGHT 1985), where the repeated sequences may be very short or even absent. In summary, the first 10 kb or so of DNA at either end of the genome is highly repetitious and encodes several polypeptides.

2.3 Strain to Strain Size Variation of the Inverted Terminal Repetition

Although some 10 of the first 30 kb of DNA is normally present as an inverted repetition within strains of vaccinia, the size of the repetition is not fixed. Studies (MOYER et al. 1980a; DUMBELL and ARCHARD 1980; ARCHARD et al. 1984; ESPOSITO et al. 1981; PICKUP et al. 1984) have shown for various poxviruses that the length of the terminal repetition can vary within the same strain from isolate to isolate from 4 to 50 kb, indicating not only a tremendous mutational and evolutionary potential, but an ability of the assembled virion to accommodate enormous variation in the size of the genome packaged. This ability to accommodate genomic excesses was perhaps best illustrated when a 25 kb fragment of phage λ DNA was cloned into vaccinia and packaged as part of the genome within mature virions (SMITH and MOSS 1983).

2.4 Deletions Within the Terminal 30 kb of Viral DNA

2.4.1 Mutants Isolated As White Pock Variants

While variability in the terminal extremes of viral DNA through an increase in genomic content caused by expansion of the inverted terminal repetitions has been well documented, this region of the genome is also subject to variations which yield net deletions of up to 30 kb of DNA. Evidence for this statement emerged in large part from the study of the spontaneous white pock variants produced by cowpox, rabbitpox, and monkeypox viruses. There has been detailed molecular and biological characterization of this class of mutants and of their biological relevance over a number of years. Fenner, some 30 years ago, examined the response in animals to infection by various poxviruses and noted that different strains of vaccinia, cowpox, or rabbitpox virus had different biological characteristics (FENNER 1958). In this initial study, some of the variant strains were classified by the type of pock produced on the chorioallantoic membrane (CAM) of embryonated chicken eggs, i.e., either hemorrhagic and ulcerated or white and nonhemorrhagic pocks. Rabbitpox and cowpox viruses produce red, hemorrhagic pocks, whereas many strains of vaccinia (including *WR*) produce white pocks.

2.4.1.1 The White Pock Mutants of Rabbitpox Virus

Variants of rabbitpox virus which produce white pocks on the CAM arise at a frequency of 1% (FENNER 1958). Studies from Fenner's laboratory (FENNER 1958; GEMMELL and FENNER 1960; FENNER and SAMBROOK 1966) and others (MCCLAIN 1965; SAMBROOK et al. 1965; MCCLAIN and GREENLAND 1965), which concentrated primarily on rabbitpox virus, were able to ascribe some novel properties to these variants. If one began with a collection of white pock variants, they could be classified into two groups depending on their host range properties, e.g., the ability to grow on pig kidney cells. Some grew on pig kidney as well as on the same normal spectrum of cell lines and were otherwise indistinguishable from the parent strain except when examined on the CAM. Others, however, had a reduced spectrum of permissive cells. Some of the mutants, when examined for the production of antigens, were observed to differ from each other in the number of antigens elaborated in a given non-permissive cell line. One mutant even elaborated differing numbers of antigens depending on which nonpermissive cell line was examined (SAMBROOK et al. 1965). Some variants synthesized DNA, whereas others did not. This apparent differential expression of genes by individual mutants in a given cell line suggested that these variants might be very useful for investigating the life cycle of the virus. Similarly, when inoculated into animals, many but not all of the white pock variants were observed to be attenuated to varying degrees as compared to wild type virus (FENNER 1958; GEMMELL and FENNER 1960).

The experimental potential of the white pock mutants of rabbitpox virus was thereafter largely ignored for 20 years until development of technology made molecular analysis of the mutants possible. It was then discovered that many of the mutants which had a restricted host range contained deletions of sequences from the left or right terminal terminal extremes of the genome. Deletions (μ) at the right-hand terminus removed approximately 15 kb of DNA. Other mutants (μ.p) were found to contain 8–30 kb deletions at the left-hand end. In addition to forming white pocks, the left-hand deletion mutants were unable to grow on a pig kidney (PK-15) cell line, although growth on chick embryo fibroblast (CEF) cells was normal. This last class of mutants, which includes some of those originally described by FENNER and SAMBROOK (1966), had apparently deleted a gene(s) involved in growth in certain cells. Mixed infections between viruses bearing a left-hand (μ.p) and a right-hand (μ) deletion produced wild type (μ^+p^+) viruses, but ($\mu \times \mu$) or ($\mu.p \times \mu.p$) crosses did not generate wild type virus (LAKE and COOPER 1980).

MOYER and ROTHE (1980) studied five white pock, host range mutants of rabbitpox virus (RPV). They contained deletions of between 5 and 30 kb at the left end of the genome, i.e., up to 17% of the viral DNA was nonessential for growth in certain cell lines. At least three deletions extended into the left terminal inverted repeat. The restriction maps of these mutants were consistent with a family of overlapping deletions. All were host range mutants unable to grow on PK-15 cells, and some had also lost the ability to grow on a rabbit cornea (RC-60) cell line (MOYER and ROTHE 1980).

Two RPV white pock mutants, *RPμ21* and *RPμ7*, which were unaffected in host range, had 15 kb deletions at the right end with an inverted transposition of 20 kb and 23 kb, respectively, at the left end (MOYER et al. 1980a). These mutants resembled the deletion/duplication variants later described for cowpox virus (ARCHARD et al. 1984; PICKUP et al. 1984 and see Sect. 2.4.1.2). A third RPV white pock mutant, *RPμ8lg*, had deleted 9 kb of the extreme left-hand end and transposed 42 kb from the right-hand end for a net gain of approximately 33 kb. *RPμhr8sm*, a segregant of *RPμ8lg*, recognized by an altered pock morphology, had a net deletion of material at the left end of the genome and simultaneously acquired a host range phenotype (MOYER et al. 1980a). These data are consistent with the presence of at least one red pock gene at either end of the wild type RPV genome and with "nonessential" host range gene(s) being located towards the left-hand terminus.

FENNER and SAMBROOK (1966) had originally reported data to suggest that different host range mutants were blocked at different steps during infection of nonpermissive PK-15 cells and that some were capable of DNA replication, whereas others were not. More recently, biochemical studies of selected white pock host range restricted variants of RPV confirmed the earlier observation (FENNER and SAMBROOK 1966) that a given host range variant might be blocked at different stages of the development cycle, allowing two distinct nonpermissive phenotypes to be described. The first (early) abortive phenotype, illustrated by mutants *RPμhr23* and *RPμhr31*, revealed that while all translation in nonpermissive PK cells had stopped by 8–10 hr postinfection, translatable viral mRNA continued to be synthesized and even overproduced (MOYER et al. 1980b; BROWN and MOYER 1983; BROWN et al. 1983). The second (late) abortive phenotype exemplified by *RPμhr28* and *RPμhr8sm*, was characterized by an inability to elaborate mature virus, even though both early and late components were elaborated within the abortively infected cell (MOYER and GRAVES 1982).

EWARDS et al. (1988) tested these four RPV host range deletion mutants for growth in different organs following i.p. (intraperitoneal) inoculation of BALB/c mice and for neurovirulence following i.c. (intracranial) injection. *RPμhr8sm* and *RPμhr28*, with deletions of 12 kb, grew normally in skin, brain, liver, lung, and spleen; *RPμhr23* and *RPμhr31*, with 30 kb deletions, showed much reduced titers from these organs. Inoculation by the i.c. route gave LD_{50} values for wild type and the deletion mutants *RPμhr8sm* and *RPμhr28* of $< 10^2$ PFU; however, for deletion mutants *RPμhr23* and *RPμhr31*, LD_{50} was $> 10^5$ PFU. Although *RPμhr23* and *RPμhr31* had markedly reduced invasiveness and neurovirulence, they were still capable of localized growth in the tail skin and provided protection against a lethal challenge with wild type RPV (EDWARDS et al. 1988). Clearly, some of the left-hand RPV deletions attenuate the virus for invasiveness, as was found for similar left-hand vaccinia virus (VV) deletions described by DALLO and ESTEBAN (1987) which are discussed later, in Sect. 2.4.2.2. The reduced neurovirulence of the left-hand RPV deletions also resembled the phenotype of the 9 kb left-hand VV deletion isolated by MOSS et al. (1981) and described in Sect. 2.4.2.1.

2.4.1.2 The White Pock Variants of Cowpox Virus

The first descriptions of white nonhemorrhagic pock variants for any of the normally hemorrhagic poxviruses were by DOWNIE and HADDOCK (1952) and VAN TONGEREN (1952) for cowpox virus (CPV). Like RPV, this virus is also subject to ready deletion, with a propensity to delete sequences from the right-most regions of the genome (ARCHARD et al. 1984; PICKUP et al. 1984). ARCHARD and MACKETT (1979) described a white pock variant of the *Brighton Red* strain of CPV. Like RPV, CPV variants arise with a frequency of up to 1% of the total population on the CAM (FENNER 1958). A comparison of the restriction maps of the parent and variant revealed that the mutant had deleted between 24 and 27 kb from the right-hand end of the genome (ARCHARD and MACKETT 1979). These results indicated that the deleted region, comprising 11%–12% of the genome, was nonessential for growth in tissue culture and presumably contained the gene(s) responsible for the red pock phenotype.

Later work (ARCHARD et al. 1984) showed that white pock variants of CPV could tolerate deletions extending inwards from the right-hand terminus between 32 and 41 kb (32, 36, and 41 kb deletions were observed). A terminal inverted repeat and covalent cross-link were restored by a compensating transposition of between 5 and 41 kb of DNA from the left-hand terminus (ARCHARD et al. 1984). The mutants were extremely unstable, with siblings isolated from a white clone differing in both the size of the deletion and the extent of the duplicated sequence.

PICKUP et al. (1984) analyzed the genomes of ten white pock variants of CPV (*Brighton Red* strain). One had a simple deletion of DNA, 12–32 kb from the right-hand terminus; nine had combinations of deletions/duplications. Some 32–38 kb of DNA from the right-hand end were deleted and replaced by an inverted copy of the left-hand terminal sequences ranging 21–50 kb in length. The right end deletions obtained extended far enough inwards (at least 32 kb) to inactivate the "red pock" gene but no further than 38 kb, presumably because longer deletions were lethal. A gene required for the red pock phenotype in CPV was subsequently isolated and its nucleotide sequence determined (PICKUP et al. 1986 and see Sect. 3.1).

2.4.1.3 The White Pock Variants of Monkeypox Virus

White pock mutants of monkeypox virus arise at a frequency of $< 0.1\%$ (DUMBELL and ARCHARD 1980). Two isolates had right-hand end deletions of 15 kb and 19 kb, respectively. Others had symmetrical rearrangements of both termini, resembling the deletion/duplication mutants seen in CPV and RPV. Some white pock mutants, unlike wild type virus, had gained the ability to grow on a pig embryo kidney (PEK) cell line (DUMBELL and ARCHARD 1980), implying that host range genes can be activated or even created by rearrangement involving both termini. ESPOSITO et al. (1981) described a white pock variant of monkeypox virus which had suffered a deletion of 21 kb adjacent to the right-

hand inverted terminal repeat. From these studies the monkeypox red pock gene appears to be located toward the right-hand end of the genome, as for CPV.

2.4.2 Deletion Variants of Vaccinia Virus

2.4.2.1 Spontaneous Deletion Mutants of Vaccinia Virus Which Affect Virulence

The ready deletion of DNA from the terminal regions of RPV, CPV, and monkeypox virus genomes is not limited to these viruses which normally generate hemorrhagic lesions on the CAM. Similar studies followed with VV (DRILLIEN et al. 1981; PANICALI et al. 1981; MOSS et al. 1981; PAEZ et al. 1985; ESTEBAN et al. 1986; PERKUS et al. 1986; DALLO and ESTEBAN 1987). Usually, these deletions arise from serial passage of VV in tissue culture and involve DNA from the left-hand end of the genome which is nonessential for growth under these conditions. MOSS et al. (1981) described variants *6/1* and *6/2* of VV *WR* which had deleted 9 kb of parental sequences. The deletion mapped near the left end in the unique region but starting very close to the junction between the inverted terminal repetition and unique sequences. The same deletion was found by PANICALI et al. (1981) when *S* (small) and *L* (large) variants of VV *WR* were examined.

Although the deletion had no effect on growth in HeLa cells (PANICALI et al. 1981), sequences encoding at least eight early proteins were eliminated (MOSS et al. 1981). Analogous sequences were also found in the *Elstree* strain of VV, RPV (*Utrecht* strain), and in the *Brighton* strain of CPV (MOSS et al. 1981). This conservation of genetic material implied that the DNA had some role in vivo which was not evident in the artificial situation created in the laboratory. BULLER et al. (1985) tested the *6/2* variant containing the spontaneous 9 kb deletion for neurovirulence by i.c. inoculation of BALB/cByJ mice. The LD_{50} for 6/2 was 6.3×10^6 PFU compared with 10 PFU for wild type *WR*; in other words, by this route of administration, the mutant was greatly attenuated.

2.4.2.2 Deletion Variants of Vaccinia Virus Which Result from Persistent Infections

Deletions at the left end of VV *WR* affecting virulence have been isolated from long-term passage of persistently infected mouse Friend erythroleukemia (FEL) cells (PAEZ et al. 1985). A 12 kb deletion starting between 3.3 and 4.8 kb from the left terminus was found which eliminated more than half of the left terminal inverted repetition. The mutants formed small plaques and were less lethal to mice following i.p. inoculation than wild type virus (PAEZ et al. 1985). The generation of deletions during persistent infections was blocked by the addition of mouse fibroblastoid interferon (IFN), which contained 15% α- and 85% β-IFN (PAEZ and ESTEBAN 1985).

DALLO and ESTEBAN (1987) showed that the deletion mutants just described also contained point mutations in the middle of the *Hin*dIII A fragment that were

responsible for the small plaque phenotype characteristic of these mutants. The deletion, coupled with the mutations in the *Hin*dIII A fragment, caused an increase in the LD_{50} in mice by the i.p. route to 1×10^9 PFU, compared with 1×10^6 PFU for wild type virus. The deletion alone caused a 2 log increase in the LD_{50}, whereas the small plaque mutation alone caused an increase of 1 log (DALLO and ESTEBAN 1987). Deletions of 21 and 22 kb, mapping near the left terminus of VV strain *IHD-W*, have obtained from persistently infected cells in a similar fashion (LAI and POGO 1989a). The *IHD-W* mutants were attenuated for female NIH Swiss mice by the i.p. route and were less efficient at inhibiting host protein synthesis following infection of mouse L cells in vitro (LAI and POGO 1989a), although plaque size was normal. Growth in liver and spleen following i.p. inoculation of mice was reduced for the mutants (LAI and POGO 1989b), which had a deletion of the vaccinia growth factor gene (Sect. 3.7).

2.5 Models for the Duplication, Transposition, and Deletion of Terminal Sequences

Based on an examination of a large number of variants which have arisen from a variety of different orthopoxviruses, the concept of a virus family, all of which have a "common" core of well conserved genes toward the center of the genome and highly divergent termini, emerges (Fig. 1). Various models have been proposed to account for the generation of the observed deletion/duplication rearrangements. For example, in Sect. 2.2 we mentioned that homologous recombination between the direct repeat elements present in the most distal portions of the genome might allow for expansion or contraction of the repeat elements. Two other major mechanisms to consider involve nonhomologous recombination and interaction between the ends during replication. Nucleotide sequence analysis of the novel junctions in four deletion/duplication variants and comparison with the unrearranged parental sequences (PICKUP et al. 1984) did not reveal any significant regions where homologous recombination could have occurred. Simple deletions could be produced by nonhomologous reciprocal recombination between two right-hand ends aligned in the same direction but out of register. Recombination between right-hand and left-hand ends aligned in opposite directions (Fig. 2) would generate deletion/duplication variants (PICKUP et al. 1984). Another model is a nonreciprocal recombination mechanism involving transfer of genetic material from one terminus to another by a gene conversion-like event (PICKUP et al. 1984). Lastly, deletions within head-to-head and tail-to-tail concatemeric replication intermediates, before genome excision and packaging, could create deletions and duplications (MOYER and GRAVES 1981).

Under certain conditions, specific genes can be selectively amplified in response to environmental changes. An example is the ribonucleotide reductase gene of VV. The ribonucleotide reductase enzyme, which was first

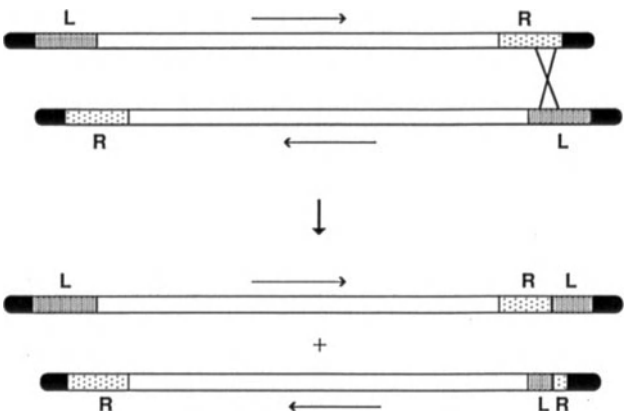


Fig. 2. An example by which deletion/duplication rearrangements of poxvirus genome terminal regions could be produced (adapted from JOKLIK et al. 1988)

described by SLABAUGH and MATHEWS (SLABAUGH et al. 1984; SLABAUGH and MATHEWS 1984), consists of two subunits. The larger subunit maps in the *Hin*dIII I fragment toward the center of the VV genome (TENGELSEN et al. 1988), whereas the smaller maps in the left-hand *Hin*dIII F fragment at or near the limits of the sequences found randomly deleted in host range mutants (SLABAUGH et al. 1988). It is quite likely that at least the smaller of the two subunits is not required for viral growth in cell culture (PERKUS et al. 1986; SLABAUGH et al. 1988). In viral mutants which have become resistant to hydroxyurea, sequence analysis has shown a selective amplification (2–15 copies) of the smaller subunit, with the copies arranged in direct tandem repeats (SLABAUGH et al. 1988). Amplification is limited to only the smaller of the two subunits, in keeping with observations noted for cellular mutants. The mechanism for this selective expansion is not known (SLABAUGH et al. 1988). Similar variations have been noted for a λtkλ/vaccinia construct as reported by BALL (1987) in which the *TK* gene is flanked by direct repeats of phage λ sequences.

2.6 Other Experimental Approaches Implicate Specific Regions of Viral DNA in the Pathogenic Process

Vaccine strains that were used in the smallpox eradication program are considerably less neurovirulent than "wild type" strains such as vaccinia *WR*. Temperature sensitive and low neurovirulent variants *LC16m0* and *LC16m8* were isolated from the *Lister* (*Elstree*) vaccine strain *LO* (*Lister Original*). By analyzing the genome structures of these three strains, SUGIMOTO et al. (1985) were able to show that *LC16m0* and *LC16m8* had acquired a new *Xhol* site when

compared with the parental *LO* virus. This site was located 18 kb from the rightmost DNA terminus, in the *Hin*dIII D fragment. TAKAHASHI-NISHIMAKI et al. (1987) attempted to show that this region was involved in both temperature sensitivity and neurovirulence. *LC16m8*, which forms small plaques and pocks on the CAM, was marker rescued to large plaque and pock size by the *Hin*dIII D fragment from the parental *LO* strain. The resulting recombinants, however, were still temperature sensitive and relatively nonneurovirulent, indicating that the relevant genes for these properties were located elsewhere.

A second way to analyze genes associated with specific characteristics of virulence is to obtain in vivo recombinants between intertypic viruses with different host specificities and then to correlate changes in pathogenicity with the regions that have been exchanged between the genomes. Early studies showed that mixed infections between alastrim and RPV or variola major and CPV readily produced recombinants (BEDSON and DUMBELL 1964a; BEDSON and DUMBELL 1964b). CHERNOS et al. (1985) selected for recombinants between VV temperature sensitive mutants and ectromelia virus (EMV). Some recombinants had acquired "double pathogenicity", i.e., were pathogenic for both mice (from EMV) and rabbits (from VV). Analysis of the *Hin*dIII and *Xho*I restriction maps indicating that the recombinants were mostly of VV origin with a centrally located region of at least 24 kb derived from EMV. More recent work, however, (SENKEVITCH and CHERNOS, personal communication) suggests that two regions of EMV, one at each end of the genome, are involved in pathogenicity to mice. Also, RPV could acquire mouse pathogenicity by recombination with a gel-purified right-hand fragment of EMV DNA (SENKEVITCH and CHERNOS, personal communication), which again is consistent with a near terminal location for pathogenicity genes.

Malignant rabbit virus (MRV) is a tumorigenic poxvirus which combines features of the leporipoxviruses Shope fibroma virus (SFV) and myxoma virus (STRAYER et al. 1983a; STRAYER et al. 1983b; STRAYER and SELL 1983). MRV appears to be a myxoma derivative which has acquired from SFV the ability to induce proliferative fibromas. Myxoma virus causes a generalized infection of rabbits with sequential involvement of the skin, lymph nodes, etc., and culminates in a widespread rash. SFV induces benign, self-limited tumors, but MRV causes immunosuppression and malignant, invasive tumors. BLOCK et al. (1985) found that over 90% of the MRV genome was derived from myxoma, while about 10 kb of myxoma DNA had been replaced by an equivalent amount of SFV DNA. The material acquired from SFV was in two regions, one in each of the terminal inverted repeats. At the left end, 4 kb of SFV DNA was located 6–10 kb from the terminus; at the right end at least 5.5 kb extended inwards beginning at 6 kb from the terminus (BLOCK et al. 1985). The authors suggested that MRV was produced by recombination between the right termini of myxoma and SFV, followed by incomplete transposition of part of the new material to the left end. The region in SFV which was transferred to myxoma has been sequenced (UPTON and MCFADDEN 1986b), and some of this region is apparently derived from a plasmid species which is present in uninfected rabbit cells (UPTON et al. 1986; UPTON and

McFADDEN 1986a). Again, one sees the demonstration of the variability of the terminal extremes of these viruses. To date, only one gene product derived from the SFV sequences of MRV is known, a growth factor similar to epidermal growth factor (EGF) and transforming growth factor α or TGFα (CHANG et al. 1987 and see Sect. 3.7).

3 Specific Gene Products That May Be Involved in Virulence

Many proteins are now known with proven or likely roles in the virulence of these viruses. We have summarized them in Table 1.

3.1 Serpin-like Proteins

The *Brighton Red* strain of CPV produces red pocks on the CAM and hemorrhagic lesions in the skin of guinea pigs, rabbits, and humans. A gene in CPV involved in the red pock phenotype has been identified by PICKUP et al.

Table 1. Specific gene products involved in virulence

Designation	Virus	Gene product/function	Reference
Red pock gene	CPV	38 kDa serpin; inhibitor of blood coagulation	PICKUP et al. 1986
SPI-2	VV	38 kDa serpin; homologue of CPV red pock gene	KOTWAL and MOSS 1989
SPI-1	VV	40 kDa serpin; unknown	KOTWAL and MOSS 1989
35 kDa gene	VV	35 kDa; complement control protein?	KOTWAL and MOSS 1988b
Human cell line hr gene	VV	29 kDa; prevents early protein synthesis shutoff?	GILLARD et al. 1986
CHO hr gene	CPV	77 kDa; prevents early protein synthesis shutoff?	SPEHNER et al. 1988
A-type inclusion protein	CPV	160 kDa; increases virion survival?	PATEL et al. 1986 FUNAHASHI et al. 1988
Thymidine kinase	VV	Nucleotide biosynthesis; required for growth in vivo?	BULLER et al. 1985
Hemagglutinin	VV	Promotes cell-to-cell spread?	FLEXNER et al. 1987 SHIDA et al. 1988
VGF	VV	Secreted growth factor; stimulates proliferation of uninfected cells	BULLER et al. 1988a, b
SFGF	SFV	Secreted growth factor?	CHANG et al. 1987
MGF	Myxoma	Secreted growth factor?	UPTON et al. 1987
14 kDa gene	VV	14 kDa envelope protein; determines plaque size; role in virus penetration?	DALLO et al. 1987 RODRIGUEZ and ESTEBAN 1987
ORF N1L	VV	13.8 kDa secreted protein; role in neurovirulence	KOTWAL et al. 1989

(1986). From analysis of the deletion endpoints of spontaneous white pock variants, the red pock gene was thought to be located about 32 kb from the right-hand end of the CPV genome (PICKUP et al. 1984) as described in Sect. 2.4.1.2. DNA fragments from this region of wild type CPV were inserted into the *TK* gene of a white pock CPV variant and the recombinants tested for production of hemorrhagic lesions on the CAM. A 5.2 kb *Eco*RI fragment containing the region 29 kb–34.2 kb from the end was found to restore the red pock phenotype (PICKUP et al. 1986). Subcloning showed that the gene was contained within a 1465 bp *Nco*I-*Hae*III fragment and was located 31–32 kb from the CPV terminus (PICKUP et al. 1986).

White pock CPV variants lacked an abundant 38 kDa early protein that was synthesized by wild type CPV (PICKUP et al. 1986). The cloned red pock gene was used to hybrid-select message from RNA made early upon infection with wild type CPV. In vitro translation of the resulting mRNA gave a protein product of 38 kDa. Nucleotide sequence analysis of the *Nco*I-*Hae*III fragment confirmed that the red pock gene encoded a 38 kDa protein (PICKUP et al. 1986).

The amino acid sequence of the CPV 38 kDa protein was found to be related to the sequences of a superfamily of eukaryotic plasma proteins which act as inhibitors of serine proteases. This group includes human antithrombin III, human α_1-antichymotrypsin, and mouse contrapsin, among others. These serpins (serine protease inhibitors) regulate the serine proteases involved in blood coagulation and other processes. Production of a similar protein by CPV and other members of the family presumably interferes with blood clotting and wound containment resulting in the production of local hemorrhage (PICKUP et al. 1986).

The CPV recombinants containing the serpin gene inserted into a white pock variant background gave an intermediate level of hemorrhage, with pink, rather than red, pocks (PICKUP et al. 1986), suggesting that other genes may be required for full inhibition of blood coagulation or full hemorrhage.

A structural analysis of the right-hand end of vaccinia *WR* revealed the presence of an ORF encoding a 40 kDa protein product also related to the serpins (KOTWAL and MOSS 1989). This gene has been designated *SPI-1* and is located 10 kb from the right-hand end, very close to the inverted terminal repetition (*ITR*). In addition, VV has a second serpin gene, *SPI-2*, which is homologous to the CPV red pock gene described by PICKUP et al. (1986); it encodes a 38 kDa protein and maps 17 kb from the right-hand end (KOTWAL and MOSS 1989).

CPV was later found to also have the homologue of the VV *SPI-1* gene located between the 38 kDa red pock ORF and the ITR (KOTWAL and MOSS 1989). The CPV *SPI-1* gene would presumably have been deleted in the white pock deletion mutants and could be the second gene needed together with the 38 kDa gene for the production of fully red pocks.

The VV *SPI-1* and *SP-2* products are very closely related, with 44% identical amino acids and very similar hydrophobicity profiles (KOTWAL and MOSS 1989), suggesting a common evolutionary origin. The sequences around a strained,

exposed domain at the C-terminus, which contains the active site, are conserved in both the *SPI-1* and *SPI-2* products, but the putative reaction centers are different for the two proteins, implying that they inhibit different proteases (KOTWAL and MOSS 1989). Although vaccinia *WR* strain does not generate red pocks, the VV *SPI-1* and *SPI-2* products could still be involved in blood coagulation. Alternatively, they may interfere with the complement cascade, which is also regulated by serpins. Another possibility is that viral serpins may inhibit the proteases which process viral antigens so that they can associate with class I MHC antigens and be recognized by cytotoxic T lymphocytes (TOWNSEND et al. 1988).

A third gene in VV may encode a serpin-like product. ORF *K1*, in the *Hin*dIII K fragment, 25 kb from the left end of the genome, encodes a 42.2 kDa protein with 25% amino acid identity to human antithrombin III in aligned regions (BOURSNELL et al. 1988). To date, no function has been assigned to this nonessential serpin-like gene.

Sequence analysis of fowlpox virus, which produces white pocks on the CAM, has identified an ORF at the ITR-unique region junction which encodes an 18.4 kDa protein with significant homology to the C-terminal half of the 38 kDa CPV red pock protein (TOMLEY et al. 1988).

The origin of the serpin-like genes in poxviruses remains obscure, although they presumably originated in the genome of the host cell. In this context, the ITR of SFV contains a region including a serpin-like gene derived from an endogeneous plasmid DNA found in uninfected rabbit cells (UPTON and MCFADDEN 1986a; UPTON et al. 1986). Possibly, genetic exchange takes place between poxvirus genomes and host plasmids within the cytoplasm of infected cells.

3.2 A Vaccinia Virus Product Resembling Complement Control Proteins

Infection with VV in vitro causes secretion into the medium of a polypeptide of approximately 35 kDa. KOTWAL and MOSS (1988b) found that this protein was absent when cells were infected with the *6/2* deletion mutant of VV (MOSS et al. 1981 and described in Sect. 2.4.2.1). By comparing the N-terminal sequence of the 38 kDa protein with each of the ORFs present in the deleted region, the relevant gene was identified.

The encoded protein has a calculated mass of 28.6 kDa and contains a signal peptide of 19 amino acids. The protein has significant homology to a superfamily of eukaryotic proteins which includes complement control proteins. The most significant homology was with the human complement C4b-binding protein (KOTWAL and MOSS 1988b) which regulates the complement pathway. This result suggests that wild type VV can inhibit the complement cascade and thus interfere with host immune responses such as the inflammatory response and killing of bacteria and viruses.

3.3 Host Range Genes

The individual genes responsible for the restricted host range of the white pock RPV deletion mutants (Sect. 2.4.1.1) have not been identified; however, host range genes have been described for VV and CPV which are necessary for growth on particular cell lines.

DRILLIEN et al. (1981) isolated a mutant of VV *Copenhagen* strain after nitrous acid mutagenesis which was unable to grow on most human cell lines, although growth was near normal on mouse, hamster, and chicken lines. Two human embryo cell lines, however, were semipermissive for the host range (*hr*) mutant, as were two monkey cell lines. On nonpermissive human cell lines, host and viral protein synthesis were shut off early after infection with the *hr* mutant. Even on permissive lines, the mutant gave smaller plaques than wild type virus and an altered cytopathic effect. The mutant contained a 12 kb deletion towards the left end of the genome (DRILLIEN et al. 1981).

By inserting DNA that had been deleted back into the *TK* gene of the *hr* mutant, GILLARD et al. (1985) found that a 5.2 kb *Eco*RI fragment restored the ability of the virus to grow on human HEp-2 cells, i.e., the wild phenotype. This activity was further localized to an 855 bp fragment and sequence analysis showed that the *hr* gene encoded a 32.5 kDa protein. A 32.5 kDa protein was observed following in vitro translation of the mRNA hybrid-selected by the cloned *hr* gene (GILLARD et al. 1986). By using a synthetic peptide corresponding to the C-terminal 18 amino acids of the *hr* protein product, antibodies were generated which recognized a 29 kDa immediate early protein from VV-infected cells (GILLARD et al. 1989).

DNA hybridization demonstrated that the VV *hr* gene is strongly conserved and in a similar location in the genomes of cowpox, variola, monkeypox, and ectromelia viruses (GILARD et al. 1989).

The reason that the vaccinia *hr* gene product is required for viral growth in some human cell lines is still unknown. From the mutant phenotype, it appears that the protein is either needed for continued protein synthesis in infected cells or antagonizes the action of a host factor which inhibits viral protein synthesis in human cells.

A second host range gene has been identified in CPV which is required for growth on Chinese hamster ovary (CHO) cells. VV is unable to grow in CHO cells, and infection results in an early arrest of both host and viral protein synthesis (DRILLIEN et al. 1978; HRUBY et al. 1980); however, CPV can grow in CHO cells and presumably contains a host range gene which VV lacks. In vivo recombinants between VV and CPV which were able to grow in CHO cells contained CPV sequences from the left end of the genome (SPEHNER et al. 1988). Marker rescue of the VV *hr* phenotype on CHO cells with successively smaller cloned CPV DNA fragments showed that a 2.3 kb fragment contained the *hr* gene. The gene maps 27 kb from the left end of the CPV genome, and its nucleotide sequence encodes a 77 kDa protein (SPEHNER et al. 1988). The presence

of the CPV hr gene product is thought to act like the VV *hr* gene product to prevent the premature cessation of protein synthesis that occurs in nonpermissive cells.

3.4 A-type Inclusion Proteins

Some pox viruses produce A-type cytoplasmic inclusions (ATI) containing virions late in infection. The proteins making up these inclusions can be considered to be involved in virulence since they presumably contribute to the stability of virus particles in nature. The best studied ATIs are those of CPV. The inclusions contain a single protein species of 160 kDa (PATEL et al. 1986) that is produced in abundant quantity very late in infection. The nucleotide sequence of the cloned gene predicts a product of 150 kDa (FUNAHASHI et al. 1988). RPV synthesizes an immunologically related protein of similar size (PATEL et al. 1986). VV makes much smaller inclusions than CPV but does produce a late 94 kDa protein related to the CPV 160 kDa protein (PATEL et al. 1986) from a gene with a similar genomic location (FUNAHASHI et al. 1988).

3.5 Thymidine Kinase (*TK*)

The *TK* gene of VV has been used most often as the insertion site for foreign genes (MOSS and FLEXNER 1987), as this gene is nonessential in tissue culture and there is a positive biochemical selection for TK^- viruses. BULLER et al. (1985), however, showed that derivatives of vaccinia *WR* containing a mutation or insertion in the *TK* gene were attenuated for BALB/cByJ mice following i.c. inoculation when compared with TK^+ virus. The LD_{50} was increased by at least 4000-fold compared with the wild type virus. Although, this effect suggests that the *TK* gene plays an important role in vivo, it may also reflect the presence of relatively small nucleotide pools in some brain cells rather than a direct role of tk in neurovirulence. TK^- and TK^+ viruses had similar immunogenicity and gave similar local replication following intradermal (i.d.) scarification of mice (BULLER et al. 1985). By contrast, in New Zealand white rabbits, a TK^- vaccinia *WR* recombinant required 10^6 times more virus than wild type vaccinia *WR* to produce the same size of skin lesion (FLEXNER et al. 1987).

3.6 Hemagglutinin

Vaccinia *WR* produces a hemagglutinin (HA) which causes erythrocytes to adsorb to infected tissue culture cells. The *HA* gene has been used in vaccinia expression vectors as a site for the insertion of foreign genes, and two studies now indicate that HA^- viruses are attenuated. FLEXNER et al. (1987) found that insertion of the *lacZ* gene into the *HA* gene of vaccinia *WR* increased the LD_{50} for

BALB/cByJ mice by the i.c. route to $1.4 \pm 0.3 \times 10^5$PFU compared with $4.0 \pm 1.0 \times 10^1$PFU for wild type. The HA^- recombinant also required 10^4 times as much virus as vaccinia *WR* to produce a skin lesion of the same size in New Zealand white rabbits (FLEXNER et al. 1987). Also, the LD_{50} for *DD Y* mice by the i.c. route was shown to be 10^2–10^4 times higher for an HA^- mutant and an HA^- recombinant containing the HTLV-1 *env* gene (SHIDA et al. 1988) than for the parental vaccinia *WR* strain.

These results suggest that the vaccinia *HA* gene product is involved in virulence in vivo, although the nature of its role is unclear. On the other hand, RPV (*Utrecht* strain) and several VV strains do not produce a functional HA, although RPV is highly virulent in animal tests (BLOOM and MOYER, unpublished work).

3.7 Growth Factors

Retrospective examination of the nucleotide sequences of part of the VV ITR (VENKATESAN et al. 1982) revealed the presence of a gene whose product was related to mammalian EGF and TGF-α (BLOMQUIST et al. 1984; BROWN et al. 1985; REISNER 1985). A viral protein with growth factor-like activity was found by several groups in the medium of infected cells. An early protein of 25 kDa was identified which bound to EGF membrane receptors and was mitogenic but was serologically distinct from EGF and TGF-α (TWARDZIK et al. 1985). Vaccinia growth factor (VGF), like EGF, induces tyrosine phosphorylation at EGF receptors (KING et al. 1986). A signal sequence present in the primary translation product and a C-terminal transmembrane sequence were absent in the secreted form of VGF (STROOBANT et al. 1985). VGF is glycosylated, unlike EGF or TGF-α (STROOBANT et al. 1985), and its biosynthetic pathway has been characterized using an antipeptide antiserum (CHANG et al. 1988).

VGF is thought to mediate binding of virus particles to cells via the EGF receptor or to stimulate the proliferation of uninfected cells at a distance and hence promote the spread of infection. On A431 cells, which have an unusually high density of EGF receptors, VV forms foci rather than plaques (BULLER et al. 1988a). Additional of monoclonal antibody to the EGF receptor blocked the appearance of foci and instead resulted in plaque formation (BULLER et al. 1988a). Inoculation of the CAM with wild type VV caused the growth of ectodermal and entodermal cells in advance of infection (BULLER et al. 1988a), as the proliferating cells did not contain virus antigen.

Wild type VV contains two copies of the VGF gene, one in each ITR. A VGF^- mutant of VV was constructed (BULLER et al. 1988b) by replacing part of one copy of the *VGF* gene with a *lacZ* gene cassette and then allowing recombination with the second copy of the VGF in the ITR. The mutant was viable, indicating that VGF was not required for virus attachment or growth. The VGF^- derivative formed plaques on A431 cells rather than foci (BULLER et al. 1988a). VGF^- virus

produces lesions on the CAM containing fewer infectious virions than VGF^+ virus (BULLER et al. 1988a), suggesting that VGF plays a role in virus dissemination in vivo.

The VGF^- virus was greatly attenuated for BALB/cByJ mice following i.c. inoculation (BULLER et al. 1988b), with an LD_{50} of 2.5×10^4 PFU compared with 1.2×10^1 PFU for wild type, and shows less replication in the brain. Production of a visible lesion after i.d. inoculation of outbred black rabbits needed 10–100 times more VGF^- virus than wild type, and there was less proliferation of epidermal cells at the margin of the lesion (BULLER et al. 1988b). Although VV is strongly cytopathic, the secretion of VGF from infected cells appears to have an augmentative role in stimulating the metabolic activity and growth of peripheral cells prior to infection.

The leporipoxviruses, SFV and myxoma virus, also contain genes encoding growth factors related to EGF and TGF-α (CHANG et al. 1987; UPTON et al. 1987). The gene for SFV growth factor (SFGF) was isolated using a degenerate oligonucleotide probe based on a conserved region of EGF, TGF-α, and VGF (CHANG et al. 1987) and maps to the unique region near the ITR at the right end of the genome. A myxoma growth factor (MGF) gene was found using the cloned SFGF gene as probe and maps to a similar position in the myxoma genome (UPTON et al. 1987 and see Sect. 2.6). SFGF and MGF are much more closely related to each other than either is to VGF (UPTON et al. 1987); both SFGF and MGF lack the C-terminal hydrophobic region present in the primary translation product of VGF and resemble the secreted form of VGF.

The SFV and myxoma growth factors are particularly interesting in that, during the generation of MRV from SFV and myxoma virus, the MGF gene was replaced by the SFGF gene (UPTON et al. 1987). The construction of growth factor negative mutants of these viruses is needed in order to test whether the proliferative diseases they cause involve expression and function of growth factors. It is also tempting to speculate that the tumorigenic properties of other poxviruses such as Yaba tumor virus (BROWN et al. 1981) and molluscum contagiosum virus (MCV) (BEARCROFT and JAMIESON 1958) require growth factor secretion. The MCV genome shows hybridization to an oligonucleotide growth factor probe (PORTER and ARCHARD 1987), suggesting that it also encodes a growth factor belonging to the EGF family.

3.8 Proteins Altered During Vaccinia Virus Persistent Infections

Persistent infections can be established in certain cell lines. Viruses isolated from these persistently infected cells have accumulated deletions and point mutations which can lead to phenotypic variants and altered viral proteins. The small plaque mutants from untreated, persistently infected FEL cells (Sect. 2.4.2.2) synthesized an envelope protein whose size was 15.5 kDa, compared with 14 kDa in wild type virus (DALLO et al. 1987). Marker rescue of the small plaque mutants with the cloned wild type gene for the 14 kDa protein showed that is was possible

to generate large plaque viruses which now made a 14 kDa protein demonstrating that this protein was responsible for the observed small plaque size (DALLO et al. 1987).

The 14 kDa protein had previously been shown to be an envelope protein recognized by a neutralizing monoclonal antibody mABC3 (RODRIGUEZ et al. 1985). This mAB blocked virus uncoating but not attachment. Similar 14 kDa proteins were found in the *Elstree* strain of VV and in RPV and CPV (RODRIGUEZ et al. 1985). The vaccinia *WR* 14 kDa protein forms trimers and is thought to be involved in virus penetration (RODRIGUEZ et al. 1987). The sequence of the gene, which is expressed late in infection, has now been determined (RODRIGUEZ and ESTEBAN 1987).

Persistent infection of FEL cells with vaccinia *WR* also results in alterations in the sizes of virus proteins of 39 and 21 kDa and in changes in the isoelectric points of proteins of 48, 27, and 14 kDa (PAEZ et al. 1987). Like the 14 kDa protein described above, the 39 and 21 kDa proteins were immunogenic in mice; however, the 39 kDa protein did not elicit neutralizing antibodies (MAA and ESTEBAN 1987).

Although the above studies have identified a region involved in virulence and specific proteins that are recognized by the immune system, the interpretation of the significance of these results is made difficult by the isolation of strains carrying multiple mutations. Construction of isogenic strains is clearly required in order to define the roles of these individual genes. A mutant virus, *48-7*, containing the 12 kb deletion (see Sect. 2.4.2.2) and two point mutations in the gene encoding the 14 kDa protein is highly attenuated, even in chemically immunosuppressed mice, and may therefore be useful as a a vaccine strain (RODRIGUEZ et al. 1989). Many of the deletions described in Sects. 2.4.2.1 and 2.4.2.2 were attenuated in vivo. Future work is likely to assess the effects of inactivating individual genes within the regions defined by attenuating deletions.

3.9 The 13.8 kDa Secreted Protein of Vaccinia Virus

Cells infected with VV secrete a protein of 12 kDa into the medium; however, this protein is absent on infection with the *6/2* VV deletion mutant described in Sect. 2.4.2.1 (MOSS et al. 1981; KOTWAL and MOSS 1988b). The rearrangement in the *6/2* variant has recently been shown more accurately to be a deletion of 12 kb with an inverted transposition of 2.1 kb from the right end (KOTWAL and MOSS 1988a). One of the genes within the deleted region encodes a 13.8 kDa protein and overlaps the junction of the *Hin*dIII N and C fragments. The in vitro translation product from message hybrid-selected by the 13.8 kDa ORF was immunoprecipitated by antibody to the 12 kDa secreted protein (KOTWAL et al. 1989). A mutant in which the *lacZ* gene was used to replace the segment encoding the first 50 amino acids of the 13.8 kDa ORF failed to synthesize a 12 kDa protein of pI 5.3 (KOTWAL et al. 1989), confirming that the missing protein is the product of the gene. The 13.8 kDa ORF deletion mutant was strongly attenuated in vivo

for BALB/c mice by the i.c. route, with a $\log_{10}$ LD_{50} of 4.95 ± 0 compared with 0.72 ± 0.23 for wild type vaccinia *WR*, although the original *6/2* variant was further attenuated with a $\log_{10}$ LD_{50} of 7.17 ± 0.26 (KOTWAL et al. 1989). Other genes involved in neurovirulence, besides that for the 13.8 kDa protein, remain to be identified in the region at the left end of the VV genome. As yet, one can only speculate on the functions of the relevant gene products in vivo.

4 Future Directions

Two points emerge from this review. The first is that DNA within the terminal regions of viral DNA is subject to extensive deletion, transposition, and rearrangement. The second point is that many of the genes within the terminal regions of the virus are related to cellular genes involved in the regulation of the immune system and homeostasis. These genes also have profound effects on the virulence and pathogenesis of the virus.

It is not clear whether the rampant transposition, deletion, and rearrangement of sequences amongst and within termini is reflective of an in vivo mechanism by which these viruses can evolve and adapt to new situations and hosts. Certainly the potential for the creation and modification of genes is present as is the elimination of genes which are without benefit or even detrimental within a given environment. It should now be possible to design Darwinian experiments to address this intriguing possibility.

In the future, genes within the terminal regions will be examined individually in attempts to discern the contributions each makes to the virulence and pathogenesis of the virus. These studies will need to be of two types. The first type involves selective inactivation of a given gene within the context of wild type virus, whereas the second type of study involves the reintroduction of the same gene(s) back into attenuated deletion mutants from which they have been naturally eliminated. Parallel studies such as these will be particularly useful to identify genes responsible for complicating side effects and undue virulence in a given host. Genes so identified can then be removed to allow the design of ever safer strains for use in the construction of live vaccine vectors. Another relevant question is whether genes in this region, associated with host range in cell culture, are also responsible for the host range/specificity of a given virus in vivo. A differently way of phrasing this question is to ask whether the host range of a particular class of poxviruses having animal reservoirs, such as Avi- or Capripoxviruses, might have a far broader host range if genes from the terminal regions of another poxvirus with a different host range, such as vaccinia, were added. This question is not a trivial one, particularly if live poxvirus vectors become accepted and generally disseminated throughout the animal and human populations offering an increased possibility for intertypic recombination with indigenous poxviruses.

In the case of virus genes in the terminal regions which are highly homologous to cellular genes, their products need to be evaluated biochemically for the specific effects suggested by relatedness to cellular proteins. It is possible that some viral proteins may find commercial application in human medicine. As an example, the serpin gene of CPV may interfere with the normal clotting process. If so, this protein may find use as an anticoagulant in individuals suffering cerebral or myocardial infarctions.

It is clear that the poxviruses will continue to be the subject of intensive study. As has been true in the past, it is likely that they will continue to produce a wealth of information and many surprises.

Acknowledgments. We would like to thank Drs. P. Bhatt, V. Chernos, M. Esteban, and B. Pogo for generously sharing their results prior to publication.

References

Archard LC, Mackett M (1979) Restriction endonuclease analysis of red cowpox virus and its white pock variant. J Gen Virol 45: 51–63

Archard LC, Mackett M, Barnes DE, Dumbell KR (1984) The genome structure of cowpox virus white pock variants. J Gen Virol 65: 875–886

Ball LA (1987) High-frequency homologous recombination in vaccinia virus DNA. J Virol 61: 1788–1795

Baroudy BM, Venkatesan S, Moss B (1982) Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. Cell 28: 315–324

Baroudy BM, Venkatesan S, Moss B (1983) Structure and replication of vaccinia virus telomeres. Cold Spring Harbor Symp Quant Biol 47: 723–729

Bearcroft WGC, Jamieson MF (1958) Yaba tumor virus. An outbreak of subcutaneous tumours in rhesus monkeys. Nature 182: 195–196

Bedson HS, Dumbell KR (1964a) Hybrids derived from the viruses of alastrim and rabbit pox. J Hyg (Lond) 62: 141–146

Bedson HS, Dumbell KR (1964b) Hybrids derived from the viruses of variola major and cowpox. J Hyg (Lond) 62: 147–158

Berns KI, Silverman C (1970) Natural occurrence of cross-linked vaccinia virus deoxyribonucleic acid. J Virol 5: 299–304

Bhatt PN, Jacoby RO (1987a) Mousepox in inbred mice innately resistant or susceptible to lethal infection with ectromelia virus III. Experimental transmission of infection and derivation of virus-free progeny from previously infected dams. Lab Anim Sci 37: 22–28

Bhatt PN, Jacoby RO (1987b) Mousepox in inbred mice innately resistant or susceptible to lethal infection with ectromelia virus I. Clinical responses. Lab Anim Sci 37: 11–15

Bhatt PN, Jacoby RO, Gras L (1988) Mousepox in inbred mice innately resistant or susceptible to lethal infection with ectromelia virus. IV. Studies with the Moscow strain. Arch Virol 100: 221–230

Binns MM, Tomley FM, Campbell J, Boursnell ME (1988) Comparison of a conserved region in fowlpox virus and vaccinia virus genomes and the translocation of the fowlpox virus thymidine kinase gene. J Gen Virol 69: 1275–1283

Blanden RV (1970) Mechanisms of recovery from a generalized viral infection: mousepox I. The effects of anti-thymocyte serum. J Exp Med 132:1035–1054

Blanden RV (1971a) Mechanisms of recovery from a generalized viral infection: mousepox III. Regression infectious foci. J Exp Med 133: 1090–1104
Blanden RV (1971b) Mechanisms of recovery from a generalized viral infection: mousepox II. Passive transfer of recovery mechanisms with immune lymphoid cells. J Exp Med 133: 1074–1089
Block W, Upton C, McFadden G (1985) Tumorigenic poxviruses: genomic organization of malignant rabbit virus, a recombinant between Shope fibroma virus and myxoma virus. Virology 140: 113–124
Blomquist MC, Hunt LT, Barker WC (1984) Vaccinia virus 19-kilodalton protein: relationship to several mammalian proteins, including two growth factors. Proc Natl Acad Sci USA 81: 7363–7367
Boursnell ME, Foulds IJ, Campbell JI, Binns MM (1988) Non-essential genes in the vaccinia virus *Hin*dIII K fragment: a gene related to serine protease inhibitors and a gene related to the 37K vaccinia virus major envelope antigen. J Gen Virol 69: 2995–3003
Brown GD, Moyer RW (1983) The white pock mutants of rabbit poxvirus: V. In vitro translation of early host range mutant mRNA. Virology 126: 381–390
Brown GD, Peluso RW, Moyer SA, Moyer RW (1983) A simple method for the preparation of extracts from animal cells which catalyze efficient in vitro protein synthesis. J Biol Chem 258: 14309–14314
Brown JP, Twardzik DR, Marquardt H, Todaro GJ (1985) Vaccinia virus encodes a polypeptide homologous to epidermal growth factor and transforming growth factor. Nature 313: 491–492
Brown ST, Nalley JF, Kraus SJ (1981) Molluscum contagiosum. Sex Transm Dis 8: 227–234
Buller RM, Smith GL, Cremer K, Notkins AL, Moss B (1985) Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. Nature 317: 813–815
Buller RM, Chakrabarti S, Moss B, Fredrickson T (1988a) Cell proliferative response to vaccinia virus is mediated by VGF. Virology 164: 182–192
Buller RM, Chakrabarti S, Cooper JA, Twardzik DR, Moss B (1988b) Deletion of the vaccinia virus growth factor gene reduces virus virulence. J Virol 62: 866–874
Chang W, Upton C, Hu SL, Purchio AF, McFadden G (1987) The genome of Shope fibroma virus, a tumorigenic poxvirus, contains a growth factor gene with sequence similarity to those encoding epidermal growth factor and transforming growth factor alpha. Mol Cell Biol 7: 535–540
Chang W, Lim JG, Hellstrom I, Gentry LE (1988) Characterization of vaccinia virus growth factor biosynthetic pathway with an antipeptide antiserum. J Virol 62: 1080–1083
Chernos VI, Antonova TP, Senkevich TG (1985) Recombinants between vaccinia and ectromelia viruses bearing the specific pathogenicity markers of both parents. J Gen Virol 66: 621–626
Cooper JA, Wittek R, Moss B (1981) Hybridization selection and cell-free translation of mRNAs encoded within the inverted terminal repetition of the vaccinia virus genome. J Virol 37: 284–294
Dallo S, Esteban M (1987) Isolation and characterization of attenuated mutants of vaccinia virus. Virology 159: 408–422
Dallo S, Rodriguez JF, Esteban M (1987) A 14 K envelope protein of vaccinia virus with an important role in virus-host cell interactions is altered during virus persistence and determines the plaque size phenotype of the virus. Virology 159: 423–432
DeFilippes FM (1982) Restriction enzyme mapping of vaccinia virus DNA. J Virol 43: 136–149
Downie AW, Haddock DW (1952) A variant of cowpox virus. Lancet I:1049–1050
Drillien R, Spehner D, Kirn A (1978) Host range restriction of vaccinia virus in Chinese hamster ovary cells: relationship to shutoff of protein synthesis. J Virol 28: 843–850
Drillen R, Koehren F, Kirn A (1981) Host range deletion mutant of vaccinia virus defective in human cells. Virology 111: 488–499
Drillien R, Spehner D, Villeval D, Lecocq JP (1987) Similar genetic organization between a region of fowlpox virus DNA and the vaccinia virus *Hin*dIII J fragment despite divergent location of the thymidine kinase gene. Virology. 160: 203–209
Dumbell KR, Archard LC (1980) Comparison of white pock (h) mutants of moneypox virus with parental monkeypox and with variola-like viruses isolated from animals. Nature 286: 29–32
Edwards KM, Andrews TC, Van Savage J, Palmer PS, Moyer RW (1988) Poxvirus deletion mutants: virulence and immunogenicity. Microbial Pathogenesis 4: 325–333
Esposito JJ, Obijeski JF, Nakano JH (1978) Orthopoxvirus DNA: strain differentiation by electrophoresis of restriction endonuclease fragmented virion DNA. Virology 89: 53–66
Esposito JJ, Cabradilla CD, Nakano JH, Obijeski JF (1981) Intragenomic sequence transposition in monkeypox virus. Virology 109: 231–243

Esposito JJ, Knight JC (1985) Orthopoxvirus DNA: a comparison of restriction profiles and maps. Virology 143: 230–251
Esteban M, Benavente J, Paez E (1986) Mode of sensitivity and resistance of vaccinia virus replication to interferon. J Gen Virol 67: 801–808
Fenner F (1948a) The pathogenesis of the acute exanthems. An interpretation based on experimental investigations with mousepox (infectious ectromelia of mice). Lancet II: 915–920
Fenner F (1948b) The clinical features of mouse-pox (infectious ectromelia of mice) and the pathogenesis of the disease. J Pathol Bacteriol 60: 529–552
Fenner F (1949a) Studies in mousepox (infectious ectromelia of mice). VI. A comparison of the virulence and infectivity of three strains of ectromelia virus. Aust J Exp Biol Med Sci 27: 31–50
Fenner F (1949b) Mouse-pox (infectious ectromelia of mice): a review. J Immunol 63: 341–373
Fenner F (1958) The biological characters of several strains of vaccinia, cowpox and rabbitpox viruses. Virology 5: 502–529
Fenner F, Cairns J (1959) Variation in virulence in relation to adaptation to new hosts. In: Burnet FM, Stanley WM (eds) The viruses. Vol 3. Academic, New York, pp 225–249
Fenner F, Sambrook JF (1966) Conditional lethal mutants of rabbitpox virus. II. Mutants (p) that fail to multiply in PK-2a cells. Virology 28: 600–609
Fenner F, Henderson DA, Arita I, Jezek Z, Ladnyi ID (1988a) Variola virus and the other orthopoxviruses. In: Smallpox and its eradication. World Health Organization, Geneva, pp 69–120
Fenner F, Henderson DA, Arita I, Jezek Z, Ladnyi ID (1988b) The pathogenesis, pathology and immunology of smallpox and vaccinia. In: Smallpox and its eradication. World Health Organization, Geneva, pp 121–168
Fenner F, Wittek R, Dumbell KR (1989) The orthopoxviruses. Academic, New York
Flexner C, Hugin A, Moss B (1987) Prevention of vaccinia virus infection in immunodeficient mice by vector-directed IL-2 expression. Nature 330: 259–262
Funahashi S, Sato T, Shida H (1988) Cloning and characterization of the gene encoding the major protein of the A-type inclusion body of cowpox virus. J Gen Virol 69: 35–47
Gangemi JD, Sharp DG (1976) Use of a restriction endonuclease in analyzing the genomes from two different strains of vaccinia virus. J Virol 20: 319–323
Garon CF, Barbosa E, Moss B (1978) Visualization of an inverted terminal repetition in vaccinia virus DNA. Proc Natl Acad Sci USA 75: 4863–4867
Gemmell A, Fenner F (1960) Genetic studies with mammalian poxviruses. III. White (u) mutants of rabbitpox virus. Virology 11: 219–235
Gershon PD, Black DN (1989) The nucleotide sequence around the capripoxvirus thymidine kinase gene reveals a gene shared specifically with leporipoxvirus. J Gen Virol. 70: 525–533
Geshelin P, Berns KI (1974) Characterization and localization of the naturally occurring cross-links in vaccinia virus DNA. J Mol Biol 88: 785–796
Gillard S, Spehner D, Drillien R (1985) Mapping of a vaccinia host range sequence by insertion into the viral thymidine kinase gene. J Virol 53: 316–318
Gillard S, Spehner D, Drillien R, Kirn A (1986) Localization and sequence of a vaccinia virus gene required for multiplication in human cells. Proc Natl Acad Sci USA 83: 5573–5577
Gillard S, Spehner D, Drillien R, Kirn A (1989) Antibodies directed against a synthetic peptide enable detection of a protein encoded by a vaccinia virus host range gene that is conserved within the Orthopoxvirus genus. J Virol 63: 1814–1817
Grady LJ, Paoletti E (1977) Molecular complexity of vaccinia DNA and the presence of reiterated sequences in the genome. Virology 79: 337–341
Hruby DE, Lynn DL, Condit RC, Kates JR (1980) Cellular differences in the molecular mechanisms of vaccinia virus host range restriction. J Gen Virol 47: 485–488
Jacoby RO, Bhatt PN (1987) Mousepox in inbred mice innately resistant or susceptible to lethal infection with ectromelia virus II. Pathogenesis. Lab Anim Sci 37: 16–22
Jacoby RO, Smith AL, Bhatt PN (1982) Viral disease research. In: The mouse in biomedical research. (Experimental biology and oncology vol 4.) Academic, New York, pp. 169–211
Jacoby RO Bhatt PN, Johnson EA, Paturzo FX (1983) Pathogenesis of vaccinia (IHD-T) virus infection in *BALB/cAnN* mice. Lab Anim Sci 33: 435–441
Joklik WK, Pickup DJ, Patel DD, Moody MD (1988) Virulence genes of poxviruses and reoviruses. Vaccine 6: 123–128
King CS, Cooper JA, Moss B, Twardzik DR (1986) Vaccinia virus growth factor stimulates tyrosine protein kinase activity of A431 cell epidermal growth factor receptors. Mol Cell Biol 6: 332–336
Kotwal GJ, Moss B (1988a) Analysis of a large cluster of nonessential genes deleted from a vaccinia virus terminal transposition mutant. Virology 167: 524–537

Kotwal GJ, Moss B (1988b) Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. Nature 335: 176–178
Kotwal GJ, Moss B (1989) Vaccinia virus encodes two proteins that are structurally related to members of the plasma serine protease inhibitor superfamily. J Virol 63: 600–606
Kotwal GJ, Hugin AW, Moss B (1989) Mapping and insertional mutagenesis of a vaccinia virus gene encoding a 13,800-Da secreted protein. Virology 171: 579–587
Lai AC, Pogo BG (1989a) Characterization of vaccinia virus deletion mutants isolated from persistently infected Friend erythroleukemia cells. Virus Res 12: 239–250
Lai AC, Pogo BG (1989b) Attenuated deletion mutants of vaccinia virus lacking the vaccinia growth factor are defective in replication in vivo. Microb Pathog 6: 219–226
Lake JR, Cooper PD (1980) Deletions of the terminal sequences in the genomes of the white pock (u) and host-restricted (p) mutants of rabbitpox virus. J Gen Virol 48: 135–147
Maa, JS, Esteban M (1987) Structural and functional studies of a 39,000-Mr immunodominant protein of vaccinia virus. J Virol 61: 3910–3919
Mackett M, Archard LC (1979) Conservation and variation in Orthopoxvirus genome structure. J Gen Virol 45: 683–701
McCarron RJ, Cabrera CV, Esteban M, McAllister WT, Holowczak JA (1978) Structure of vaccinia DNA: analysis of the viral genome by restriction endonucleases. Virology 86: 88–101
McClain ME (1965) The host range and plaque morphology of rabbitpox virus (RPu^+) and its u mutants on chick fibroblast, PK-2a, and L929 cells. Aust J Exp Biol Med Sci 42: 31–44
McClain ME, Greenland RM (1965) Recombination between rabbitpox virus mutants in permissive and nonpermissive cells. Virology 25: 516–522
Mims CA (1964) Aspects of the pathogenesis of virus diseases. Bacteriol Rev 28: 30–71
Moss B, Flexner C (1987) Vaccinia virus expression vectors. Annu Rev Immunol 5: 305–324
Moss B, Winters E, Cooper JA (1981) Deletion of a 9,000-base-pair segment of the vaccinia virus genome that encodes nonessential polypeptides. J Virol 40: 387–395
Moyer RW, Graves RL (1981) The mechanism of cytoplasmic orthopoxvirus DNA replication. Cell 27: 391–401
Moyer RW, Graves RL (1982) The white pock mutants of rabbit poxvirus IV. The late white pock (mu) host range (hr) mutants of rabbit poxvirus are blocked in morphogenesis. Virology 119: 332–346
Moyer RW, Rothe CT (1980) The white pock mutants of rabbit poxvirus. I. Spontaneous host range mutants contain deletions. Virology 102: 119–132
Moyer RW, Graves RL, Rothe CT (1980a) The white pock (mu) mutants of rabbit poxvirus III. Terminal DNA sequence duplication and transposition in rabbit poxvirus. Cell 22: 545–553
Moyer RW, Brown GD, Graves RL (1980b) The white pock mutants of rabbit poxvirus II. The early white pock (mu) host range (hr) mutants of rabbit poxvirus uncouple transcription and translation in nonpermissive cells. Virology 106: 234–249
Muller HK, Wittek R, Schaffner W, Schumperli D, Menna A, Wyler R (1978) Comparison of five poxvirus genomes by analysis with restriction endonucleases *Hin*dIII *Bam*I and *Eco*RI. J Gen Virol 38: 135–147
Paez E, Esteban M (1985) Interferon prevents the generation of spontaneous deletions at the left terminus of vaccinia virus DNA. J Virol 56: 75–84
Paez E, Dallo S, Esteban M (1985) Generation of a dominant 8-MDa deletion at the left terminus of vaccinia virus DNA. Proc Natl Acad Sci USA 82: 3365–3369
Paez E, Dallo S, Esteban M (1987) Virus attenuation and identification of structural proteins of vaccinia virus that are selectively modified during virus persistence. J Virol 61: 2642–2647
Panicali D, Davis SW, Mercer SR, Paoletti E (1981) Two major DNA variants present in serially propagated stocks of the *WR* strain of vaccinia virus. J Virol 37: 1000–1010
Patel DD, Pickup DJ, Joklik WK (1986) Isolation of cowpox virus A-type inclusions and characterization of their major protein component. Virology 149: 174–189
Pedrali Noy G, Weissbach A (1977) Evidence of a repetitive sequence in vaccinia virus DNA. J Virol 24: 406–407
Perkus ME, Panicali D, Mercer S, Paoletti E (1986) Insertion and deletion mutants of vaccinia virus. Virology 152: 285–297
Pickup DJ, Ink BS, Parsons BL, Hu W, Joklik WK (1984) Spontaneous deletions and duplications of sequences in the genome of cowpox virus. Proc Natl Acad Sci USA 81: 6817–6821
Pickup DJ, Ink BS, Hu W, Ray CA, Joklik WK (1986) Hemorrhage in lesions caused by cowpox virus

is induced by a viral protein that is related to plasma protein inhibitors of serine proteases. Proc Natl Acad Sci USA 83: 7698–7702
Porter CD, Archard LC (1987) Characterization and physical mapping of Molluscum contagiosum virus DNA and location of a sequence capable of encoding a conserved domain of epidermal growth factor. J Gen Virol 68: 673–682
Reisner AH (1985) Similarity between the vaccinia virus 19K early protein and epidermal growth factor. Nature 313: 801–803
Rodriguez D, Rodriguez JR, Rodriguez JF, Trauber D, Esteban M (1989) Highly attenuated vaccinia virus mutants for the generation of safe recombinant viruses. Proc Natl Acad Sci USA 86: 1287–1291
Rodriguez JF, Esteban M (1987) Mapping and nucleotide sequence of the vaccinia virus gene that encodes a 14-kilodalton fusion protein. J Virol 61: 3550–3554
Rodriguez JF, Janeczko R, Esteban M (1985) Isolation and characterization of neutralizing monoclonal antibodies to vaccinia virus. J Virol 56: 482–488
Rodriguez JF, Paez E, Esteban M (1987) A 14,000-Mr envelope protein of vaccinia virus is involved in cell fusion and forms covalently linked trimers. J Virol 61: 395–404
Sambrook JF, Mcclain ME, Easterbrook KB, McAuslan BR (1965). A mutant of rabbitpox virus defective at different stages of its multiplication in three cell types. Virology 26: 738–745
Shida H, Hinuma Y, Hatanaka M et al. (1988) Effects and virulences of recombinant vaccinia viruses derived from attenuated strains that express the human T-cell leukemia virus type I envelope gene. J Virol 62: 4474–4480
Slabaugh MB, Mathews CK (1984) Vaccinia virus-induced ribonucleotide reductase can be distinguished from host cell activity. J Virol 52: 501–506
Slabaugh MB, Johnson TL, Mathews CK (1984) Vaccinia virus induces ribonucleotide reductase in primate cells. J Virol 52: 507–514
Slabaugh M, Roseman N, Davis R, Mathews C (1988) Vaccinia virus-encoded ribonucleotide reductase: sequence conservation of the gene for the small subunit and its amplification in hydroxyurea-resistant mutants. J Virol 62: 519–527
Smith GL, Moss B (1983) Infectious poxvirus vectors have capacity for at least 25,000 base pairs of foreign DNA. Gene 25: 21–28
Spehner D, Gillard S, Drillien R, Kirn A (1988) A cowpox virus gene required for multiplication in Chinese hamster ovary cells. J Virol 62: 1297–1304
Strayer DS, Sell S (1983) Immunohistology of malignant rabbit fibroma virus—a comparative study with rabbit myxoma virus. JNCI 71: 105–116
Strayer DS, Cabirac G, Sell S, Leibowitz JL (1983a) Malignant rabbit fibroma virus: observations on the culture and histopathologic characteristics of a new virus-induced rabbit tumor. JNCI 71: 91–104
Strayer DS, Skaletsky E, Cabirac GF et al. (1983b) Malignant rabbit fibroma virus causes secondary immunosuppression in rabbits. J Immunol 130: 399–404
Stroobant P, Rice AP, Gullick WJ, Cheng DJ, Kerr IM, Waterfield MD (1985) Purification and characterization of vaccinia virus growth factor. Cell 42: 383–393
Sugimoto M, Yasuda A, Miki K et al. (1985) Gene structures of low-neurovirulent vaccinia virus LC16m0, LC16m8, and their Lister original (*LO*) strains. Microbiol Immunol 29: 421–428
Takahashi-Nishimaki F, Suzuki K, Morita M et al. (1987) Genetic analysis of vaccinia virus Lister strain and its attenuated mutant LC16m8: production of intermediate variants by homologous recombination. J Gen Virol 68: 2705–2710
Tengelsen LA, Slabaugh MB, Bibler JK, Hruby DE (1988) Nucleotide sequence and molecular genetic analysis of the large subunit of ribonucleotide reductase encoded by vaccinia virus. Virology 164: 121–131
Tomley F, Binns M, Campbell J, Boursnell M (1988) Sequence analysis of an 11.2 kilobase, near-terminal, *Bam*HI fragment of fowlpox virus. J Gen Virol 69: 1025–1040
Townsend A, Bastin J, Gould K et al. (1988) Defective presentation to class I-restricted cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen. J Exp Med 168: 1211–1224
Twardzik DR, Brown JP, Ranchalis JE, Todaro GJ, Moss B (1985) Vaccine virus-infected cells release a novel polypeptide functionally related to transforming and epidermal growth factors. Proc Natl Acad Sci USA 82: 5300–5304
Upton C, Mcfadden G (1986a) DNA sequence homology between the terminal inverted repeats of Shope fibroma virus and an endogenous cellular plasmid species. Mol Cell Biol 6: 265–276

Upton C, Mcfadden G (1986b) Tumorigenic poxviruses: analysis of viral DNA sequences implicated in the tumorigenicity of Shope fibroma virus and malignant rabbit virus. Virology 152: 308–321
Upton C, Mcfadden G (1986c) Identification and nucleotide sequence of the thymidine kinase gene of Shope fibroma virus. J Virol 60: 920–927
Upton C, Carrell RW, McFadden G (1986) A novel member of the serpin superfamily is encoded on a circular plasmid-like DNA species isolated from rabbit cells. FEBS Lett 207: 115–120
Upton C, Macen JL, McFadden G (1987) Mapping and sequencing of a gene from myxoma virus that is related to those encoding epidermal growth factor and transforming growth factor alpha. J Virol 61: 1271–1275
Van Tongeren HAE (1952) Spontaneous mutation of cowpox-virus by means of egg passage. Arch Gesamte Virusforsch 5: 35.
Venkatesan S, Gershowitz A, and Moss B (1982) Complete nucleotide sequences of two adjacent early vaccinia virus genes located within the terminal repetition J Virol 44, 637–646
Wittek R, Moss B (1980) Tandem repeats within the inverted terminal repetition of vaccinia virus DNA. Cell 21: 277–284
Wittek R, Menna A, Schumperli D, Stoffel S, Muller HK, Wyler R (1977) *Hin*dIII and *Sst* I restriction sites mapped on rabbit poxvirus and vaccinia virus DNA. J Virol 23: 669–678
Wittek R, Menna A, Muller HK, Schumperli D, Boseley PG, Wyler R (1978) Inverted terminal repeats in rabbit poxvirus and vaccinia virus DNA J Virol 28: 171–181
Wittek R, Barbosa E, Cooper JA, Garon CF, Chan H, Moss B (1980) Inverted terminal repetition in vaccinia virus DNA encodes early mRNAs. Nature 285: 21–25
Wittek R, Cooper JA, Moss B (1981) Transcriptional and translational mapping of a 6.6-kilobase-pair DNA fragment containing the junction of the terminal repetition and unique sequence at the left end of the vaccinia virus genome. J Virol 39: 722–732

Recombinant Vaccinia Viruses As Vectors for Studying T Lymphocyte Specificity and Function

J. R. BENNINK and J. W. YEWDELL

Laboratory of Viral Diseases, NIAID, 12441 Parklawn Drive, Rockville, MD 20852, USA

Current Topics in Microbiology and Immunology, Vol. 163

1 Introduction

Over the past 20 years, a steadily increasing portion of immunological research has been directed towards understanding the specificity and function of T lymphocytes. Vaccinia virus (VV) has proven to be the preferred vector for determining the specificity of T lymphocytes for individual gene products derived from a variety of organisms, including viruses, protozoa, and mammalian cells (we refer to these genes as "extrinsic genes", their products as "extrinsic antigens" and their origin as "source organisms") and for studying the processing and presentation of antigens to T lymphocytes by antigen presenting cells (APCs). The popularity of VV recombinants has two sources. First, the technology of producing VV recombinants is relatively straightforward and VV recombinants consistently provide high levels of expression of extrinsic antigens. Second, studies of T lymphocytes require the expression of antigens in histocompatible cells. The ability of VV to infect mice and a broad variety of mouse and human cell lines of diverse lineages that can function as APCs make them ideal vectors for studies of T lymphocytes.

Our aims for the present review are threefold: (1) to illustrate how VV recombinants can be used to study questions in T lymphocyte recognition and function, (2) to impart practical information regarding the use of VV recombinants, and (3) to indicate the pitfalls associated with using VV recombinants.

2 Basic Features of T Lymphocytes

For readers with a limited immunological background, we provide a very abbreviated review of T lymphocyte biology, a topic treated in detail in review articles (BERZOFSKY et al. 1987; SCHWARTZ 1986; ZINKERNAGEL and DOHERTY 1979) and textbooks (KLEIN 1986, PAUL 1984). The distinguishing and most remarkable characteristic of T lymphocytes is that they recognize foreign antigens only in conjunction with molecules of the major histocompatibility complex (MHC; termed HLA in man and H-2 in mice). Two classes of "restriction" molecules are encoded by the MHC. Class I molecules consist of an integral membrane glycoprotein heavy chain (M_r 44K) noncovalently complexed with $\beta 2$ microglobulin (M_r 12K; a protein that also exists in

noncomplexed form in serum). Class I molecules are expressed on virtually all somatic cells. Mouse cells produce two or three (and maybe more) class I gene products that can serve as T lymphocyte restriction elements. The number of human class I restriction elements is less certain, but there appear to be three loci encoding class I genes. In both humans and mice, a large number of alleles exist for each class I gene. In mice, combinations of alleles that occur in certain strains (termed haplotypes) are arbitrarily assigned superscript letters (e.g., $H\text{-}2^k$), as are the alleles present at the individual loci in these mice (e.g., K^k).

Class II molecules consist of two noncovalently bound integral membrane glycoproteins, termed $\alpha(M_r 34K)$ and β ($M_r 28K$) subunits. Unlike class I molecules, class II molecules are normally expressed on a limited number of cell types including macrophages and B lymphocytes. Class II molecules are also expressed on T lymphocytes in humans but not in mice. As with class I molecules, a large number of alleles exist for each class II gene.

T lymphocytes are divided into two major classes, termed cytotoxic T lymphocytes (T_C), and helper lymphocytes (T_H). This terminology reflects what were thought to be absolute functional differences between the classes. In recent years these differences have blurred somewhat. While it was thought that T_H functioned solely to secrete lymphokines with important roles in B lymphocyte and T_C growth and differentiation, it is now clear that T_H can also lyse target cells in an antigen specific, MHC restricted manner (WAGNER et al. 1977; KAPLAN et al. 1984; LUKACHER et al. 1985; FLEISCHER et al. 1985). Similarly, it is also known that, in addition to lysing cells expressing foreign antigens, T_C release lymphokines (such as γ-interferon) that have major biological effects (ENNIS 1982; MORRIS et al. 1982; KLEIN et al. 1984). These overlaps in function should not, however, obscure at least three important functional differences between T_C and T_H subsets. First, and most absolutely, T_C recognize antigen in conjunction with class I MHC molecules, while T_H recognize antigen in conjunction with class II molecules. Second, T_C recognize proteins biosynthesized by APCs, while T_H can recognize exogenously provided antigens. Thus, to induce anti-viral T_C responses or to sensitize target cells for lysis by virus-specific T_C, it is generally necessary to use infectious virus, while T_H responses to viral structural proteins can be induced with either noninfectious virus or simply with material containing the foreign antigen (reviewed in YEWDELL and HACKETT 1989). Third, in mediating immunity, it is likely that T_C function directly to reduce replication of the source organism, while T_H function to maximize antibody and T_C responses against the source organism.

Recognition of foreign antigens by T lymphocytes is mediated by a single receptor composed of multiple subunits, two of which, α and β, confer antigen specificity. T_H and T_C cells apparently utilize the same pool of α and β genes (HEDRICK et al. 1985). The differential MHC restriction of T_H and T_C is due, at least in part, to interactions between MHC molecules expressed on APCs and accessory molecules uniquely expressed by T_H and T_C. The T_H specific CD4 molecule interacts with class II MHC molecules (DOYLE and STROMINGER 1987), while the T_C specific CD8 molecule interacts with class I MHC molecules

(CONNOLLY et al. 1988). Consistent with their sharing of T cell receptor genes, both T_H and T_C recognize linear sequences of foreign proteins that can be substituted by synthetic peptides of 5–15 residues (BUUS et al. 1987; BABBITT et al. 1985; TOWNSEND et al. 1986). Peptides determinants have been directly shown to bind to MHC class I and class II molecules (BABBITT et al. 1985; BUUS et al. 1986; CHEN and PARHAM 1989; KANE et al. 1989; WATTS et al. 1984). Solution of the three-dimensional structure of a class I MHC molecule reveals a groove at the distal portion believed to be the antigen binding site (BJORKMAN et al. 1987a, b). A similar groove can be located in a class II molecule whose structure is modeled by primary sequence and structural homologies to class I molecules (BROWN et al. 1988).

The manner by which proteins are processed into antigenic determinants, transported into a compartment containing MHC molecules, and bound by MHC molecules is a topic of intense current interest. Although many important aspects of antigen processing pathways await discovery, the broad outlines of the schemes appear to be defined.

Exogenously provided antigens recognized by T_H are internalized into endosomes where they are partially degraded by acid proteases into fragments containing antigenic determinants. The ability of lysosomotropic amines to block this process serves as a simple indicator of the functioning of the endosomal pathway since they do not affect the class I processing pathway. The site of association of endosomally processed antigens with class II molecules is not firmly established but would appear to be either an endosomal compartment or regions of the Golgi complex (GC) that are transited by contents of the endosomal compartment.

The pathway for processing biosynthesized antigens into determinants recognized by T_C begins in the cytosol of virus infected cells. By delivering proteins to the cytosol of APCs it is possible to bypass the apparent requirement of antigen biosynthesis for T_C recognition (MOORE et al. 1988; YEWDELL et al. 1988). Although direct evidence is lacking, it is suspected that cytosolic proteolysis is involved in T_C processing pathway (TOWNSEND et al. 1988). Recent results indicate that determinants processed from the cytosol associate with newly synthesized class I molecules, most likely in the endoplasmic reticulum (ER) of APCs (COX et al. 1990; NUCHTERN et al. 1989; TOWNSEND et al. 1989; YEWDELL and BENNINK 1989).

3 Practical Aspects of Using Vaccinia Virus Recombinants to Study T Lymphocyte Responses

3.1 Virus Stocks

The genetic engineering, growth, and characterization of VV recombinants has been detailed in a number of articles and chapters (MACKETT et al. 1985a;

MACKETT and SMITH 1986; PICCINI and PAOLETTI 1988), to which we refer those desiring to learn the technology of VV recombinant production. It is worth mentioning a few tips, however, that pertain specifically to the use of VV for study of cellular immune responses. First, mycoplasma can be lethal to T lymphocytes grown in short- or long-term culture, and every effort should be taken to ensure that the cell line used to propagate VV stocks is free of mycoplasma. Second, although a number of investigators use only purified VV stocks for T lymphocyte studies, purification of virus is not required for in vivo or in vitro stimulation of T lymphocytes or for sensitization of target cells. Therefore, we recommend the use of purified virus only in special circumstances (for example, to distinguish between presentation of endogenous versus exogenously provided antigen).

3.2 Immunization of Mice

T_C responses are much more vigorous and consistent when mice are free of infection from natural pathogens such as Sendai virus and mouse hepatitis virus. Therefore, if possible, animals should be maintained in a specific pathogen free colony. Under optimal conditions, mice are housed in microisolator cages; all cage, food, and bedding materials are autoclaved before and after use; and all animal handling (including cage changes) is performed in a laminar flow biosafety cabinet using aseptic techniques. Mice can be immunized with VV by a number of routes, including intravenous, intraperitoneal, and epidermal scarification. Scarification is no more effective than other routes of immunization and is associated with unnecessary exposure of animal handlers to virus present in exposed lesions. We therefore recommend immunization by intraperitoneal or intravenous routes. We have found that intravenous immunization with 5×10^6 plaque forming units of VV recombinants results in good priming of splenic T_C precursors for subsequent in vitro responses to extrinsic antigens. Although intraperitoneal immunization can be accomplished more rapidly and is considerably less demanding technically than intravenous injection, we have favored intravenous immunization based on its better stimulation of T_C following adoptive transfer of splenocytes into lethally irradiated mice. We must mention, however, that we have encountered no difficulties in obtaining good T_C responses to extrinsic antigens when mice have been immunized via intraperitoneal immunization.

3.3 In Vitro Stimulation of T_C

Although it is possible to obtain T_C following primary or secondary immunization of mice, the most vigorous and reproductible responses are generally obtained when splenocytes from primed animals are stimulated in vitro. In vitro stimulation is also an essential part of producing T_C lines or clones. Splenocytes should be taken from mice no less than 2 weeks following immunization with VV recombinants. Although responses can be obtained for at least a year following

immunization, it is preferable to use animals immunized with VV recombinants no more than 6 months previously. Stimulation is accomplished using APCs that express the extrinsic antigen in association with histocompatible class I MHC molecules. Either tissue culture cell lines or cells derived from the sacrificed animal can be used as APCs. We have found that for a number of viruses (VV, influenza, vesicular stomatitis, respiratory syncytial), good stimulation results from using autologous virus infected splenocytes as stimulators at a ratio of 1:2 with noninfected splenocytes. T_C activity usually peaks after 6–7 days of culture at 37° C. There is considerable variation in the abilities of different lots of fetal bovine sera to support T_C responses. Serum suppliers are willing to provide samples of their available lots for testing prior to purchase. Samples should be tested under standard stimulation and culture conditions for the vigor and antigen specificity of T_C activity. Often difficulties with generating secondary anti-viral T_C using virus infected APCs are related to virus stocks used for infection of APCs. This can be due to a number of factors. First, the virus may not be of sufficient infectivity for the APCs utilized. Second, the virus might kill or paralyze T_C themselves or T_H required for T_C stimulation. Third, virus stocks might contain mycoplasma, which can efficiently block in vitro T_C responses, probably by directly killing T lymphocytes. For stimulation with VV or VV recombinants, we routinely use autologous splenocytes infected with 2 plaque forming units of VV per cell. We have noticed that a few stocks of VV are toxic for secondary in vitro cultures. We strongly suspect that the toxicity is due to contamination of stocks with mycoplasma. Given a toxic VV stock, efforts should first be made to obtain a seed stock known to be mycoplasma free. If this is not available, it is possible to reduce to eliminate contaminating mycoplasma by filtration of virus stocks through 0.22 μ filters or, for recombinants with inactivated thymidine kinase (*TK*) genes (a popular protocol for producing VV recombinants results in inactivating the *TK* gene), by plaquing virus in TK^- cells in the presence of 5-bromodeoxyuridine. 5-Bromodeoxyuridine, which is toxic to TK^+ cells, can subsequently be diluted to nontoxic levels by growing virus from individual plaques. Should the toxicity of VV stocks prove intractable, it is possible to use APCs infected with VV for 12–24 h and then fixed by incubation with aldehydes following protocols developed for stimulation of T_H using fixed APCs (EISENLOHR et al. 1987; ALLEN and UNANUE 1984).

3.4 Measuring T_C Activity

The activity of T_C populations or clones is measured by their ability to lyse APCs. For T_C clones it is also possible to use proliferation as a measure of antigen recognition. Target cell lysis is generally measured by release of a marker retained by viable cells. Based on cost, simplicity, and sensitivity, $Na^{51}CrO_4$ is the most widely used marker of cell viability, but other radioactive or nonradioactive markers can be used if circumstances preclude the use of ^{51}Cr. A large number of cell lines derived from inbred mouse strains are available for use as target cells

in ^{51}Cr release assays. Highly adherent cell lines generally make poor target cells due to clumping and high spontaneous release of isotope. It is therefore best to use non- or semi-adherent cell lines as target cells. When using a cell line for the first time as target cells, it is a good idea to check for: (a) expression of potential T_C target antigens, or if not possible, susceptibility to infection with VV or the source organism; (b) expression of class I MHC molecules, as detected by monoclonal antibodies specific for each class I allele contained by the cell. In many cases, lack of T_C recognition is due to poor class I expression. This can often be partially or completely remedied by treating cells for 24–48 h with γ-interferon, which does not inhibit subsequent infection with VV; and (c) expression of class II MHC molecules, to eliminate the possibility that lysis of target cells is due to recognition by T_H with cytotoxic activity.

The most popular MHC haplotypes for T_C studies are H-2^d, H-2^k, and H-2^b. Cell lines that make good target cells following infection with VV recombinant for T_C restricted to these haplotypes are, respectively, P815, L929, and MC57G. We hasten to add that there is considerable heterogeneity between subclones of these cell lines in class I expression, susceptibility to VV infection, and degree of lysis by T_C. Investigators experiencing difficulties using these or other cell lines reported to be good target cells should obtain the lines from laboratories that have described their successful use.

In first characterizing T_C responses to extrinsic antigens it is imperative to demonstrate that the recognition observed is class I restricted. This is quite easy to accomplish using mouse T_C, due to the availability of H-2 congenic mouse strains and target cells that express class I but not class II molecules. The outbred nature of human populations makes determination of class I restriction much more difficult, particularly since in many instances the only histocompatible cell lines available are B lymphocytes transformed by Epstein Barr virus, which express both class I and class II MHC molecules. As a first step, the MHC restriction of human T lymphocytes should be determined using class I and class II matched and mismatched target cells. Next, blocking studies should be performed with antibodies specific for class I or class II molecules. Last, whenever possible, MHC restriction should be confirmed using cells that express cloned class I heavy chain genes.

4 Analysis of T Lymphocyte Specificity Using Vaccinia Virus Recombinants

4.1 T_C Responses

4.1.1 Basic Strategies

A major use of VV recombinants is to determine which extrinsic antigens are recognized by T_C specific for the source organism. This is essential for

understanding the effect of immunization with various viral and subviral components on a subsequent challenge with infectious virus and for eventually producing vaccines that elicit effective cellular immunity. It is also obvious that this is a necessary first step towards understanding the processing and presentation of antigens by APCs.

VV recombinants can be used in two basic strategies to establish the specificity of anti-viral T_C (BENNINK et al. 1984). With either strategy, an alternative method of expressing the gene product in histocompatible APCs is required. This can be infection of APCs with the source organism or another virus vector (e.g., herpes simplex virus), transfection of APCs with the gene encoding the extrinsic antigen, or the physical delivery of the extrinsic antigen into the cytosol of APCs. Additionally, if a linear epitope has been defined for the extrinsic antigen, APCs can simply be incubated with a synthetic peptide that contains the determinant (TOWNSEND et al. 1986b).

In the first strategy, recombinant VV infected cells are tested for recognition by T_C induced by immunization with the source organism, non-VV vector, or APCs expressing the extrinsic antigen. This has the advantage of directly identifying the gene product recognized by T_C induced by the source organism. In the second strategy, VV recombinants are used to stimulate T_C that are then tested for activity against APCs expressing the extrinsic antigen. This is of particular use for generating T_C populations or clones specific for individual gene products. These T_C can then be used to study questions involving antigen processing and presentation or the biological function of T_C. VV recombinants can be used in three protocols to generate T_C specific for the extrinsic antigen: (1) T_C are simply recovered from animals inoculated 6–10 days previously with the VV recombinants, (2) VV recombinants are used for in vitro stimulation of T_C precursors derived from primed mice, and (3) T_C precursors obtained from animals immunized with VV recombinants are restimulated in vitro. Each of these protocols is hampered to some extent by the magnitude of the anti-VV T_C response in mice. To put this into perspective, the frequency of VV precursors in spleens following immunization with VV is 10–100-fold higher than the frequency of influenza virus precursors following immunization with influenza virus, which is by no means a poor stimulator of T_C. Thus, the first protocol is rarely of use since splenocytes derived from mice immunized with VV recombinants generally exhibit low or undetectable recognition of the extrinsic antigen. The second protocol is much more useful but is variably accompanied by the stimulation of primary anti-VV T_C, since VV is one of a select group of antigens able to induce in vitro primary responses. This can be a problem when testing T_C on VV recombinant infected APCs, the severity of which is inversely proportional to the strength of the T_C response for the extrinsic antigen. Thus, using this protocol, it is best to test T_C on non-VV infected APCs. In this case, it is possible to use VV recombinants to both prime and stimulate T_C, although the magnitude of the VV response generally overwhelms all but the strongest responses to extrinsic antigens. The last protocol usually result in a T_C response with minimal anti-VV activity whose specificity for the source organism is limited to the gene

product expressed by the VV recombinant used for priming. We have found this to be the most useful method of generating T_C populations specific for individual viral gene products.

4.1.2 Lessons from Studying Anti-Influenza T_C

VV recombinants were first used to study the specificity of anti-influenza T_C, and most of the experience with VV recombinants has accumulated in this system. In this section we describe the influenza system and provide the salient findings.

Influenza A virions contain eight segmented genes that code for ten known proteins (LAMB 1989). These include two integral membrane glycoproteins, hemagglutinin (HA) and neuraminidase (NA); a peripheral membrane protein that forms the inner shell of the virus (M1); and the proteins of the ribonucleoprotein complex, which is comprised of nucleoprotein (NP) and small amounts of three viral polymerases (P_A, P_{B2}, P_{B1}). Three additional proteins are synthesized by influenza virus infected cells that appear to be largely excluded from virions. Nonstructural protein 1 (NS1) and NS2 are located in the cytosol and nuclei of infected cells and are produced via alternative splicing from the same gene segment. M2 is an integral membrane protein produced via alternative splicing from the gene segment that encodes M1. We used a panel of VV recombinants that encode each of the ten known gene products to define the T_C responses in seven mouse strains with unique H-2 haplotypes. It was necessary to produce individual VV recombinants containing cDNAs from influenza virus genes normally produced by splicing, since the cytosolic site of VV mRNA synthesis precludes splicing (SMITH et al. 1987). This is a positive feature of the VV vectors, as it allows for discrimination of protein products produced by alternative splicing of common precursor RNAs without extensive genetic manipulation.

Each of the VV recombinants produced a gene product reactive with polyclonal antisera against influenza virus proteins that migrated with the expected M_r in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SMITH et al. 1987). For proteins in which panels of monoclonal antibodies were available (HA, NA, NP, M1, NS1, P_{B2}), we found that gene products produced by VV recombinants were immunochemically indistinguishable from their influenza virus derived counterparts.

4.1.2.1 Most, If Not All Influenza Virus Proteins Are Processed for T_C Recognition

HA, NA, NP, NS1, NS2, and the three polymerases are recognized by T_C derived from at least one of the seven mouse strains tested (BENNINK et al. 1986; BENNINK and YEWDELL, unpublished work; BRACIALE et al. 1984; REAY et al. 1989; TOWNSEND et al. 1984; WYSOCKA and HACKETT 1990; YEWDELL et al. 1985). Thus many different types of proteins, including type I and type II integral

membrane proteins (HA and NA, respectively), nucleic acid binding proteins with nuclear localization sequences (NP and the polymerases), and nonstructural viral proteins (NS1 and NS2) can be processed and presented to T_C. We failed to detect T_C recognition of M1 and M2 by mouse T_C induced either by immunization with influenza virus or the corresponding VV recombinants. This suggests that APCs might not process all proteins for T_C recognition, perhaps because of resistance to proteolysis or an inability of the protein or its fragments to be transported across internal membranes. On the other hand, since M1 is a predominant target for T_C derived from humans with a particular class I allele (GOTCH et al. 1987), the recognition of M1 and M2 by mouse T_C might reflect other defects in the presentation and recognition process, including association with class I molecules or the absence of antigen receptors that recognize the MHC-antigen complex.

4.1.2.2 T_C Recognition of Internal Viral Proteins Is Independent of Their Relative Abundance

Despite the fact that the polymerases are produced in very low amounts during an influenza virus infection, they can be recognized with equal efficiency as NP or NS1, which are produced in 100–1000-fold higher amounts. Thus, low abundance proteins can be major target antigens for T_C.

4.1.2.3 Crossreactive T_C Recognition of Related Proteins Generally Reflects Their Degree of Amino Acid Homology

Original studies of anti-influenza T_C found that T_C could be divided into two populations, a minor population specific for the immunizing strain and other closely related strains ("strain specific") and a major population able to lyse cells infected with any human influenza A virus ("crossreactive") (BRACIALE 1977; CAMBRIDGE et al. 1976; EFFROS et al. 1977; ZWEERINK et al. 1977). The identity of the viral protein(s) recognized by crossreactive T_C sparked much of the original interest in anti-influenza T_C recognition. Using the panel of VV recombinants we found that the vast majority of crossreactive T_C recognized the highly conserved nonglycoprotein structural and nonstructural proteins. Similarly, we showed that T_C crossreactive between two serotypes of vesicular stomatitis virus predominantly recognized the highly conserved nucleocapsid proteins, while the more variable glycoproteins were recognized in a largely strain specific manner (YEWDELL et al. 1986). This is not to say that recognition of internal proteins is always crossreactive; it is now clear that at least two of the influenza virus internal proteins, P_{B2} (BENNINK et al. 1982) and NP (TOWNSEND et al. 1984), can be recognized in a highly strain specific manner. Indeed, it has been found that, under some conditions, most strain specific T_C recognize internal viral proteins (KEES and KRAMMER 1984). Conversely, a minority of crossreactive clones are specific for the highly variable HA, which is generally recognized in a strain specific manner (BENNINK et al. 1984, 1986; BRACIALE et al. 1984).

4.1.2.4 Epitopes Can Be Mapped by Expressing Fragments of Proteins in Vaccinia Virus Recombinants

The feasibility of this approach was originally demonstrated using transfected cells expressing fragments of NP (TOWNSEND et al. 1985). Subsequently, VV recombinants were used to map epitopes recognized on HA (BRACIALE et al. 1987, 1989). Recent work, in which a 15 amino acid sequence of NP expressed in a VV recombinant was shown to be recognized by anti-NP T_C, suggests that T_C epitopes do not require additional sequences to be properly transported and complexed with class I molecules (TOWNSEND et al. 1989). In many instances, however, it is probably more expedient to define epitopes by narrowing the site to 50–100 residues using VV recombinants and precisely locating the site using a nested set of synthetic peptides composed of 10–20 residues (BASTIN et al. 1987).

4.1.2.5 Individual Proteins Contain Very Few Determinants Recognized by T_C Restricted to an Individual Class I Allele

Even large proteins such as NP (498 residues) have only one, or occasionally two, determinants that naturally elicit T_C responses (BASTIN et al. 1987). This contrasts with the recognition of influenza virus proteins by T_H, which recognize multiple determinants on proteins in association with individual class II alleles.

4.1.2.6 Responsiveness to Individual Proteins Is Highly Controlled by the MHC

When T_C responses are examined at the level of individual viral proteins, the frequency of nonresponder class I alleles ranges from roughly 50% for high responder (e.g., NP) to less than 10% (e.g., NS2) (BENNINK et al. 1988; BENNINK and YEWDELL, unpublished work; PALA and ASKONAS 1986). Responses to individual determinants on proteins are even more restricted, to the extent that determinants recognized in conjunction with more than a single class I allele have yet to be identified. Thus even when proteins are recognized in association with multiple class I alleles, T_C restricted to these alleles recognize different determinants. It is unclear whether this is due to defects in antigen presentation, deficiencies in the repertoire of T_C antigen receptors, or both. From the practical standpoint, responsiveness to an individual antigen in conjunction with a given class I allele must be determined empirically.

4.1.2.7 Other Unknown Factors Contribute to the Prevalence of T_C Specific for Individual Proteins

It must be emphasized that lack of a detectable T_C response for a given protein must always be considered as a tentative finding. For example, we repeatedly failed to demonstrate T_C responses to the NA using standard protocols for generating T_C. WYSOCKA and HACKETT 1990 recently found, however,

that NA specific T_C can easily be detected if NA-VV primed mice are boosted in vivo with influenza and then stimulated in vitro with influenza virus. Additional observations suggest that the magnitude of T_C responses to individual proteins is under complicated regulation. First, even in high responder mouse strains, T_C recognition of NP has been found to vary widely between individuals (PALA et al. 1986). Whether this is due to specific suppression of anti-NP responses or to fundamental differences between individual T_C repertoires is unknown. Second, we have found that, although NS1-VV is recognized by T_C derived from BALB/c mice (H-2^d), it cannot prime BALB/c mice for NS1-specific T_C responses. This is unlikely to be related to some peculiarity of the NS1-VV stock since it primes CBA mice (H-2^k) for T_C responses (BENNINK et al. 1987). Third, the frequency of nonresponder MHC alleles to individual proteins would appear to be decidedly nonrandom. HA and especially NP are recognized in association with approximately 50% of class I alleles, while the polymerases, which contain twice the number of residues and presumably twice the number of potential T_C epitopes, are only recognized in association with approximately 20% of class I alleles.

4.1.2.8 Vaccinia Virus Inhibits Presentation of Some Influenza Virus Determinants

In some unpublished early experiments, we found that co-infection of cells with VV and influenza virus inhibited recognition by an influenza specific T_C clone. The effect appeared to be specific for the determinant recognized by the clone since the same co-infected target cells were recognized perfectly well by secondary anti-influenza T_C population. In what appears to be a similar phenomenon, certain target cell lines infected by NP-VV are poorly recognized by a H-2^b-restricted, NP-specific T_C (TOWNSEND et al. 1987). The same cells were recognized when NP was expressed in lower quantities by DNA-mediated transfection. Although VV inhibition of antigen presentation is a nuisance when the goal is determination of T_C specificity, it is potentially an invaluable key for unlocking the mysteries of antigen processing and presentation, a topic treated in Sect. 6. The available evidence suggests that VV inhibition of presentation of proteins to T_C is an exceptional occurrence. First, we know of no other discrepancies between results obtained with VV recombinants and other expression vectors. Second, the overall frequency of anti-influenza T_C precursors as determined by limiting dilution analysis is in good agreement with the summation of the frequencies of T_C specific for individual influenza proteins expressed by VV recombinants (WYSOCKA and BENNINK 1988).

4.2 T_H Responses

VV recombinants have not been extensively used in specificity analyses of T_H responses. In large part this is due to the ability of T_H to recognize APCs exposed to non infectious antigens. Thus, it has been possible to study T_H specificity using

proteins purified from virions or virus infected cells. There are, however, two compelling reasons for using VV recombinants to characterize T_H. First, many viral antigens, particularly minor structural components or nonstructural proteins, cannot be obtained in either suitable quantity or purity for studies of T_H specificity. Second, recent findings indicate that a subset of determinants recognized by T_H arise only when proteins are biosynthesized by APCs (EISENLOHR and HACKETT 1989; JACOBSEN et al. 1989; WEISS and BOGEN 1989). Although many immunologists believe that this population represents only a minor fraction of the T_H repertoire, this must be firmly established by experiment and not by sentiment.

VV recombinants can be used in several ways to study the specificity of T_H responses. First, they can simply be used in noninfectious form as a source of genetically isolated antigen. Lysates or homogenates of VV recombinant infected cells can be used either to immunize animals for T_H responses or tested for their ability to sensitize APCs for stimulation of T_H populations or clones. The use of such crude antigen containing preparations will to some extent be associated with nonspecific stimulation of T_H. The severity of this problem will vary with a number of parameters, including the strain of mice and the potency of the foreign antigens as an inducer of T_H. Should levels of nonspecific stimulation be unacceptable, partial purification of the antigen by standard biochemical techniques is in order. Second, VV recombinants can be used as a vector to synthesize the antigen in APCs (MORRISON et al. 1986). Crude preparations of infectious VV contain considerable quantities of extrinsic antigen that will be processed and presented by APCs via the endosomal pathway. To solely measure presentation of antigen biosynthesized by APCs, it is necessary to use purified VV recombinants. Although it is commonly found that VV virions do not incorporate sufficient foreign proteins to sensitize APCs for T_H recognition, this point must be established by blocking the ability of VV to direct biosynthesis of the extrinsic antigen, either by inactivating viral infectivity (conveniently by UV irradiation) or by addition of a protein synthesis inhibitor.

5 Use of Vaccinia Virus Recombinants to Study the Role of T Lymphocytes in Protection Against Source Organisms

The excitement that greeted the introduction of VV recombinant technology was largely due to the potential use of VV recombinants as vaccines to induce immunity in humans and other mammals to source organisms. The importance of characterizing the cellular immune response to the VV recombinant varies directly with the importance of cellular immunity in providing protection against the source organism. Since for many organisms this is undetermined, studies using VV recombinants can provide basic information regarding not only the role of individual gene products in providing immunity, but also the relative contributions to immunity of humoral versus cellular effector functions.

Table 1. Recombinant VV protection experiments

Source organism	Extrinsic antigen	Species	Protection	Reference
Bacteria				
Group A streptococci	M6	Mice	+	FISCHETTI et al. 1989
Parasites				
Schistosoma mansoni	GP38, P28-I, P28-II	Rats, hamsters, baboons	+	PIERCE et al. 1987
Tumors				
Human melanoma	p97	Mice	+	ESTIN et al. 1988
	p97	Mice	+	HU et al. 1988
Rat neu oncogene encoded protein	p185	Mice	+	BERNARDS et al. 1987
Polyoma virus	Large, middle and small T antigens	Rats	+ and −	LATHE et al. 1987 CLERTANT et al. 1988
Viruses				
Arenaviridae				
Lassa	N	Guinea pigs	+	CLEGG and LLOYD 1987
	G	Guinea pigs	+	AUPERIN et al. 1988
	NP, G1, G2	Guinea pigs	+	MORRISON et al. 1989
	G	Monkeys	+	FISHER-HOCH et al. 1989
Lymphocytic choriomeningitis	G, NP	Mice	+	HANY et al. 1989
Hepadnaviridae				
Hepatitis B	HBsAg	Chimpanzees	+	MOSS et al. 1984
Herpesviridae				
Epstein-Barr	gp-340	Cottontop tamarrins	+	MORGAN et al. 1988
Cytomegalovirus	pp89,	Mice	+	KOSZINOWSKI et al. 1987
	pp89	Mice	+	JONJIC et al. 1988
Herpes simplex 1	gD	Mice	+	PAOLETTI et al. 1984
	gD	Mice	+	CREMER et al. 1985a, b
	gB	Mice	+	CANTIN et al. 1987
	gD	Mice	+	MARTIN and ROUSE 1987
	gD	Guinea pigs	+	WACHSMAN et al. 1987
	gB, gD	Mice	+	MARTIN et al. 1988, 1989
	gB	Mice	+	WILLEY et al. 1988
	gD	Mice	+	WACHSMAN et al. 1988
	gD	Mice	+	ROONEY et al. 1988
Pseudorabies	gp50	Mice and swine	+	MARCHIOLI et al. 1987

Rhabdoviridae				
Rabies	G	Mice	+	KIENY et al. 1984
	G	Foxes	+	WIKTOR et al. 1984
	G	Rabbits and mice	+	DIETZSCHOLD et al. 1985
	G	Raccoons	+	RUPPRECHT et al. 1986, 1988
	G	Foxes, dogs, cats	+	BLANCOU et al. 1986, 1989
	G	Skunks	+	TOLSON et al. 1987
	G	Mice, dogs	+	ESPOSITO et al. 1987
	G	Foxes	+	BROCHIER et al. 1988, 1989
	G	Foxes	+	KIENY et al. 1988
	G	Foxes	+	TOLSON et al. 1988
	G	Raccoons	+	ESPOSITO et al. 1988
	G	Raccoons	+	HANLON et al. 1989
Vesicular stomatitis	G	Mice, cattle	+	MACKETT et al. 1985b
Orthomyxoviridae				
Influenza	HA	Hamsters	+	SMITH et al. 1983
	HA	Mice	+	SMALL et al. 1985
	HA, NP	Mice	+ and +/−	ANDREW et al. 1986, 1987
	HA	Mice	+	ROTA et al. 1987, 1989
	HA	Chickens	+	CHAMBERS et al. 1988
	HA	Chickens	+	DE et al. 1988
	NA	Chickens	+	WEBSTER et al. 1988
	HA, NP	Mice	+ and −	ANDREW and COUPAR 1988*
	HA, P_{B1}, P_{B2}, P_A, NA, NP, NS1, NS2	Ferrets	+ and −	JAKEMAN et al. 1989
	HA	Mice	+	HIOE and HINSHAW 1989*
Paramyxoviridae				
Measles	HA, F	Mice	+	DRILLIEN et al. 1988
Parainfluenza	F and HN	Cotton rats	+	SPRIGGS et al. 1987
	HN	Monkeys	+	SPRIGGS et al. 1988
Respiratory syncytial	F, G	Cotton rats	+	OLMSTED et al. 1986
	G	Cotton rats	+	ELONGO et al. 1986
	G, F, N	Mice, cotton rats	+	STOTT et al. 1986, 1987
	N	Mice	+	KING et al. 1987
	F	Mice	+	WERTZ et al. 1987
	F, G	Monkeys	+	OLMSTED et al. 1988
	F, G	Cotton rats	+	MURPHY et al. 1988
	G truncations	Cotton rats	+ and −	OLMSTED et al. 1989

Table 1 (*Continued*)

Table 1 (*Continued*)

Source organism	Extrinsic antigen	Species	Protection	Reference
Simian 5	F, HN	Hamsters	+	PATTERSON et al. 1987
Rinderpest	HA, F	Cattle	+	YILMA et al. 1988
	F	Rabbits	+	BARRETT et al. 1989
Retroviridae				
HIV	gp160	Chimpanzees	+/−	HU et al. 1987
	gp160, p25, and F/3′ orf	Chimpanzees	−	VAN EEDENBURG et al. 1989
HTLV-1	env	Rabbits	+	SHIDA et al. 1987
Friend murine leukemia	env	Mice	+	MORRISON et al. 1987
Togaviridae				
Venezuelan equine encephalitis	E1, E2 and capsid	Mice	+ and −	KINNEY et al. 1988
Dengue	C, M, and E	Monkeys	−	DEUBEL et al. 1988
	C, pre-M, E, and NS1, NS2a	Mice	+	BRAY et al. 1989
Special				
Bivalent				
Herpes simplex virus and influenza	Herpes D and influenza HA	Mice	+	FLEXNER et al. 1988
Lymphokines				
Murine cutaneous leishmanias	GM-CSF	Mice	−	CORCORAN et al. 1988
Vaccinia	IL-2	Mice	+	FLEXNER et al. 1987
		Mice	+	RAMSHAW et al. 1987

Protection (+) refers to the ability of VV recombinants to alter the outcome, in a manner beneficial to the host, of a subsequent challenge of immunized animals with the source organism

References marked with an asterisk include adoptive transfer studies to directly examine the role of T lymphocytes in immunity

Many studies have been performed to date to assess the ability of VV recombinants to protect a variety of host species against a challenge with source organisms. Not surprisingly, the outcome of immunization with VV recombinants is dependent on the specific characteristics of the immune response to each extrinsic antigen and the role of this type of immunity in protection against the source organism. The information obtained from these studies is beyond the scope of this review, and for those interested in further information we summarize the studies and provide references in Table 1. In this section we limit our discussion to some general difficulties commonly encountered in using VV recombinants to assess the role of T lymphocytes in host immunity to the source organism.

5.1 Protective Effects Following Recombinant Vaccinia Virus Immunization Must Be Demonstrated To Be Due To Immune Recognition of the Extrinsic Antigen

Although this point may seem trivial, self-delusion is possible in the absence of stringent controls. To cite an example, we tested the ability of VV recombinants to protect mice against a lethal intranasal challenge with influenza virus. In contrast to immunization with wild type VV, mice immunized with the recombinants demonstrated significant protection against influenza virus-induced mortality. These data appeared promising until we included additional control recombinants that contained genes derived from vesicular stomatitis virus; these recombinants provided a similar degree of protection as the recombinants containing influenza virus genes.

5.2 The Route of Immunization May Be Important

In the event that VV recombinants fail to protect against a challenge with the source organism, it is worth considering different routes of immunization. It is possible to obtain immune responses to VV by inoculation by epidermal, aerosol, intradermal, intraperitoneal, intravenous, intranasal, intracerebral, and intramuscular routes. Although it has not been carefully examined, it would be surprising if varying the route of immunization with VV recombinants did not alter the induction of local cellular immune responses. It is logical that a route be chosen that best approximates the delivery of the source organism or promises the best opportunity to induce a local immune response in the organ in which replication of the source organism occurs.

5.3 Separating the Roles of Humoral and Cellular Immunity

The contribution of humoral immunity to protection can be assessed by testing sera for the presence of antibodies that inhibit replication of the source organism

or, more conclusively, by examining the capacity of purified B lymphocytes or serum to confer immunity to recipients. Conversely, the contribution of cellular immunity can be assessed by testing the ability of purified T lymphocyte populations to confer immunity to naive recipients or the effect on immunity by in vivo depletion of T lymphocytes; the latter can be accomplished simply by treating animals with T lymphocyte-specific monoclonal antibodies.

5.4 Separating the Roles of T_H and T_C

In many studies investigators fail to consider the possible contribution of T_H to cellular immunity. Priming of T_H by VV recombinants can protect against challenge with source organisms in a number of ways including. (a) magnifying antibody responses against the extrinsic antigen and even source organism antigens not produced by the VV recombinant used for immunization since, for example, T_H specific for internal viral proteins provide "intrastructural, intermolecular help" for antibody responses to virus receptor glycoproteins upon challenge with intact virions (RUSSELL and LIEW 1979; SCHERLE and GERHARD 1986, 1988); (b) amplifying the T_C response (ASHMAN and MULLBACHER 1979; ASKONAS et al. 1982; BIDDISON et al. 1981; MILLER and REISS 1984; MOLL et al. 1985; REISS and BURAKOFF 1981); (c) releasing lymphokines that activate "nonspecific" immune cells such as macrophages and natural killer cells; and (iv) directly limiting source organism replication by either lysing class II bearing cells or releasing lymphokines with anti-source organism activity.

The relative roles of primed T_H versus T_C in mediating protection can be estimated most easily, and unfortunately, least definitively, by comparing the effect of priming with infectious versus noninfectious antigens since in most cases only the former will prime for T_C responses. More rigorously, CD4- or CD8-specific monoclonal antibodies can be used to deplete T_H or T_C from VV recombinant-primed mice prior to challenge with the source organism. Alternatively, transfer experiments can be performed using T_H and T_C populations purified from splenocytes derived from VV recombinant-primed animals. Using animals mismatched at MHC class I or class II loci, it is possible to separate the function of T_C and T_H using nonfractionated splenocytes, although these experiments can be complicated by the responses of both host and graft to MHC alloantigens.

5.5 Vaccinia Virus Might Alter the Normal Handling of the Extrinsic Antigen by the Immune System

Factors that could influence the immune response include differences between VV and the source organism in: their tropism, their ability to modify antigen processing pathways in APCs, and the substitution of intrinsic VV antigens for

those of the source organism. VV does not necessarily have to influence the immune response to the extrinsic antigen in a negative manner; there are clearly cases (immunodeficiency viruses, for example) where immunization with VV recombinants might result in better immunity than immunization with the source organism. Should immunization with VV recombinants result in suboptimal cellular immunity to the extrinsic antigen, this might be improved by including gene products that modulate the immune response, such as interleukins (CORCORAN et al. 1988; FLEXNER et al. 1987; RAMSHAW et al. 1987).

5.6 Information Derived from Experiments Utilizing Vaccinia Virus Recombinants Is Limited to Secondary T Lymphocyte Responses

This is also important information; however, T lymphocytes may play a different role in primary responses associated with infection of naive animals with the source organism.

5.7 Immune Responses Are Complex

The vertebrate immune system has evolved numerous mechanisms with overlapping functions to combat a constant onslaught of pathogens. Many of these mechanisms are only poorly understood and some, no doubt, remain to be discovered. Without diminishing the importance of attempting to dissect immunity into its constituent parts, it should be considered that the role of any single component in protection against a pathogen can be overestimated when it is the sole object of investigation. To fairly gauge the importance of individual effector mechanisms, experiments should be balanced between positive and negative manipulations, for example, testing both the effect of transferring antigen-specific T_C to naive recipients and depleting T_C precursors from animals. To inject a final note of caution, it is usually best to carry a healthy sense of humility and skepticism into in vivo systems.

6 Use of Recombinant Vaccinia Virus to Study the Processing of Antigens for T Lymphocyte Recognition

The qualities that make VV recombinants useful for studying the specificity of T lymphocytes make them equally attractive for studies of antigen processing. In this section we summarize the various ways in which VV recombinants have been or might be used to study the processing and presentation of proteins to T lymphocytes.

6.1 Generation of Specific T_C Populations

In dissecting biological processes it is almost always advantageous to use the most discriminating probe possible. In the case of processing and presentation of antigens for class I restricted recognition, this dictates the use of T_C specific for individual epitopes. As discussed above, most antigens characterized to date have a single immunodominant epitope recognized by T_C derived from any given individual. Thus, using T_C raised to an individual protein is in most cases equivalent to using T_C specific for individual determinants. VV recombinants have proven to be valuable for this purpose, since they are able to induce potent, highly specific T_C responses, especially when used to prime lymphocytes for in vitro secondary responses elicited with the source organism. We routinely use T_C populations generated in this manner to study antigen processing and presentation (YEWDELL et al. 1988; YEWDELL and BENNINK 1989).

6.2 Expression of Altered Protein Forms

An important tool for studying antigen processing is the use of altered forms of proteins. In perhaps the first application of this approach, it was shown that removal of the leader sequence responsible for transporting the influenza HA into the ER had no effect on the presentation of HA to T_C (TOWNSEND et al. 1986a). Thus, even for proteins that are normally targeted to the ER in what is believed to be a cotranslational process, it is likely that processing for T_C recognition originates in the cytosol. Most recently, VV recombinants were used to identify the minimal sequence requirements for antigen presentation; gene products consisting of 22 and 15 residues containing defined epitopes were shown to be presented to T_C (WHITTON and OLDSTONE 1989; GOULD et al. 1989). In a variation of this approach, VV recombinants were used to express chimeric proteins consisting of the influenza NP and a cellular protein, ubiquitin (TOWNSEND et al. 1988); this experiment is described in Sect. 6.5.

6.3 Expression of MHC Restriction Molecules in APCs

It has been shown that cells infected with a VV recombinant expressing H-2 K^d (COUPAR et al. 1986b) are recognized by K^d restricted, VV-specific T_C, and to a lesser extent by alloreactive T_C. It should also be possible to express class II molecules using VV recombinants; in this case it will be necessary to either include both α and β genes in the recombinant or, probably better, simply to co-infect APCs with recombinants expressing α or β genes. By choosing MHC molecules that serve as restriction elements for VV-specific T lymphocytes, the need to introduce additional foreign antigens into APCs is obviated. Alternatively, APCs can be co-infected with VV recombinants expressing an extrinsic antigen and its respective MHC restriction molecule.

VV recombinants expressing MHC restriction molecules can be used in at least three ways; First, as vectors for expressing genetically engineered MHC molecules; second, to allow study of the antigen processing and presentation capacity of xenogeneic and allogeneic cell lines with defined biochemical defects, and third, as a means of expressing MHC molecules in relatively large quantities in a regulated manner. Studies utilizing the first approach have yet to appear, but this situation will certainly be remedied when the utility of VV for expressing histocompatibility molecules is recognized. Using the K^d-VV recombinant produced by COUPAR et al. (1986b) we have found the second and third approaches to be very productive. For example, we have been able to study the ability of a hamster cell line with a temperature sensitive defect in ubiquitin-targeted cytosolic proteolysis to present VV antigens to K^d restricted T_C (COX, unpublished). We have also been able to explore the effect of a pharmacologic inhibitor of exocytosis on the intracellular trafficking of nascent class I molecules (YEWDELL and BENNINK 1989).

While extolling the use of VV recombinants expressing MHC molecules, it is necessary to inject a slightly sobering technical difficulty; most genes encoding MHC restriction elements are available off the shelf only in genomic form. To engineer many of the potential VV recombinants, it will be necessary to isolate cDNAs that encode the coveted restriction molecule.

6.4 Expression of Extrinsic Gene Products That Influence Antigen Processing

Two types of gene products can be studied, cellular genes implicated in antigen processing and presentation and foreign genes implicated in blocking antigen processing or presentation. Using VV in this manner to study cellular genes awaits the identification of cells with genetically defined defects in antigen processing (see preceding paragraph), in which case VV could be used to determine whether a given gene product reverses the defect in antigen presentation. Less speculatively, we have recently used VV to determine how the adenovirus E19 glycoprotein acts to prevent presentation of antigens to T_C (COX et al. 1990). E19 is a resident glycoprotein of the ER that specifically binds class I molecules and prevents their egress from the ER (ANDERSSON et al. 1985; BURGERT and KVIST 1985; PAABO et al. 1983, 1986, 1987; SIGNAS et al. 1982; WOLD et al. 1985). Difficulties exist in synchronously and rapidly attaining high levels of E19 expression in antigen presenting cells suitable for detailed analysis of T_C recognition. Thus, the effect of E19 on antigen presentation has been examined only to the extent of documenting that T_C recognition of E19 expressing cells is diminished in parallel with a decrease in cell surface class I expression (BURGERT et al. 1987; RAWLE et al. 1989; TANAKA and TEVETHIA 1988). To surmount these problems we produced a VV recombinant containing the E19 gene under the control of the VV 7.5K promoter. Like most recombinants constructed in this manner, the recombinant (E19-VV) rapidly produced large

amounts of the extrinsic gene product in mouse cells following infection. The co-expression of E19 with VV or influenza virus proteins, introduced by co-infection with other VV recombinant viruses, had a profound effect on the presentation of VV or influenza virus antigens to T_C. The magnitude to this effect is governed by the identity of the restricting class I molecule. Recognition by L^d and K^d restricted specific T_C was greatly reduced, while the effect on recognition by D^d, D^k, and K^k restricted T_C ranged from slight to nondetectable. E19-mediated inhibition of antigen presentation could not be attributed to diminished levels of K^d or L^d expression at the cell surface. Rather, biochemical experiments revealed that the effect of E19 on antigen presentation paralleled its effect on class I transport from the ER. Experiments in which cells were co-infected with E19-VV and the K^d-VV recombinant discussed above provided additional confirmation that E19 prevents the surface expression of newly synthesized K^d. These findings were important for conclusively establishing that presentation of protein antigens processed from the cytosol requires the transport of newly synthesized class I molecules from the ER. Finally, note that E19-VV and other VV recombinants containing extrinsic genes that alter antigen processing or presentation could prove valuable in assessing the role of T_C in host immunity to VV.

6.5 Characterization of Vaccinia Virus Gene Products That Influence Antigen Processing

As we noted in Sect. 4.1.2.8, VV itself can interfere with the presentation of some determinants to T_C. This phenomenon was initially reported by COUPAR et al. (1986a) who noted that cells infected with VV recombinants expressing the influenza HA under the control of the VV 11K late promoter (extrinsic gene transcription only following VV DNA replication) were poorly recognized by anti-influenza T_C. This was not due to insufficient production of HA since T_C recognized the same APCs infected with a VV recombinant producing HA at lower levels but under control of the VV PF early promoter (extrinsic gene transcription only until VV DNA replication commences). The VV blockade of antigen presentation was subsequently characterized in more detail (TOWNSEND et al. 1988). To summarize the conclusions of this study: (a) The blockade is not specific for individual proteins per se, but for individual determinants. For a protein with two determinants restricted by different class I alleles, presentation of one might be severely inhibited while the other is unaffected. The blockade is not class I allele specific, however, since determinants from other proteins recognized in association with the blockade-associated allele are not affected; (b) The blockade can occur for extrinsic antigens under the control of early promoters but is more complete when gene products are synthesized under the control of late VV promoters; and (c) The blockade can be partially or completely bypassed by expressing forms of the proteins that are rapidly degraded. This includes a protein produced from a chimeric gene consisting of the ubiquitin gene fused to the amino terminus of an influenza NP gene whose amino terminal

methionine codon is altered to a lysine codon. Based on these findings the authors speculated that the VV blockade might be related to either inhibition of class I synthesis or to production of an inhibitor of antigen fragmentation. The first possibility is unlikely since we have found that protein synthesis inhibitors do not prevent presentation of the influenza HA in the same APCs used by COUPAR et al. 1986a (YEWDELL and BENNINK, unpublished results). Furthermore, VV does not inhibit the synthesis of class I molecules in these cells (Cox et al. 1990). Direct experimental evidence for the second possibility has not been reported, but it is consistent with the recent discovery that VV encodes at least three proteins whose amino acid sequence places them squarely within the serine protease inhibitor family (KOTWAL and MOSS 1989; PICKUP et al. 1986).

7 Concluding Remarks

VV recombinants have played a central role in moving the study of T lymphocyte recognition of foreign antigens from its phenomenological roots to the firmer ground of molecular and cellular biology. Like so many other advances in science, this is an unexpected benefit from basic research into an apparently unrelated field. Immunologists are indebted to the cadre of virologists who doggedly maintained their interest in poxviruses despite the eradication of smallpox.

Looking ahead, it is clear that VV recombinants will continue to be used to characterize the specificity of T_C and to dissect the molecular basis for the processing and presentation of antigens to T_C. Increasingly, they will be used to study similar aspects of T_H biology. They will also serve as an essential tool in a more complicated problem: sorting out the relative contributions of subsets of immune cells to immunity and recovery from disease.

As useful as they have proven to be, VV recombinants are not perfect expression vectors for probing the specificity and function of T lymphocytes or for the induction of protective cellular immunity, for that matter. For these purposes, it would be advantageous to engineer new VVs that express the minimal amount of VV genes required for expression of extrinsic antigens. A first step in this direction could be a VV that did not display the block in antigen presentation discussed in Sect. 6. Perhaps it might even be possible to create a VV that was not cytopathic. Although we hesitate to advance heretical ideas in this volume devoted to pox-viruses, it is possible that other viral- or DNA-based vectors that alter the metabolism of APCs less severely than VV might eventually supplant VV as vectors for immunological studies. So pox virologists may rest only briefly on their laurels if they wish to maintain their virtual monopoly on expression vectors for studies of cellular immunology.

Acknowledgments. We have had the good fortune of collaborating with Geoff Smith (Cambridge University, Cambridge, England) and Bernie Moss (National

Institute of Allergy and Infectious Diseases, Bethesda, MD), who produced all of the VV recombinants that enabled our studies of T_C recognition of individual influenza virus proteins.

References

Allen PM, Unanue ER (1984) Differential requirements for antigen processing by macrophages for lysozyme-specific T cell hybridomas. J Immunol 132: 1077–1079

Andersson M, Paabo S, Nilsson T, Peterson PA (1985) Impaired intracellular transport of class I MHC antigen as a possible means for adenoviruses to evade immune surveillance. Cell 43: 215–222

Andrew ME, Coupar BE (1988) Efficacy of influenza haemagglutinin and nucleoprotein as protective antigens against influenza virus infection in mice. Scand J Immunol 28: 81–85

Andrew ME, Coupar BE, Ada GL, Boyle DB (1986) Cell-mediated immune responses to influenza virus antigens expressed by vaccinia virus recombinants. Microb Pathog 1: 443–452

Andrew ME, Coupar BE, Boyle DB, Ada GL (1987) The roles of influenza virus haemagglutinin and nucleoprotein in protection: analysis using vaccinia virus recombinants. Scand J Immunol 25: 21–28

Ashman RB, Mullbacher A (1979) A T helper cell for anti-viral cytotoxic T cell responses. J Exp Med 150: 1277–1282

Askonas BA, Mullbacher A, Ashman RB (1982) Cytotoxic T-memory cells in virus infection and the specificity of helper T cells. Immunology 45: 79–84

Auperin DD, Esposito JJ, Lange JV, Bauer SP, Knight J, Sasso DR, McCormick JB (1988) Construction of a recombinant vaccinia virus expressing the Lassa virus glycoprotein gene and protection of guinea pigs from a lethal Lassa virus infection. Virus Res 9: 233–248

Babbitt BP, Allen PM, Matsueda G, Haber E, Unanue ER (1985) Binding of immunogenic peptides to Ia histocompatibility molecules. Nature 317: 359–361

Barrett T, Belsham GJ, Subbarao SM, Evans SA (1989) Immunization with a vaccinia recombinant expressing the F protein protects rabbits from challenge with a lethal dose of rinderpest virus. Virology 170: 11–18

Bastin J, Rothbard J, Davey J, Jones I, Townsend A (1987) Use of synthetic peptides of influenza nucleoprotein to define epitopes recognized by class I-restricted cytotoxic T lymphocytes. J Exp Med 168: 1935–1939

Bennink JR, Yewdell JW (1988) Murine cytotoxic T lymphocyte recognition of individual influenza virus proteins: high frequency of nonresponder MHC class I alleles. J Exp Med 168: 1935–1939

Bennink JR, Yewdell JW, Gerhard W (1982) A viral polymerase involved in recognition of influenza virus-infected cells by a cytotoxic T-cell clone. Nature 296: 75–76

Bennink JR, Yewdell JW, Smith GL, Moller C, Moss B (1984) Recombinant vaccinia virus primes and stimulates influenza haemagglutinin-specific cytotoxic T cells. Nature 311: 578–579

Bennink JR, Yewdell JW, Smith GL, Moss B (1986) Recognition of cloned influenza virus hemagglutinin gene products by cytotoxic T lymphocytes. J Virol 57: 786–791

Bennink JR, Yewdell JW, Smith GL, Moss B (1987) Anti-influenza virus cytotoxic T lymphocytes recognize the three viral polymerases and a nonstructural protein: responsiveness to individual viral antigens is major histocompatibility complex controlled. J Virol 61: 1098–1102

Bernards R, Destree A, McKenzie S, Gordon E, Weinberg RA, Panacali D (1987) Effective tumor immunotherapy directed against an oncogene-encoded product using a vaccinia virus vector. Proc Natl Acad Sci USA 84: 6854–6858

Berzofsky JA, Cease KB, Cornette JL, Spouge JL, Margalit H, Berkower IJ, Good MF, Miler LH, Delisi C (1987) Protein antigenic structures recognized by T cells: potential applications to vaccine design. Immunol Rev 98: 9–52

Biddison WE, Sharrow SO, Shearer GM (1981) T cell subpopulations required for the human cytotoxic T lymphocyte response to influenza virus: evidence for T cell help. J Immunol 127: 487–491

Bjorkman PJ, Saper MA, Samraoui B, Bennet WS, Strominger JL, Wiley DC (1987a) Structure of the human class I histocompatibility antigen, HLA-A2. Nature 329: 506–512

Bjorkman PJ, Saper MA, Samraoui B, Bennet WS, Strominger JL, Wiley DC (1987b) The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigen. Nature 329: 512–518

Blancou J, Kieny MP, Lathe R, Lecocq JP, Pastoret PP, Soulebot JP, Desmettre P (1986) Oral vaccination of the fox against rabies using a live recombinant vaccinia virus. Nature 322: 373–375

Blancou J, Artois M, Brouchier B, Thomas I, Pastoret PP, Desmettre P, Lanquet B, Kieny MP (1989) Safety and efficacy of an antirabies vaccine consisting of recombinant vaccinia-rabies virus administered orally to fox, dog and cat. Ann Rech Vet 20: 195–204

Braciale TJ (1977) Immunologic recognition of influenza virus-infected cells. I. Generation of a virus-strain specific and a cross-reactive subpopulation of cytotoxic T cells in the response to type A influenza viruses of different subtypes. Cell Immunol 33: 423–436

Braciale TJ, Braciale VL, Henkle TJ, Sambrook J, Gething MJ (1984) Cytotoxic T lymphocyte recognition of the influenza hemagglutinin gene product expressed by DNA-mediated gene transfer. J Exp Med 159: 341–354

Braciale TJ, Braciale VL, Winkler M, Stroynowski I, Hood L, Sambrook J, Gething MJ (1987) On the role of the transmembrane anchor sequence of influenza hemagglutinin in target cell recognition by class I MHC-restricted, hemagglutinin-specific cytolytic T lymphocytes. J Exp Med 166: 678–692

Braciale TJ, Sweetser MT, Morrison LA, Kittlesen DJ, Braciale VL (1989) Class I major histocompatibility complex-restricted cytolytic T lymphocytes recognize a limited number of sites on the influenza hemagglutinin. Proc Natl Acad Sci USA 86: 277–281

Bray M, Zhao BT, Markoff L, Eckels KH, Chanock RM, Lai CJ (1989) Mice immunized with recombinant vaccinia virus expressing dengue 4 virus structural proteins with or without nonstructural protein NS1 are protected against fatal dengue virus encephalitis. J Virol 63: 2853–2856

Brochier BM, Languet B, Blancou J, Kieny MP, Lelocq JP, Costy F, Desmettre P, Pastoret PP (1988) Use of recombinant vaccinia-rabies virus for oral vaccination of fox cubs (*Vulpes vulpes*) against rabies. Vet Microbiol 18: 103–108

Brochier BM, Blancou J, Aubert MF, Kieny MP, Desmettre P, Pastoret PP (1989) Interaction between rabies infection and oral administration of vaccinia-rabies recombinant virus to foxes (*Vulpes vulpes*). J Gen Virol 70: 1601–1604

Brown JH, Jardetzky T, Saper MA, Samraoui B, Bjorkman PJ, Wiley DC (1988) A hypothetical model of the foreign antigen binding site of class II histocompatibility molecules [published erratum appears in Nature 333: 786] Nature 332: 845–850

Burgert HG, Kvist S (1985) An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigens. Cell 41: 987–997

Burgert H-G, Maryanski JL, Kvist S (1987) "E3/19" protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigens. Proc Natl Acad Sci USA 84: 1356–1360

Buus S, Colon S, Smith C, Freed JH, Miles C, Grey HM (1986) Interaction between a "processed" ovalbumin peptide and Ia molecules. Proc Natl Acad Sci USA 83: 3968–3971

Buus S, Sette A, Colon SM, Miles C, Grey HM (1987) The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunologic peptides. Science 235: 1353–1358

Cambridge G, Mackenzie JS, Keast D (1976) Cell-mediated immune response to influenza virus infections in mice. Infect Immun 13: 36–43

Cantin EM, Eberle R, Baldick JL, Moss B, Willey DE, Notkins AL, Openshaw H (1987) Expression of herpes simplex virus 1 glycoprotein B by a recombinant vaccinia virus and protection of mice against lethal herpes simplex virus 1 infection. Proc Natl Acad Sci USA 84: 5908–5912

Chambers TM, Kawaoka Y, Webster RG (1988) Protection of chickens from lethal influenza infection by vaccinia-expressed hemagglutinin. Virology 167(2): 414–421

Chen BP, Parham P (1989) Direct binding of influenza peptides to class I HLA molecules. Nature 337: 743–745

Clegg JC, Llyod G (1987) Vaccinia recombinant expressing Lassa-virus internal nucleocapsid protein protests guinea pigs against Lassa fever. Lancet 2: 186–188

Clertant P, Kieny MP, Lecocq JP, Guizani I, Chambon P, Cuzin F, Lathe R (1988) Recombinant polyoma-vaccinia viruses: T antigen expression vectors and anti-tumor immunization agents. Biochimie 70: 1075–1087

Connolly JM, Potter TA, Wormstall EM, Hansen TH (1988) The Lyt-2 molecule recognizes residues in the class I alpha-3 domain in allogeneic cytotoxic T cell responses. J Exp Med 168: 325–341.

Corcoran LM, Metcalf D, Edwards SJ, Handman E (1988) GM-CSF produced by recombinant vaccinia virus or in GM-CSF transgenic mice has no effect in vivo on murine cutaneous leishmaniasis. J Parasitol 74: 763–767

Coupar BEH, Andrew ME, Both BW, Boyle DB (1986a) Temporal regulation of influenza hemagglutinin expression in vaccinia virus recombinants and effects on the immune response. Eur J Immunol 16: 1479–1487

Coupar BEH, Andrew ME, Boyle DB, Blanden RV (1986b) Immune response to H-2K^d antigen expressed by recombinant vaccinia virus. Proc Natl Acad Sci USA 83: 7879–7882

Cox JH, Yewdell JW, Eisenlohr LC, Johnson PR, Bennink JR (1990) Antigen presentation requires transport of MHC class I molecules from the endoplasmic reticulum. Science 247: 715–718

Cremer K, Wohlenberg C, Mackett M, Moss B, Notkins AL (1985a) Infectious vaccinia virus recombination express herpes simplex virus glycoprotein D and protect against lethal and latent infections of HSV. In: Quinnan GV (ed) Vaccinia viruses as vectors for vaccine antigens. Elsevier, New York, pp 153–162

Cremer KJ, Mackett M, Wohlenberg C, Notkins AL, Moss B (1985b) Vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D prevents latent herpes in mice. Science 228: 737–740

De BK, Shaw MW, Rota PA, Harmon MW, Esposito JJ, Rott R, Cox NJ, Kendal AP (1988) Protection against virulent H5 avian influenza virus infection in chickens by an inactivated vaccine produced with recombinant vaccinia virus. Vaccine 6: 257–261

Deubel V, Kinney RM, Esposito JJ, Cropp CB, Vorndam AV, Monath TP, Trent DW (1988) Dengue 2 virus envelope protein expressed by a recombinant vaccinia virus fails to protect monkeys against dengue. J Gen Virol 69: 1921–1929

Dietzschold B, Wiktor TJ, Koprowski H (1985) Induction of virus neutralizing antibodies and protection against rabies using a vaccinia rabies glycoprotein recombinant. In: Quinnan GV (ed) Vaccinia viruses as vectors for vaccine antigens. Elsevier, New York, pp 163–167

Doyle C, Strominger JL (1987) Interaction between CD4 and class II MHC molecules mediates cell adhesion. Nature 330: 256–259

Drillien R, Spehner D, Kirn A Giraudon P, Buckland R, Wild F, Lecocq J-P (1988) Protection of mice from fatal measles encephalitis by vaccination with vaccinia virus recombinants encoding either the hemagglutinin or the fusion protein. Proc Natl Acad Sci USA 85: 1252–1256

Earl PL, Moss B, Morrison RP, Wehrly K, Nishio J, Chesebro B (1986) T-lymphocyte priming and protection against Friend leukemia by vaccinia-retrovirus env gene recombinant. Science 234: 728–731

Effros R, Doherty PC, Gerhard W, Bennink J (1977) Generation of both cross-reactive and virus-specific T-cell populations after immunization with serologically distinct influenza A viruses. J Exp Med 145: 557–568

Eisenlohr LC, Hackett CJ (1989) Class II major histocompatibility complex-restricted T cells specific for a virion structural protein that do not recognize exogenous influenza virus. Evidence that presentation of labile T cell determination is favored by endogenous antigen synthesis. J Exp Med 169: 921–931

Eisenlohr LC, Gerhard W, Hackett CJ, (1987) Role of receptor-binding activity of the viral hemagglutinin molecule in the presentation of influenza virus antigens to helper T cells. J Virol 61: 1375–1383

Elango N, Prince GA, Murphy BR, Venkatesan S, Chanock RM, Moss B (1986) Resistance to human respiratory syncytial virus (RSV) infection induced by immunization of cotton rats with a recombinant vaccinia virus expressing the RSV G glycoprotein. Proc Natl Acad Sci USA 83: 1906–1910

Ennis FA (1982) Some newly recognized aspects of resistance against and recovery from influenza. Arch Virol 73: 207–215

Esposito J, Brechling K, Baer G, Moss B (1987) Vaccinia virus recombinants expressing rabies virus glycoprotein protect against rabies. Virus Genes 1: 7–21

Esposito JJ, Knight JC, Shaddock JH, Novembre FJ, Baer GM (1988) Successful oral rabies vaccination of raccoons with raccoon poxvirus recombinants expressing rabies virus glycoprotein. Virology 165: 313–316

Estin CD, Stevenson US, Plowman GD, Hu SL, Sridhar P, Hellstrom I, Brown JP, Hellstrom KE (1988) Recombinant vaccinia virus vaccine against the human melanoma antigen p97 for use in immunotherapy. Proc Natl Acad Sci USA 85: 1052–1056

Fisher-Hoch SP, McCormick JB, Auperin D, Brown BG, Castor M, Perez G, Ruo S, Conaty A, Brammer L, Bauer S (1989) Protection of rhesus monkeys from fatal Lassa fever by vaccination with a recombinant vaccinia virus containing the Lassa virus glycoprotein gene. Proc Natl Acad Sci USA 86: 317–321

Fischetti VA, Hodges WM, Hruby DE (1989) Protection against streptococcal pharyngeal colonization with a vaccinia: M protein recombinant. Science 244: 1487–1490

Fleisher B, Becht H, Rott R (1985) Recognition of viral antigens by human influenza A virus-specific T lymphocyte clones. J Immunol 135: 2800–2804

Flexner C, Hugin A, Moss B (1987) Prevention of vaccinia virus infection in immunodeficient mice by vector-directed IL-2 expression. Nature 330: 259–262

Flexner C, Murphy BR, Rooney JF, Wohlenberg C, Yuferow V, Notkins, AL, Moss B (1988) Successful vaccination with a polyvalent live vector despite existing immunity to an expressed antigen. Nature 335: 259–262

Gotch F, Rothbard J, Howland K, Townsend A, McMichael A (1987) Cytotoxic T lymphocytes recognized a fragment of influenza virus matrix protein in association with HLA-A2. Nature 326: 881–882

Gould KC, Cossins J, Bastin J, Brownlee GG, Townsend ARM (1989) A 15 amino acid fragment of influenza nucleoprotein synthesized in the cytoplasm is presented to class I-restricted cytotoxic T lymphocytes. J Exp Med 170: 1051–1056

Hanlon CA, Ziemer EL, Hamir AN, Rupprecht CE (1989) Cerebrospinal fluid analysis of rabid and vaccinia-rabies glycoprotein recombinant, orally vaccinated raccoons (*Procyon lotor*). Ann J Vet Res 50: 364–367

Hany M, Oehen S, Schulz M, Hengartner H, Mackett M, Hishop DHL, Overton H, Zinkernagel RM (1989) Anti-viral protection and prevention of lymphocytic choriomeningitis or of the local footpad swelling reaction in mice by immunization with vaccinia-recombinant virus expressing LCMV-WE nucleoprotein or glycoprotein. Eur J Immunol 19: 417–424

Hedrick S, Germain RN, Bevan MJ Dorf M, Engel I, Fink P, Gascoigne N, Heber-Katz E, Kapp J, Kauffmann Y, Kaye J, Melchers F, Pierce C, Schwartz RH, Sorenson C, Taniguchi M, Davis MM (1985) Rearrangement and transcription of a T-cell receptor beta-chain gene in different T-cell subsets. Proc Natl Acad Sci USA 82: 531–535

Hioe CE, Hinshaw VS (1989) Induction and activity of class II-restricted, Lyt-2 + cytolytic T lymphocytes specific for the influenza H5 hemagglutinin. J Immunol 142: 2482–2488

Hu SL, Fultz PN, McClure HM, Eichberg JW, Thomas EK, Zarling J, Singhal MC, Kosowski SG, Swenson RB, Anderson DC, Todaro G (1987) Effect of immunization with a vaccinia-HIV env recombinant on HIV infection of chimpanzees. Nature 328: 721–723

Hu SH, Plowman GD, Sridhar P, Stevenson US, Brown JP, Estin CD (1988) Characterization of a recombinant vaccinia virus expressing human melanoma-associated antigen p97. J Virol 62: 176–180

Jacobson S, Sekaly RP, Jacobson CL, McFarland HF, Long EO (1989) HLA class II-restricted presentation of cytoplasmic measles virus antigens to cytotoxic T cells. J Virol 63: 1756–1762

Jakeman KJ, Smith H, Sweet C (1989) Mechanism of immunity to influenza: maternal and passive neonatal protection following immunization of adult ferrets with a live vaccinia-influenza virus haemagglutinin recombinant but not with recombinants containing other influenza virus proteins. J Gen Virol 70: 1523–1531

Jonjic S, del Val M, Keil GM, Reddehase MJ, Koszinowski UH (1988) A nonstructural viral protein expressed by a recombinant vaccinia virus protects against lethal cytomegalovirus infection. J Virol 62: 1653–1658

Kane KP, Vitiello A, Sherman LA, Mescher MF (1989) Cytolytic T-lymphocyte response to isolated class I H-2 proteins and influenza peptides. Nature 340: 157–159

Kaplan DR, Griffith R, Braciale VL, Braciale TJ (1984) Influenza virus specific human cytotoxic T cell clones: heterogeneity in antigen specificity and restriction by class II products. Cell Immunol 188: 193–206

Kees U, Krammer PH (1984) Most influenza A virus-specific memory cytotoxic T lymphocytes react with antigenic epitopes associated with internal virus determinants. J Exp Med 159: 365–377

Kieny MP, Lathe R, Drillien R, Spehner D, Skory S, Schmitt D, Wiktor T, Koprowski H, Lecocq JP (1984) Expression of rabies virus glycoprotein from a recombinant vaccinia virus. Nature 312: 163–166

Kieny MP, Blancou J, Lathe R, Pastoret PP, Soulebot JP, Desmettre P, Lelocq JP (1988) Development of animal recombinant DNA vaccine and its efficacy in foxes. Rev Infect Dis [Suppl 4] 10: S799–802

King AM, Stott EJ, Langer SJ, Young KK, Ball LA, Wertz GW (1987) Recombinant vaccinia viruses carrying the N gene of human respiratory syncytial virus: studies of gene expression in cell culture and immune response in mice. J Virol 61: 2885–2890

Kinney RM, Esposito JJ, Mathews JH, Johnson BJ, Roehrig JJ, Barrett ADT, Trent DW (1988) Recombinant vaccinia virus/Venezuelan equine encephalitis (VEE) virus protects mice from peripheral VEE virus challenge. J Virol 62: 4697–4702

Klein J (1986) Natural history of the major histocompatibility complex. Wiley, New York

Klein JR, Raulet DH, Pasternack MS, Bevan MJ (1982) Cytotoxic T lymphocytes produce immune interferon in response to antigen or mitogen. J Exp Med 155: 1198–

Koszinowski UH, Reddehase MJ, Keil GM, Volkmer H, Jonjic S, Messerle M, del Val M, Mutter W, Munch K, Buhler B (1987) Molecular analysis of herpesviral gene products recognized by protective cytotoxic T lymphocytes. Immunol Lett 16: 185–192

Kotwal GJ, Moss B (1989) Vaccinia virus encodes two proteins that are structurally related to members of the plasma serine protease inhibitor superfamily. J Virol 63: 600–606

Lamb RA (1989) Genes and proteins of the influenza viruses. In: Krug RM (ed) The Influenza Viruses. Plenum Press, New York, pp 1–87

Lathe R, Kieny MP, Gerlinger P, Clertant P, Guizani I, Cuzin F, Chambon P (1987) Tumor prevention and rejection with recombinant vaccinia. Nature 326: 878–880

Lukacher AE, Morrison LA, Braciale VL, Malissen B, Brachiale TJ (1985) Expression of specific cytolytic activity by H-2I region-restricted influenza virus specific T lymphocyte clones. J Exp Med 162: 171–187

Mackett M, and Smith GL (1986) Vaccinia virus expression vectors. J Gen Virol 67: 2067–2082

Mackett M, Smith GL, Moss B (1985a) The construction and characterization of vaccinia virus recombinants expressing foreign genes. In: Glover DM (ed) DNA cloning: a practical approach, vol II. IRL, Oxford, pp 191–211

Mackett M, Yilma T, Rose JK, Moss B (1985b) Vaccinia virus recombinants: expression of VSV genes and protective immunization of mice and cattle. Science 227: 433–435

Marchioli CC, Yancey RJ Jr, Petrovskis EA, Timmins JG, Post LE (1987) Evaluation of pseudorabies virus glycoprotein gp50 as a vaccine for Aujeszky's disease in mice ans swine: expression by vaccinia virus and Chinese hamster ovary cells. J Virol 61: 3977–3982

Martin S, Rouse BT (1987) The mechanisms of antiviral immunity induced by a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: clearance of local infection. J Immunol 138: 3431–3437

Martin S, Cantin E, Rouse BT (1988) Cytotoxic T lymphocytes. Their relevance in herpesvirus infections. Ann NY Acad Sci 532: 257–272

Martin S, Cantin E, Rouse BT (1989) Evaluation of antiviral immunity using vaccinia virus recombinants expressing cloned genes for herpes simplex virus type 1 glycoproteins. J Gen Virol 70: 1359–1570

Miller RA, Reiss CS (1984) Limiting dilution cultures reveal latent influenza virus-specific helper T cells in virus-primed mice. J Mol Cell Immunol 1: 357–368

Moll H, Elchmann K, Simon MM (1985) Immunoregulation by mouse T-cell clones. II. The same H-Y-specific clones can provide help for the generation of cytotoxic lymphocytes and antibody-secreting cells. Immunology 54: 255–264

Moore MW, Carbone FR, Bevan MJ (1988) Introduction of soluble protein into the class I pathway of antigen processing and presentation. Cell 54: 777–785

Morgan AJ, Mackett M, Finerty S, Arrand JR, Sculion FT, Epstein MA (1988) Recombinant vaccinia virus expressing Epstein-Barr virus glycoprotein gp340 protects cottontop tamarins against EB virus-induced malignant lymphomas. J Med Virol 25: 189–195

Morris AG, Lin YL and Askonas BA (1982) Immune interferon release when a cloned cytotoxic T-cell line meets its correct influenza-infected target cell. Nature 295: 150–152

Morrison LA, Lukacher AE, Braciale VL, Fan D, Braciale TJ (1986) Differences in antigen presentation of MHC class I and class II-restricted influenza A virus-specific cytolytic T lymphocyte clones. J Exp Med 163: 903–921

Morrison RP, Earl PL, Nishio J, Lodmell DL, Moss B, Chesebro B (1987) Different H-2 subregions influence immunization against retrovirus and immunosuppression. Nature 329: 729–732

Morrison HG, Bauer SP, Lange JV, Esposito JJ, McCormic JB, Auperin DD (1989) Protection of guinea pigs from Lassa fever by vaccinia virus recombinants expressing the nucleoprotein or the envelope glycoprotein of Lassa virus. Virology 171: 179–188

Moss B, Smith GL, Gerin JL, Purcell RH (1984) Live recombinant vaccinia virus protects chimpanzees against hepatitis B. Nature 311: 67–69

Murphy BR, Olmsted RA, Collins PL, Chanock RM, Prince GA (1988) Passive transfer of respiratory syncytial virus (RSV) antiserum suppresses the immune response to the RSV fusion (F) and large (G) glycoprotein expressed by recombinant vaccinia viruses. J Virol 62: 3907–3910

Nuchtern JG, Bonifacino JS, Biddison WE, Klausner RD (1989) Brefeldin A implicates egress from endoplasmic reticulum in class I restricted antigen presentation. Nature 339: 223–226

Olmsted RA, Flango N, Prince GA, Murphy BR, Johnson PR, Moss B, Chanock R, Collins PL (1986) Expression of the F glycoprotein of respiratory syncytial virus by a recombinant vaccinia virus: comparison of the individual contributions of the F and G glycoproteins to host immunity. Proc Natl Acad Sci USA 83: 7462–7466

Olmsted RA, Buller RM, Collins PL, London WT, Beeler JA, Prince GA, Chanock RM, Murphy BR (1988) Evaluation in non-human primates of the safety, immunogenicity and efficacy of recombinant vaccinia viruses expressing the F or G glycoprotein of respiratory syncytial virus. Vaccine 6: 519–524

Olmsted RA, Murphy BR, Lawrence LA, Elango N, Moss B, Collins PL (1989) Processing surface expression, and immunogenicity of carboxy-terminally truncated mutants of G protein of human respiratory syncytial Virol. J Virol 63: 411–420

Paabo S, Weber F, Kampe O, Schaffner W, Peterson PA (1983) Association between transplantation antigens and a viral membrane protein synthesized from a mammalian expression vector. Cell 35: 445–453

Paabo S, Weber F, Nilsson T, Schaffner W, Peterson P (1986) Structural and functional dissection of a MHC class I antigen binding adenovirus glycoprotein. EMBO J 5: 1921–1927

Paabo S, Bhat BM, Wold WSM, Peterson PA (1987) A short sequence in the COOH- terminus makes an adenovirus membrane glycoprotein a resident of the endoplasmic reticulum. Cell 50: 311–317

Pala P, Askonas BA (1986) Low responder MHC alleles for Tc recognition of influenza nucleoprotein. Immunogenetics 23: 379–384

Pala P, Townsend ARM, Askonas BA (1986) Viral recognition by influenza A virus cross-reactive cytotoxic T (Tc) cells: the proportion of Tc cells that recognize nucleoprotein varies between individual mice. Eur J Immunol 16: 193–198

Paoletti E, Lipinskas BR, Samsonoff C, Mercer S, Panicali D (1984) Construction of live vaccines using genetically engineered poxviruses: biological activity of vaccinia virus recombinants expressing the hepatitis B virus surface antigen and the herpes simplex virus glycoprotein D. Proc Natl Acad Sci USA 81: 193–197

Patterson RG, Lamb RA, Moss B, Murphy BR (1987) Comparison of the relative roles of the F and HN surface glycoproteins of the paramyxovirus simian virus 5 in inducing protective immunity. J Virol 61: 1972–1977

Paul WE (1984) Fundamental immunology. Raven, New York

Piccini A, Paoletti E (1988) Vaccinia: virus, vector, vaccine. Adv Virus Res 34: 43–64

Pickup DJ, Ink BS, Hu W, Ray CA, Joklik WK (1986) Hemorrhage in lesions caused by cowpox virus is induced by a viral protein that is related to plasma protein inhibitors of serine proteases. Proc Natl Acad Sci USA 81: 6817–6821

Pierce RJ, Balloul JM, Grzych JM, Dissou C, Auriault C, Boulanger D, Capron M, Soundermeyer P, Lecocq JP, Capron A (1987) GP38, P28-I and P28-II candidates for a vaccine against schistosomia. Mem Inst Oswaldo Cruz 82 [Suppl 4]: 111–114

Ramshaw IA, Andrews ME, Phillips SM, Boyle DB, Coupar BEH (1987) Recovery of immunodeficient mice from a vaccinia virus/IL-2 recombinant infection. Nature 329: 545–546

Rawle FC, Tollefson AE, Wold WSM, Gooding LR (1989) Mouse anti-adenovirus cytotoxic T lymphocytes: inhibition of lysis by E3 gp19 K but not E3 14.7 K. Immunol 143: 2031–2037

Reay PA, Jones IM, Gotch FM, McMichael AJ, Brownlee GG (1989) Recognition of the PB1, neuraminidase, and matrix proteins of influenza virus A/NT/60/68 by cytotoxic T lymphocytes. Virol 170: 477–485

Reiss CS, Burakoff SJ (1981) Specificity of the helper T cell for the cytolytic T lymphocyte response to influenza viruses. J Exp Med 154: 541–546

Rooney JF, Wohlenberg C, Cremer KJ, Moss B, Notkins AL (1988) Immunization with a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: long-term protection and effect of revaccination. J Virol 62: 1530–1534

Rota PA, Shaw MW, Kendal AP (1987) Comparison of the immune response to variant influenza type B hemagglutinins expressed in vaccinia virus. Virology 161: 269–275

Rota PA, Shaw MW, Kendal AP (1989) Cross-protection against microvariants of influenza virus type B by vaccinia viruses expressing haemagglutinins from egg- or MDCK cell-derived subpopulations of influenza virus type B/England/222/82. J Gen Virol 70: 1533–1537

Rupprecht CE, Wiktor TJ, Johnston DH, Hamir AN, Dietzschold B, Wunner WH, Glickman LT, Koprowski H (1986) Oral immunization and protection of raccoons (*Procyon lotor*) with a vaccinia-rabies glycoprotein recombinant virus vaccine. Proc Natl Acad Sci USA 83: 7947–7950

Rupprecht CE, Hamir AN, Johnston DH, Koprowski H (1988) Efficacy of a vaccinia-rabies glycoprotein recombinant virus vaccine in raccoons (*Procyon lotor*). Rev Infect Dis 10: S803–809

Russell SM, Liew FY (1979) T cells primed by influenza virion internal components can cooperate in the antibody response to hemagglutinin. Nature 280: 147–148

Scherle PA, Gerhard WU (1986) Functional analysis of influenza-specific helper T cell clones in vivo. T cells specific for internal viral proteins provide cognate help for B cell responses to hemagglutinin. J Exp Med 164: 1114–1129

Scherle PA, Gerhard WU (1988) Differential ability of B cells specific for external vs. internal influenza virus proteins to respond to help from influenza virus-specific T-cell clones in vivo. Proc Natl Acad Sci USA 85: 4446–4450

Schwartz RH (1986) Immune response (Ir) genes of the murine major histocompatibility complex. Adv Immunol 38: 31–201

Shida H, Tochikura T, Sato T, Konno T, Hirayoshi K, Ito Y, Hatanaka M, Hinuma Y, Sugimoto M, Takahasi-Nishimaki F, Maruyama T, Miki K, Suzuki K, Morita M, Sashiyama H, Yoshimura N, Hayami M (1987) Effect of the recombinant vaccinia viruses that express HTLV-I envelope gene on HTLV-I infection. EMBO J 6: 3379–3384

Signas C, Katze MG, Persson H, Philipson L (1982) An adenovirus glycoprotein binds heavy chains of class I transplantation antigens from man and mouse. Nature 299: 175–178

Small PA, Smith GL, Moss B (1985) Intranasal vaccination with recombinant vaccinia containing influenza hemagglutinin prevents both influenza virus pneumonia and nasal infection: intradermal vaccination prevents only viral pneumonia. In: Quinnan GV (ed) Vaccinia viruses as vectors for vaccine antigens. Elsevier, New York, pp 175–177

Smith GL, Murphy BR, Moss B (1983) Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. Proc Natl Acad Sci USA 80: 7155–7159

Smith GL, Levin JZ, Palese P, Moss B (1987) Synthesis and cellular location of the ten influenza polypeptides individually expressed by recombinant viruses. Virology 160: 336–345

Spriggs MK, Murphy BR, Prince GA, Olmsted RA, Collins PL (1987) Expression of the F and HN glycoproteins of human parainfluenza virus type 3 by recombinant vaccinia viruses: contributions of the individual proteins to host immunity. J Virol 61: 3416–3423

Spriggs MK, Collins PL, Tierney E, London WT, Murphy BR (1988) Immunization with vaccinia virus recombinants that express the surface glycoproteins of humans parainfluenza virus type 3 (PIV3) protects patas monkeys against PIV3 infection. J Virol 62: 1293–1296

Scott EJ, Ball LA, Young KK, Furze J, Wertz GW (1986) Human respiratory syncytial virus glycoprotein G expressed from a recombinant vaccinia virus vector protects mice against live-virus challenge. J Virol 60: 607–613

Stott EJ, Taylor G, Ball LA, Anderson K, Young KKY, King AMQ, Wertz GW (1987) Immune and histopathological responses in animals vaccinated with recombinant vaccinia viruses that express individual genes of human respiratory syncytial virus. J Virol 61: 3855–3861

Tanaka Y, Tevethia SS (1988) Differential effect of adenovirus 2 E3/19 K glycoprotein on the expression of H-2K^b- and H-2D^b-restricted SV40-specific CTL-mediated lysis. Virology 165: 357–366

Tolson ND, Charlton KM, Stewart RB, Campbell JB, Wiktor TJ (1987) Immune response in skunks to a vaccinia virus recombinant expressing the rabies virus glycoprotein. Can J Vet Res 51: 363–366

Tolson ND, Charlton KM, Casey GA, Knowles MK, Rupprecht CE, Lawson KF, Campbell JB (1988) Immunization of foxes against rabies with a vaccinia recombinant virus expressing the rabies glycoprotein. Arch Virol 102: 297–301

Townsend ARM, McMichael AJ, Carter NP, Haddleston JA, Brownlee GG (1984) Cytotoxic T cell recognition of the influenza virus nucleoprotein, hemagglutinin expressed in transfected mouse L cells. Cell 39: 13–25

Townsend ARM, Gotch FM, Davey J (1985) Cytotoxic T cells recognize fragments of the influenza nucleoprotein. Cell 42: 457–467

Townsend A, Bastin J, Gould K, Brownlee G (1986a) Cytotoxic T lymphocytes recognize influenza hemagglutinin lacking a signal sequence. Nature 324: 575–577

Townsend ARM, Rothbard J, Gotch FM, Bahadur G, Wraith D, McMichael AJ (1986b) The epitopes of influenza nucleoprotein recognized by cytotpxic T lymphocytes can be defined with short peptides. Cell 44: 959–968

Townsend A, Bastin J, Gould K, Brownlee G, Andrew M, Coupar B, Boyle D, Chan S, Smith G (1988) Defective presentation to class I-restricted cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen J Exp Med 168: 1211–1224

Townsend ARM, Ohlen C, Bastin J, Ljunggren H, Foster L, Karre K (1989) Association of class I major histocompatibility heavy and light chains induced by viral peptides. Nature 340: 443–448

Van Eendenburg JP, Yagello M, Girard M, Kieny MP, Lelocq JP, Muchmore E, Fultz PN, Riviere Y, Montagnier L, Gluckman JC (1989) Cell-mediated immune proliferative responses to HIV-1 of chimpanzees vaccinated with different vaccinia recombinant viruses. AIDS Res Hum Retroviruses 5: 41–50

Wachsman M, Aurelian L, Smith CC, Lipinskas BR, Perkus ME, Paoletti E (1987) Protection of guinea pigs form primary and recurrent herpes simplex virus (HSV) type 2 cutaneous disease with vaccinia virus recombinants expressing HSV glycoprotein D. J Infect Dis 155: 1188–1197

Wachsman M, Aurelian L, Hunter JC, Perkus ME, Paoletti E (1988) Expression of herpes simplex virus glycoprotein D on antigen presenting cells infected with vaccinia recombinants and protective immunity. Biosci Rep 8: 323–334

Wagner H, Starzinski-Powitz A, Jung H, Rollinghoff M (19877) Induction of I-region restricted hapten-specific cytotoxic T lymphocytes. J Immunol 119: 1365–1368

Watts TH, Brian AA, Kappler J, Marrack P, McConnel HM, (1984;) Antigen presentation by supported planar membranes containing affinity-purified I-A^d. Proc Natl Acad Sci USA 81: 7564–7568

Webster RG, Reay PA, Laver WG (1988) Protection against lethal influenza with neuraminidase. Virology 164: 230–237

Weiss S, Bogen B (1989) B-lymphoma cells process and present their endogenous immunoglobulin to major histocompatibility complex-restricted T cells. Proc Natl Acad Sci USA 86: 282–286

Wertz GW, Stott EJ, Young KK, Anderson K, Ball LA (1987) Expression of the fusion protein of human respiratory syncytial virus from recombinant vaccinia virus vectors and protection of vaccinated mice. J Virol 61: 293–301

Whitton JL, Oldstone MBA (1989) Class I MHC can present an endogenous peptide to cytotoxic T lymphocytes. J Exp Med 170: 1033–1038

Wiktor TJ, Macfarlan RI, Reagan KJ, Dietzschold B, Curtis P, Wunner WH, Kieny MP, Lathe R, Lecocq JP, Mackett M, Moss B, Koprowski H (1984) Protection from rabies by a vaccinia virus recombinant containing the rabies virus glycoprotein gene. Proc Natl Acad Sci USA 81: 7194–7198

Willey DE, Cantin EM, Hill LR, Moss B, Notkins AL, Openshaw H (1988) Herpes simplex virus type 1-vaccinia virus recombinant expressing glycoprotein B: protection from acute and latent infection. J Infect Dis 158: 1382–1386

Wold WS, Cladaras C, Deutscher, SL, Kapoor QS (1985) The 19 kDa glycoprotein coded by region E3 of adenovirus. J Biol Chem 260: 2421–2431

Wysocka M, Bennink JR (1988) Limiting dilution analysis of memory cytotoxic T lymphocyte specific for individual influenza virus gene products. Cell Immunol 112: 425–429

Wysocka M, Hackett CJ (1990) Class I H-2^d-restricted cytotoxic T lymphocytes recognize the neuraminidase glycoprotein of influenza virus subtype N1. J Virol 64: 1028–1030

Yewdell JW, Bennink JR (1989) Brefeldin A specifically inhibits presentation of protein antigens to cytotoxic T lymphocytes. Science 244: 1072–1075

Yewdell JW, Hackett CJ (1989) The specificity and function of T lymphocytes induced by influenza A viruses. In: Krug R (ed) The influenza viruses, Plenum, New York.

Yewdell JW, Bennink JR, Smith GL Moss B (1985) Influenza A virus nucleoprotein is a major target antigen for crossreactive anti-influenza A virus cytotoxic T lymphocytes. Proc Natl Acad Sci USA 82: 1785–1789

Yewdell JW, Bennink JR, Mackett M, Lefrancois L, Lyles DS, Moss B (1986) Recognition of cloned vesicular stomatitis virus internal and external gene products by cytotoxic T lymphocytes. J Exp Med 163: 1529–1538

Yewdell JW, Bennink JR, Hosaka Y (1988) Cells process exogenous proteins for recognition by cytotoxic T lymphocytes. Science 239: 637–640

Yilma T, Hsu D, Jones L, Owens S, Grubman M, Mebus C, Yamanaka M, Dale B (1988) Protection of cattle against rinderpest with vaccinia virus recombinants expressing the HA or F gene. Science 242: 1058–1061

Zinkernagel RM, Doherty PC (1979) MHC-restricted cytotoxic T cells. Adv Immunol 27: 51–77

Zweerink HJ, Courtneidge SA, Skehel JJ, Crumpton MJ, Askonas BA (1977) Cytotoxic T cells kill influenza virus infected cells but do not distinguished between serologically distinct type A viruses. Nature 267: 354–356

Posttranslational Modification of Vaccinia Virus Proteins

J. K. VANSLYKE and D. E. HRUBY

1 Introduction

1.1 Vaccinia Life Cycle

The major features of the replication cycle of vaccinia virus (VV) are outlined in Fig. 1 (MOSS 1974). For the sake of this discussion, note that expression of the viral genetic program can be subdivided into two distinct major phases, early and late, which are delineated by replication of the viral DNA molecule. Prior to DNA synthesis, early VV genes, representing about one-half of the viral genetic potential, are transcribed by enzymes and factors present within the incoming virion into distinct 5′-capped/3′-polyadenylated mRNAs, which encode a variety of enzymatic activities including those involved in nucleotide metabolism and DNA replication. Once the VV early genes have been expressed, the core particle disintegrates, liberating the viral DNA which is then replicated within large

Department of Microbiology, Centre for Gene Research, Oregon State University, Corvallis, OR 97331-3804, USA

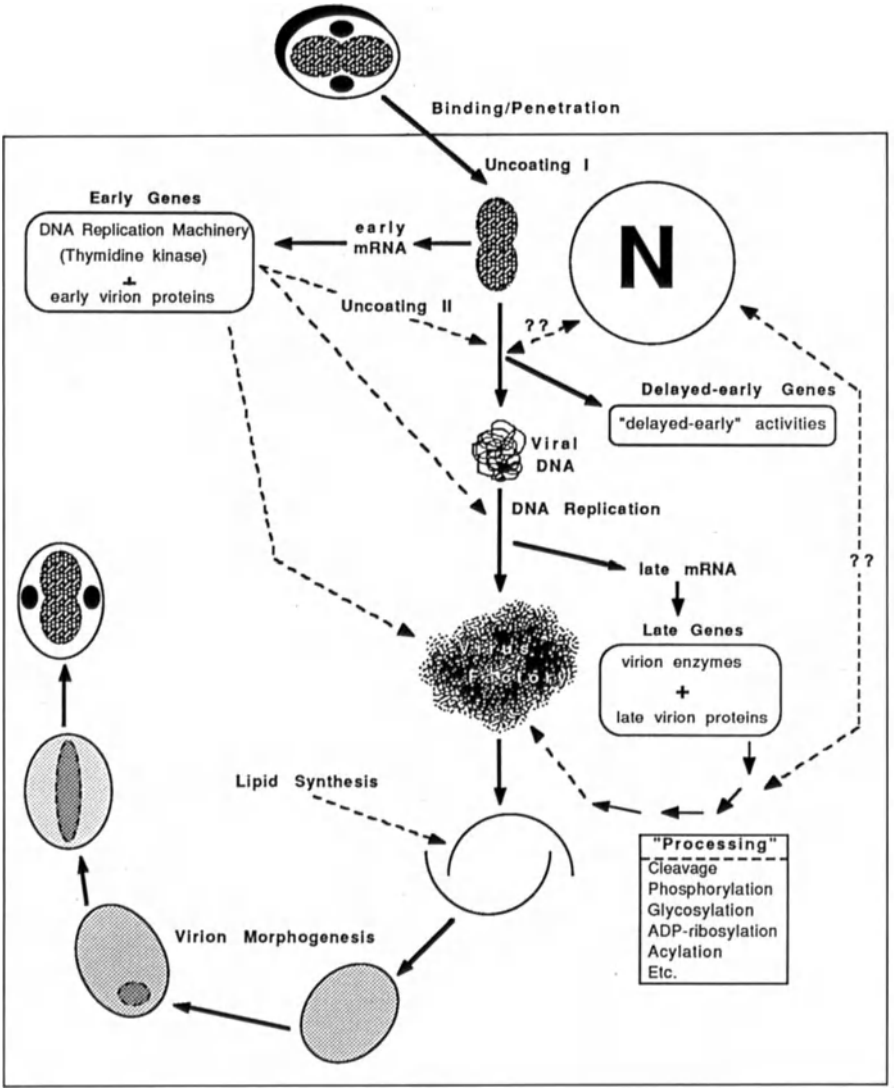


Fig. 1. Vaccinia virus replication strategy. The events that take place during infection of a host cell that lead to production of mature vaccinia virions

cytoplasmic inclusion bodies known as "virosomes" or "virus factories". The virosome, which consists of large aggregates of viral protein and catenated VV DNA, is also the site of subsequent assembly of immature viral particles. Concomitant with the onset of viral DNA replication, the transcription of early genes is attenuated and the expression of VV late genes is initiated. VV late RNAs have two unique structural features, namely, no distinct 3′-ends and the presence of a short (30 nt) 5′-poly(A) leader (Weir and Moss 1984; Schwer et al. 1987). The

mechanisms which are responsible for the transcriptional specificity of VV early and late genes, i.e., *cis*- and *trans*-acting elements, are not yet clearly understood although this area is currently the subject of intense research interest (BROYLES et al. 1988; MINER and HRUBY 1989; WILSON et al. 1988). It is sufficient to say that the information available at present indicates that poxvirus transcriptional regulatory mechanisms are substantially different from other, previously characterized, eukaryotic systems (HANGGI et al. 1986). The VV late RNA is translated into approximately 100 different polypeptides including enzymes and a number of structural proteins which are apparently processed by posttranslational modification prior to participating in the multistep process of virion morphogenesis. Although the general features of the VV life cycle are evident, a molecular understanding of how this highly regulated complex process occurs in vivo remains to be accomplished.

1.2 Vaccinia Virus As a Model System

On the basis of the discussion above, it should be obvious that in many respects VV represents an ideal model eukaryotic system with which to investigate a variety of questions pertaining to development, assembly of macromolecular structures, virus-host cell interactions, DNA replication, and the regulation of gene expression. In particular, a study of the basic mechanisms used to regulate the expression of a complex genetic program would appear to be most appropriate to the VV system since this virus apparently encodes more than 200 viral genes whose expression is tightly regulated in a temporal fashion during the viral replicative cycle (WITTEK 1982; ESSANI and DALES 1979; MOSS 1985). As an experimental system, VV provides a number of significant advantages for this type of study. First, the linear 185 kbp DNA genome of the virus contains approximately 90% unique sequences (BAROUDY et al. 1982). This renders the molecule functionally haploid and makes it a convenient substrate for both classical and directed genetic approaches (BERTHOLET et al. 1986; CONDIT and MOTYCZKA 1981; see also CONDIT and NILES, this volume). Second, the genomic DNA has been cloned and is available in a variety of plasmid and bacteriophage libraries. At the present time, about 60% of the VV genome has been sequenced, and it is reasonable to assume that the rest will be completed in the very near future (VII International Poxvirus/Iridovirus Workshop). Having the sequence of most of the viral genome available obviously facilitates the identification, excision, and manipulation of individual genes. Third, the methodologies of marker transfer (NAKANO et al. 1982) and transient expression (COCHRAN 1985) have been developed. This allows reverse genetics to be used to specifically alter the nucleotide sequence of viral genes (or the amino acid sequence of their encoded gene products). The biological activity of the mutated genes can then be assessed by recombination back into the viral genome (marker transfer) or expressed within the context of the infected cell (transient expression). This approach is now being used to undertake detailed structure-function studies of

VV genes of known function, such as thymidine kinase (HRUBY and BALL 1981; WILSON et al. 1989). Finally, unlike other DNA-containing animal viruses (e.g., adenovirus or herpesvirus), VV does not replicate within the nucleus of infected cells (PENNINGTON and FOLLET 1974). VV apparently completes most, if not all, of its replicative cycle within the cytoplasmic compartment of infected cells. This necessitates that VV contain or encode many of the enzymes and regulatory factors required to replicate the viral genome and to express and modify viral gene products since cognate cellular enzymes are primarily located in the nuclear compartment and/or do not interact with viral substrates (MOSS 1985). Thus, in theory, using VV as a model system, one should have ready access to viral genes encoding essential enzymatic and structural functions and to the factors which regulate or modulate their expression in vivo.

1.3 Regulation of Gene Expression

In eukaryotic organisms, regulation of gene expression can be exerted at a number of different levels including: chromatin structure; DNA methylation; gene copy number; transcriptional initiation; RNA processing, transport, and stability; translation; posttranslational modifications; and assembly of individual monomers into active macromolecular structures (Fig. 2; CREIGHTON 1984) Since VV expresses its genetic program within the confines of a host cell, one would presume that many of these same mechanisms will be employed by the virus. The only VV gene which has been examined in any detail with regard to

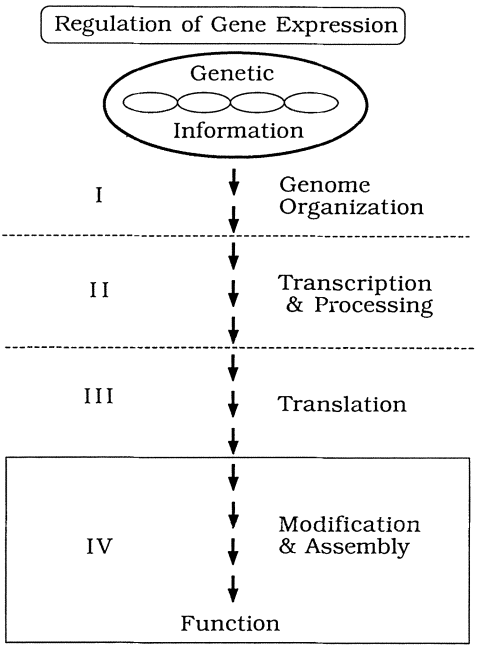


Fig. 2. Levels of regulation of gene expression during vaccinia's replicative cycle

these questions is the VV thymidine kinase gene (HRUBY and BALL 1981; HRUBY et al. 1983). As predicted, evidence has been obtained to indicate that the expression of thymidine kinase activity in VV-infected cells is complex, being regulated at the levels of transcription, translation, RNA turnover, and feedback inhibition (HRUBY 1985; WILSON et al. 1989). Most of the current research efforts into understanding the important elements of VV gene regulation are focused on the process of transcription (ROSEL et al. 1986; ROHRMAN et al. 1986; YUEN et al. 1987; MARS and BEAUD 1987; BROYLES et al. 1988; MINER and HRUBY 1989); however, it is likely that host cell factors, protein-protein interactions, and posttranslational modifications may prove to be equally important features of the overall regulatory scheme employed by VV.

1.4 Posttranslational Modification of Proteins

The spectrum of the different types of potential posttranslational modifications which can occur is virtually endless. In general, protein modifications can occur before, during, or after polymerization of amino acids into peptide chains. Moreover, modifications can occur on tRNA-associated amino acids (amino acylated tRNAs), on amino acids associated with ribosomes in a growing amino acid polymer, or on the polymerized chain after translation has been completed (WOLD 1983). This review will be limited to considering primarily those posttranslational modifications which occur after polymerization, which is the most widely accepted definition of "posttranslational". Some of the more common themes in protein modification are proteolytic processing, modification of the amino or carboxyl termini, glycosylation, hydroxylation of prolines or lysines, iodination, covalent addition of non-amino acid groups, phosphorylation, methylation, ADP-ribosylation and disulfide linkages (CREIGHTON 1984).

A variety of posttranslational modifications of VV polypeptides have already been reported, including proteolytic cleavage (MOSS and ROSENBLUM 1973), glycosylation and phosphorylation (GARON and MOSS 1971; MOSS et al. 1971; ROSEMOND and MOSS 1973), ADP-ribosylation (CHILD et al. 1988), and acylation (HILLER and WEBER 1985; FRANKE et al. 1989). It has become evident that posttranslational modifications can play an important regulatory role in biological systems. For example, with regard to ADP-ribosylation, the addition and/or removal of this prosthetic group from substrate proteins is known to be involved in a large number of essential reactions including: activation-inactivation of enzymes such as adenylate cyclase (LEFKOWITZ 1983) and transducin (STRYER 1983), proteins which participate in signal transduction pathways; the switch-on switch-off of DNA topoisomerase I (FERRO and OLIVERA 1984); and regulation of gene expression in general (RASTL and SWETLY 1978). In eukaryotic systems, however, our mechanistic understanding of posttranslational modifications is limited with regard to the manner in which the correct sites of modification are selected, the enzymology of the reactions, the regulation of substrate/enzyme interaction, and the effect of the modification on the

structure and function of the substrate protein. This is clearly ample justification for a detailed molecular genetic examination of modified VV proteins. First, based on the biological precedents, such as those described above, these reactions are likely to play an important role in the viral life cycle. Second, given the many advantages of VV as a model eukaryotic system it should be possible to carry out defined and detailed structural studies of VV substrate proteins, and perhaps the enzymes which modify them, in order to address some of the basic questions which are of general interest and importance in all biological systems.

The goal of this contribution is to provide an overview of what is known concerning the posttranslational modifications of VV proteins within the context of: (a) regulation of viral gene expression and (b) structure-function relationships of viral encoded proteins. It is hoped that, by reviewing different post-translational modifications which occur during the VV replicative cycle, some new and perhaps different insights into viral morphogenesis will be obtained. At the same time, it is hoped that a better understanding of the substrate proteins that are subjected to these posttranslational modifications might lead to predictions of the purpose of these modifications and the functions of modified proteins.

2 Proteolytic Processing

Many proteins are synthesized initially in a precursor or inactive form, referred to as the "pro-protein", and subsequently cleaved to expose active forms. This cleavage event can be either a single-step or multi-step process and can involve both endo- or exoproteolytic enzymes. Proteolysis is responsible for the post-translational removal of the initiating methionine from most proteins and for clipping the leader sequences that serve as a signal for membrane translocation of membrane-bound or secreted proteins. Furthermore, proteolytic processing of polyproteins is intimately involved in a number of other important pathways such as the production of functional neuropeptides or the genomic expression of poliovirus (CREIGHTON 1984). Along this latter line, proteolytic maturation of polio virus structural proteins from larger molecular weight precursor polypeptides during virion morphogenesis is a common theme in many viral life cycles (KRAUSSLICH and WIMMER 1988; WELLINK and VAN KAMMEN 1988). In addition, most of the responsible proteases are virally encoded and are themselves synthesized as precursors, inactive until a cleavage event activates their proteolytic activity (CREIGHTON 1984).

The genes expressed at late times during a VV infection (after DNA synthesis) include most of the structural proteins required for assembly of progeny virions. The first indication that VV structural proteins might be subject to proteolytic processing occurred when HOLOWCZAK and JOKLIK (1967) noted differences in the apparent molecular weights of the radioactively-labeled proteins present in

VV-infected cells compared to those found in purified virions. Following this initial observation, pulse-labeling of VV-infected cells was used to demonstrate that a precursor protein with an estimated molecular weight of 125 kDa could be "chased" to a smaller 76 kDa VV core polypeptide, with the concomitant disappearance of the larger sized protein (KATZ and MOSS 1970a). This phenomenon could be specifically inhibited by rifampicin with no apparent effect on the synthesis of the precursor. This precursor protein was subsequently designated as P4a and the proteolytically processed product called 4a (KATZ and MOSS 1970b). Additional pulse-chase experiments revealed that several other VV structural polypeptides, in addition to P4a, were apparently subject to cleavage during the late phases of the VV replication cycle (MOSS and ROSENBLUM 1973). These proteolytically processed proteins, referred to by the SAROV and JOKLIK (1972) designation of virion proteins, include 4a, 4b, 8, 9, and 10. This may in fact represent a conservative estimate of the number of VV late proteins which are produced by cleavage, as PENNINGTON (1974) reported that eleven proteins synthesized late in infection disappeared during pulse-chase experiments and seven new proteins appeared. 4a and 4b are the most abundant proteins in the VV particle, together constituting about 25% of the mass of the virion. Tryptic peptide mapping of P4a, 4a, P4b, and 4b has confirmed the nature of the precursor to product relationships (MOSS and ROSENBLUM 1973). The proteolytic processing of VV structural proteins is believed to be absolutely essential for the formation of infectious progeny virions. This conclusion is based on the fact that there are a variety of different drug treatments (e.g., rifampicin, α-amanitin) and conditional-lethal mutations in the VV genome which apparently inhibit proteolysis and subsequent virion assembly without affecting overall viral protein synthesis (MOSS and ROSENBLUM 1973; SILVER and DALES 1982; VILLARREAL et al. 1984). In contrast to the situation with VV late proteins, little is currently known concerning the status of proteolytic processing during the early phases of VV replication.

The recent analyses of the VV major structural proteins have centered around mapping and sequencing the VV genes that encode the precursor core polypeptides (WITTEK et al. 1984; ROSEL and MOSS 1985; VAN MEIR and WITTEK 1988). The precursor proteins, P4a and P4b, exhibit approximate molecular weights of 94 and 66 kDa, respectively, when subjected to denaturing gel electrophoresis, whereas the proteolytically processed products (4a and 4b) are estimated to be 62 and 60 kDa in size. The third major core protein, VP8, makes up approximately 7% of the VV virion and is undoubtedly encoded by the VV late gene mapped and sequenced by WEIR and MOSS (1985). This gene is believed to encode a 28 kDa precursor which is cleaved to a 25 kDa product found in purified virions. These three proteins and their precursor-product relationships can be easily identified using monospecific antisera in immunoblots of VV-infected cell extracts (Fig. 3). Using the predicted amino acid sequences derived from the DNA sequence of the respective open reading frames, N-terminal microsequencing has been employed to attempt to identify potential cleavage sites within each of the core protein precursors. YANG et al. (1989) reported the residues located at the N-terminus of

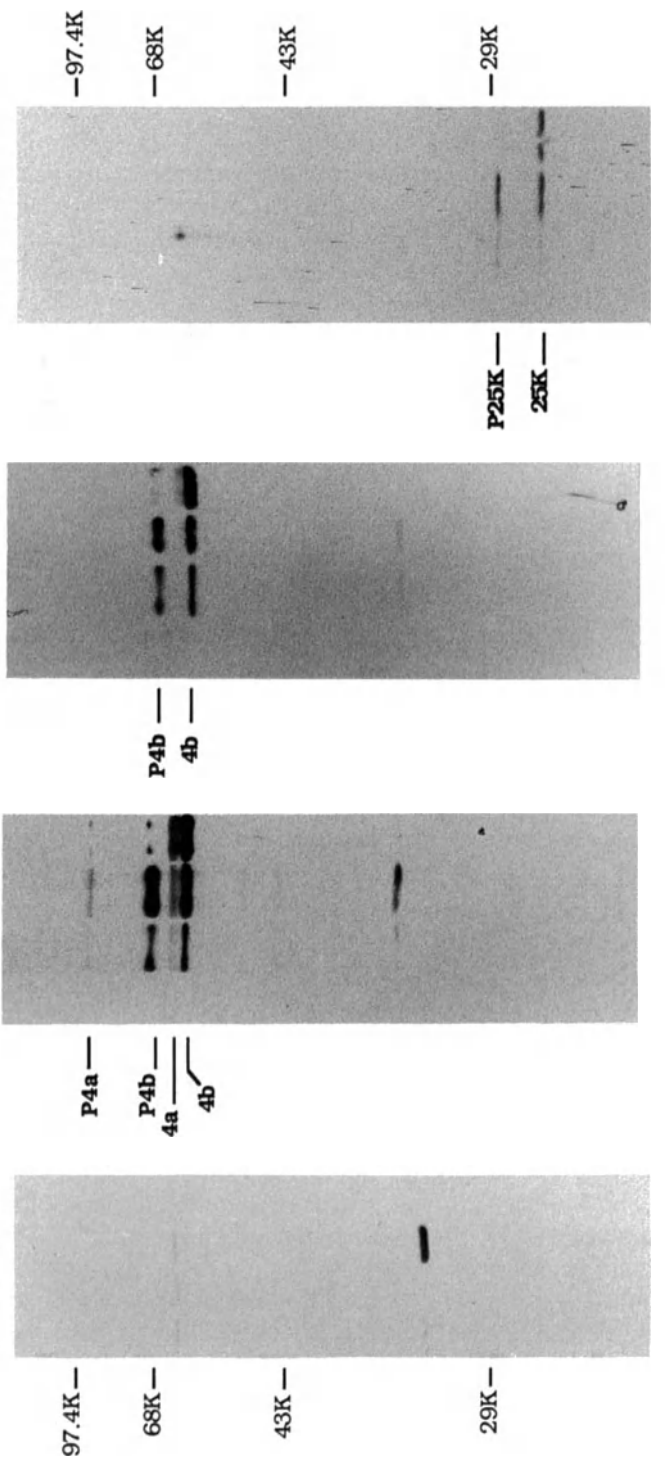


Fig. 3. Western blot analysis of infected cell extracts and purified virions with polyclonal antisera. Extracts of uninfected (*U*) and infected (*P* = harvested 8 hours post infection, *C* = harvested 12 hours post infection) BSC_{40} cells and denatured purified virion (*PV*) were immunoblotted with pre-immune serum and antiserum made against 4a, 4b and P25K. Antiserum to 4a recognizes 4a and 4b and anti-P25K was a gift from JP Weir

VP8 (or 25K) and aligned them with the precursor sequence, showing that the proteolyzed product had lost 32 amino acids from the N-terminus, apparently being cleaved between Gly-32 and Ala-33. These results have been confirmed and extended by VANSLYKE and HRUBY, (manuscript in preparation) who identified the N-terminal sequences of 25K and 4b. Alignment of the amino acids surrounding the potential cleavage sites in both proteins revealed a conserved Ala-Gly-Ala sequence at the N-termini, with cleavage apparently occurring between the glycine and alanine residues. Additional positions of identity and similarity which flanked the apparent cleavage site were also noted, and it is possible that these conserved residues constitute the "protease recognition signal". Interestingly, attempts thus far to determine the N-terminus of 4a by microsequencing have proved unsuccessful. Furthermore, a scan of the predicted P4a amino acid sequence revealed no "protease recognition signal". One interpretation of these data is that P4b and P25K may be processed by a different pathway and/or by different enzymes than is P4a. A schematic summary of the precursor-product relationships between the three major core proteins and what we know about their processing is shown in Fig. 4. Obviously, a great deal remains to be learned concerning the proteolytic pathways, about the enzymes which carry out the reactions, and the kinetics and subcellular localization of these events.

Several other nonstructural VV proteins that are secreted from the infected host cell also undergo proteolytic processing prior to relase into the surrounding medium. VV growth factor, a nonessential protein made early in vaccinia replication, is synthesized, glycosylated, integrated into the plasma membrane,

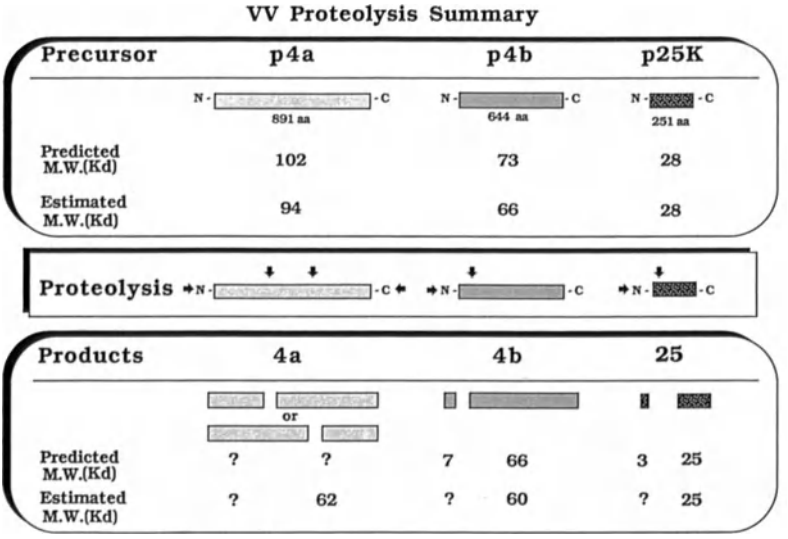


Fig. 4. Summary of proteolytic processing of vaccinia's three major core polypeptides, P4A, P4B, and P25K

proteolytically processed, and released. This cleavage process results in the loss of a signal sequence located near the N-terminus of the protein and of a proposed transmembrane spanning region near the C-terminus. The proteases responsible for these reactions may be viral or cellular in origin (STROOBANT et al. 1985; CHANG et al. 1988). Another secreted protein, with an apparent molecular weight of 19 kDa, was recently identified and shown to be missing 19 amino acid residues from the predicted N-terminus, suggesting proteolytic cleavage was involved in the removal of a signal sequence (KOTWAL and MOSS 1988). Hemagglutinin (HA) is a viral-encoded glycoprotein located in the extracellular envelope of VV particles and on the plasma membrane of infected cells (SHIDA and DALES 1981). As with many integral membrane proteins, HA contains a putative leader sequence (VON HEIJNE 1983) that is most likely cleaved after insertion into the phospholipid bilayer. This hypothesis is supported by the discrepancy between the molecular weights of HA synthesized in a cell-free system versus that expressed in an in vivo system. A protein with a slower mobility was synthesized in the reticulocyte lysate presumably due to the presence of the signal sequence (SHIDA 1986). Finally, an enzyme isolated from viral cores which has a DNA-dependent nicking-joining activity appears to be activated by proteolytic processing. This 50 kDa pronuclease is proposed to be cleaved to an active 44 kDa form, possible after virus penetration of infected cells (REDDY and BAUER 1989).

Evidence for a protease packaged within VV virions was reported by ARZOGLOU et al. (1979). When virus particles were disrupted under alkaline conditions, a proteolytic activity was detected, but no further information is available concerning the identity or function of this enzyme. A trypsin-like protease isolated from infected cells was able to uncoat VV in vitro (PEDLEY and COOPER 1987). This protease activity copurified with a protein of approximately 23 kDa, but its origin is unknown. Other than these preliminary reports, little is known concerning the protease(s) involved in proteolytic maturation of VV-encoded polypeptides. Based on the precedents from other viral systems, one would predict that at least some of these activities will prove to be specified by the virus.

3 Glycosylation

The covalent addition of carbohydrates to proteins is usually a modification associated with polypeptides that are destined to be membrane-associated or secreted. The sugar units are attached to the polypeptide backbone through an N- or O-linked bond, depending on whether linkage is through the amino group of an asparagine or the hydroxyl groups of serine or threonine. N-linked glycosylation usually occurs at a consensus site of Asn-X-(Ser/Thr), whereas O-linked glycosylation can apparently occur at any serine or threonine present in the amino acid sequence. N-linked glycosylation reactions initially occur on

newly synthesized polypeptides (or cotranslationally) at the site of protein synthesis, followed by attachment of more carbohydrates to preexisting sugars by glycosyltransferases usually found associated with the Golgi membranes in eukaryotic cells. These are the same enzymes responsible for O-linked glycosylation (CREIGHTON 1984).

The existence of virion and/or nonvirion VV glycoproteins was investigated initially by incubating VV-infected cells in media containing radioactively-labeled sugars and then analyzing the radiolabeled proteins by gel electrophoresis. A number of non-virion glycoproteins were found mainly associated with infected cell membranes, but whether these polypeptides were viral-encoded or viral-induced was not established (MOSS et al. 1971). By comparing the incorporation of labeled carbohydrates in VV-infected versus noninfected cells and noting that labeling was inhibited in the presence of actinomycin D and cycloheximide (RNA and protein synthesis inhibitors, respectively), evidence was obtained to suggest that the labeled proteins were viral in origin. Only a single glycoprotein was found associated with the virus membrane fraction which could be glycosylated with glucosamine, probably in N-acetylglucosamine units (GARON and MOSS 1971). Alternate labeling procedures, using radioactive mannose, galactose, or fucose, failed to label this 40 kDa protein. This polypeptide, when compared to SAROV and JOKLIK's designation (1972) of capsid proteins, is likely to represent VP6a, which has an estimated molecular weight of 41 kDa. GARON and MOSS (1971) suggested that the 40 kDa protein might represent a different, minor, structural protein migrating slower than 6a or that 6a is actually a mixture of differentially glycosylated forms. Along those lines, SAROV and JOKLIK (1972) reported that both 6a and 6b were glycopolypeptides, with the latter species exhibiting a molecular weight of 39 kDa. Both proteins were labeled with glucosamine, supporting the suggestion that they represent alternatively glycosylated forms of a single protein.

Certain combinations of VV strains and tissue culture cell lines result in the production of considerable amounts of extracellular enveloped virus (EEV). More often VV remains with the infected cell until it is harvested. This intracellular form is referred to as intracellular naked virus (INV) but does contain an envelope. EEV acquires a second membrane upon exiting the cell and is important in virus dissemination in an infected host animal (PAYNE 1980). By comparing the polypeptide composition of the intracellular and extracellular forms of VV, eight proteins were initially reported present in EEV but not in INV (PAYNE 1978). This count was later increased to ten, nine of which were identified as glycoproteins (labeled with glucosamine) and one 37 kDa protein showing no such modification (PAYNE 1979). These polypeptides ranged in size from 210 kDa to 20 kDa, including one 89 kDa protein identified as vaccinia HA.

It has been suggested that the glycosylation of HA might be important for antibody recognition and biological activity (WEINTRAUB et al. 1977). SHIDA and DALES (1981) carefully analyzed the nature of the carbohydrate moieties on this protein and showed them to be attached through a combination of N- and O-linkages. By using tunicamycin, an inhibitor of an intermediate in N-linked

glycosylation, HA was shown to be modified by approximately 75% N-linkages and 25% O-linkages. The 65 kDa form of HA, less its N-glycosidic attachments, was still biologically active, and therefore the O-linked carbohydrates seem to be most important to the protein's function. In addition to being a virion protein, this polypeptide is also found associated with the plasma membrane of the infected host cell. The gene encoding the VV HA protein has been mapped and sequenced. The predicted amino acid sequence contained five potential N-linked glycosylation sites and, interestingly, showed that 25% of the total amino acids were either serine or threonine residues, all potential sites for O-linked glycosylation reactions (SHIDA 1986).

Another VV polypeptide known to undergo glycosylation is the VV growth factor (VGF) which is secreted into the medium of infected tissue culture cells (STROOBANT et al. 1985). This protein undergoes obligatory proteolysis during its release from the cell. The pathway from the synthesis of the VGF precursor to the appearance of the mature product in the surrounding medium is apparently quite complex. From the predicted amino acid sequence of VGF, derived from the DNA sequence of its open reading frame (VENKATESAN et al. 1982), this protein has a predicted molecular weight of 15.5 kDa and contains two potential N-linked glycosylation sites at amino acids residues 34 and 96. In vivo the protein exhibits an estimated molecular weight closer to 23 kDa, along with a 28 kDa form believed to be the result of glycosylation. Treatment of the protein with endoglycosidase H and neuraminidase, which remove "high mannose type N-linked" carbohydrates and sialic acid, respectively, did not cause a change in migration of VGF on SDS-polyacrylamide gels. Endoglycosidase F, which removes the same components as the H form plus complex carbohydrates, increased the relative mobility of VGF. STROOBANT et al. (1985) subsequently proposed that VGF must be modified by N-glycosidic bridges to complex sugars. The released form of VGF is identified as a protein with an apparent 23 kDa molecular weight. CHANG et al. (1988) followed the posttranslational modifications of VGF closely using anti-VGF antiserum and showed that this protein is initially synthesized as a 19 kDa protein. It is then N-glycosylated with a high mannose type of sugar into a 22 kDa polypeptide still located inside the infected cell. Further glycosidic modifications are proposed to take place as mannose units are removed and complex oligopolysaccharides are attached, leading to a 25 kDa protein which becomes membrane-bound. This modified form then proceeds to undergo proteolysis, allowing its release into the surrounding medium where it exists again as a 22 kDa protein. CHANG et al. (1988) also found VGF to be sensitive to neuraminidase, indicating that, in addition to these steps, the protein is also sialated at some point.

One other VV protein thought to be a glycoprotein is the 14 kDa fusion polypeptide, which is apparently essential for the effective replication of VV and initial attachment of virus to host cell membranes prior to penetration (RODRIGUEZ and ESTEBAN 1987). This protein is located on the lipid membrane (formed *de novo* during vaccinia's life cycle) of virus particles and also in plasma membranes of VV-infected cells. This polypeptide has a potential N-linked

glycosylation site and a difference in predicted versus observed molecular weights, 12.5 kDa and 14 kDa, respectively. Whether glycosylation or possibly acylation is responsible for this mobility shift has not been firmly established.

4 Phosphorylation

A common mechanism of activating-deactivating enzymatic or binding functions of a protein is by phosphorylation, usually with ATP serving as the phosphate donor in a reaction mediated by a protein kinase. Phosphates are usually attached to the hydroxyl groups of serine and threonine, but tyrosine, histidine, and lysine can also serve as phosphate acceptors (CREIGHTON 1984).

The first report of phosphorylated VV proteins was published by SAROV and JOKLIK in a study of the capsid proteins of VV virions (1972). Seven labeled proteins were found in purified virus grown in the presence of radioactive orthophosphate, with most of the label associated with an 11 kDa protein complex. Three other proteins with approximate molecular weights of 46.5, 31.5, and 17 kDa were labeled to a lesser extent. Following the report of a protein kinase found packaged within VV virions, serious attention was given to phosphorylation of VV proteins as a form of posttranslational regulation (KLEIMAN and MOSS 1975a, b). The origin (viral or cellular) of this protein kinase has not been determined, but vaccinia acceptor proteins could be phosphorylated in vitro by partially purified kinase fractions when the reaction was supplemented with Mg^{2+}, ATP, and protamine. The VV acceptor proteins modified in this reaction had relative molecular weights of 38.5 and 11.7 kDa and therefore are thought to be different than the phosphoproteins described previously. The covalent attachment of phosphate groups to viral proteins has been suggested to participate in the inhibition of host cell protein synthesis and/or the activation of viral proteins after VV infection of a host cell. Evidence has been obtained showing that phosphorylated protein (VP11b) is associated with ribosomes in infected cells (SAGOT and BEAUD 1979). Another study of VV phosphoproteins labeled in vivo with radioactive orthophosphate and incorporated into mature virions demonstrated a phosphorylated protein with a molecular weight of 11–12 kDa which was associated primarily with the core of vaccinia virions and the site of phosphate attachment appeared to be at a serine residue (ROSEMOND and MOSS 1973).

Some confusion exists concerning the identity of the VV 11 kDa protein(s) which have been reported as being phosphorylated in VV-infected cells or able to be phosphorylated in vitro by protein kinase. HILLER and WEBER (1982) reported that an 11 kDa phosphoprotein (11K) was present on the surface of mature virions and associated with cellular actin-containing microfilaments in VV-infected host cells. This information suggested that this protein might be involved with transport of intracellular viruses. VP11, another 11 kDa phosphorylated

species, was identified and isolated from the cores of VV particles (KAO et al. 1981). This protein was initially investigated for its DNA-binding properties and has been proposed to be involved with DNA condensation within vaccinia virions. N-terminal sequencing of VP11 (KAO and BAUER 1987) indicates that it is a different protein than 11K, whose amino acid sequence was deduced after the genomic location of this gene was mapped and sequenced (WITTEK et al. 1984; BERTHOLET et al. 1985). POGO et al. (1975) described a 11 kDa phosphoprotein, rich in arginine residues, associated with virion cores and viral factories within infected cells. Another 11 kDa polypeptide located with the viral core was able to be phosphorylated by a thymidine kinase associated with VV (PERSON-FERNANDEZ and BEAUD 1986). Most of these proteins are basic in nature, but the relationships between these 11 kDa proteins remain to be established.

The most detailed molecular information about phosphorylation of a VV protein is from the investigation of VP11 (KAO and BAUER 1987). Through phosphoamino acid analysis, phosphate groups were shown to be attached to serine residues. Tryptic peptide mapping indicated there were two sites of phosphorylation. An estimated 85% of the total VP11 protein made was phosphorylated, leaving 15% that was not posttranslationally modified in this

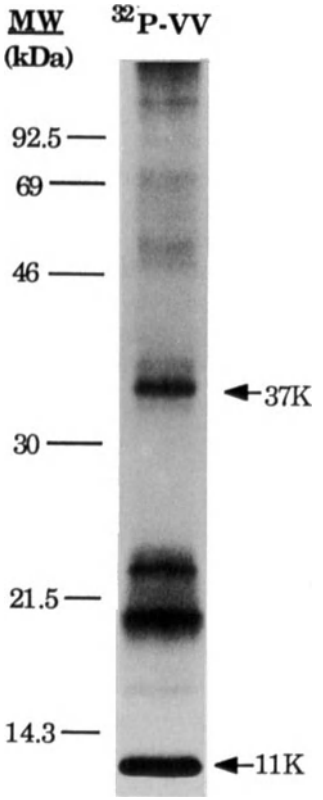


Fig. 5. Vaccinia virion proteins, labeled with radioactive orthophosphate and separated by SDS-polyacrylamide gel electrophoresis. A prominent 11 K protein is evident, along with other labeled proteins of varying molecular weights

manner. Analysis of purified virus indicated that only the phosphorylated form of the 11 kDa protein became associated with the virion core. Pulse-chase labeling experiments indicated the presence of non-, mono-, and diphosphorylated forms of VP11 in varying amounts at different times post infection. This led to the hypothesis that after this protein is synthesized late in VV infection, modification takes place first at one serine residue (either one) and then at the second residue. Interestingly, the 11K protein investigated by BERTHOLET et al. (1985) had a single phosphorylation site, occurring at the sequence Arg-Arg-Pro-Ser. This fits the criteria for recognition by cAMP-dependent protein kinases that phosphorylate at the serine residue in the sequence Arg-Arg-X-Ser, in which X is any amino acid (BERTHOLET et al. 1985).

Further analysis of phosphorylation of proteins is needed to determine exactly which viral proteins (structural and nonstructural) are modified, the biosynthetic pathway of this process, and the factors involved. The phosphorylation of one or more 11 kDa proteins is an established fact, but, as seen in Fig. 5, other less intensively labeled ^{32}P-labeled proteins are present in vaccinia virions. These could be phosphoproteins or ADP-ribosylated proteins, another form of posttranslational modification discussed below.

5 ADP-Ribosylation

Besides serving as an energy source through oxidation and reduction, NAD serves as an ADP-ribose donor in ADP-ribosylation reactions. This ADP-ribose moiety can be transferred to an appropriate protein or to a preexisting ADP-ribose attached to a protein, producing mono- or poly-ADP-ribosylated polypeptides. ADP-ribosyltransferases can attach the prosthetic group to an arginine, histidine, glutamate, aspartate, cysteine, lysine or dipthamide (modified histidine) within the peptide backbone. This modification leads to the activation of some enzymes (like adenylate cyclase and topoisomerase) but it can have inhibitory effects as well. For example, diphtheria toxin causes the ADP-ribosylation of elongation factor II with a resultant inhibition of protein synthesis (VAUGHAN and MOSS 1983; ALTHAUS and RICHTER 1987).

This form of posttranslational modification is relatively new to the poxvirus literature, but a recent analysis using [^{3}H]adenosine to label the proteins associated with purified virions suggested that eight VV proteins are ADP-ribosylated (CHILD et al. 1988). Several of these proteins comigrated with known VV proteins including the structural proteins 4a, 4b, and 25 kDa and a VV-encoded topoisomerase, characterized by SHAFFER and TRAKTMAN (1987); however, the verification of the identity of these putative ADP-ribosylated VV proteins remains to be experimentally established. Interestingly, in the presence of nicotinamide (an inhibitor of ADP-ribosylation) proteolytic processing of the VV structural proteins was inhibited, suggesting that this modification may be

linked in some as yet undefined manner to proteolysis. Information is currently unavailable concerning the acceptor sites in the VV acceptor proteins, the extent of ADP-ribosylation on these proteins (mono- or poly-ADP-ribosylated), and the enzymes (viral or cellular) responsible for this modification.

6 Acylation

Covalent attachment of fatty acid residues to polypeptides has received a great deal of attention recently because of the role of this modification in directing the correct localization of proteins to membranes. The acyl groups most often attached to proteins are myristic and palmitic acid with occasional addition of stearic, linoleic, and oleic acids. Palmitate is usually covalently attached via a thioester or ester bond to a cysteine or threonine found internally within the protein. Myristylation commonly occurs at an N-terminal glycine by an amide bond and occurs cotranslationally (SCHULTZ et al. 1988).

HILLER and WEBER (1985) have described an acylated protein associated with the outermost envelope present around the EEV forms of VV. This 37 kDa polypeptide (p37K) was labeled with radioactive palmitic acid when VV-infected cells were incubated in the presence of the tritiated fatty acid. Unlike many other acylated proteins, p37K is apparently not glycosylated. Most acylated proteins are membrane polypeptides, as is p37K, but the process by which it becomes associated with the VV extracellular envelope is unique. P37K is synthesized at late times post infection, presumably at the rough endoplasmic reticulum, and eventually accumulates at the membranes of Golgi bodies. Intracellular virus particles come in contact with and are surrounded by Golgi vesicles, already beginning to break up as a result of the VV infection. The vesicles surround the virions, leaving them enwrapped in a double Golgi membrane with the integrated p37K. At this time the virion essentially has three membranes or envelopes, but when the virus exits the cell, it does so by fusing with the plasma membrane, losing its outermost envelope. The extracellular form of vaccinia consists of a double membrane-wrapped virus, with p37K exposed on the surface. This protein is thought to be involved in membrane fusion events, but it also might be involved with recognition or penetration of host cells (PAYNE 1980).

Two other acylated VV structural proteins have been recently discovered which contain covalently attached myristic acid moieties (FRANKE et al. 1989). The two polypeptides, M25 and M35 (25 and 35 kD, respectively; Fig. 6), are associated with the intracellular virus particle's envelope and the virion core periphery, respectively. They both appear to be linked to their fatty acid group through an amide linkage, and they are distinct from the palmitated 37 kD protein previously described. It has been demonstrated that when the M25 and M35 proteins are labeled in vivo with [^{3}H]myristic acid and the acyl groups are analyzed by HPLC, although the proteins are initially modified by myristylation, a substantial proportion of the protein associated label becomes interconverted

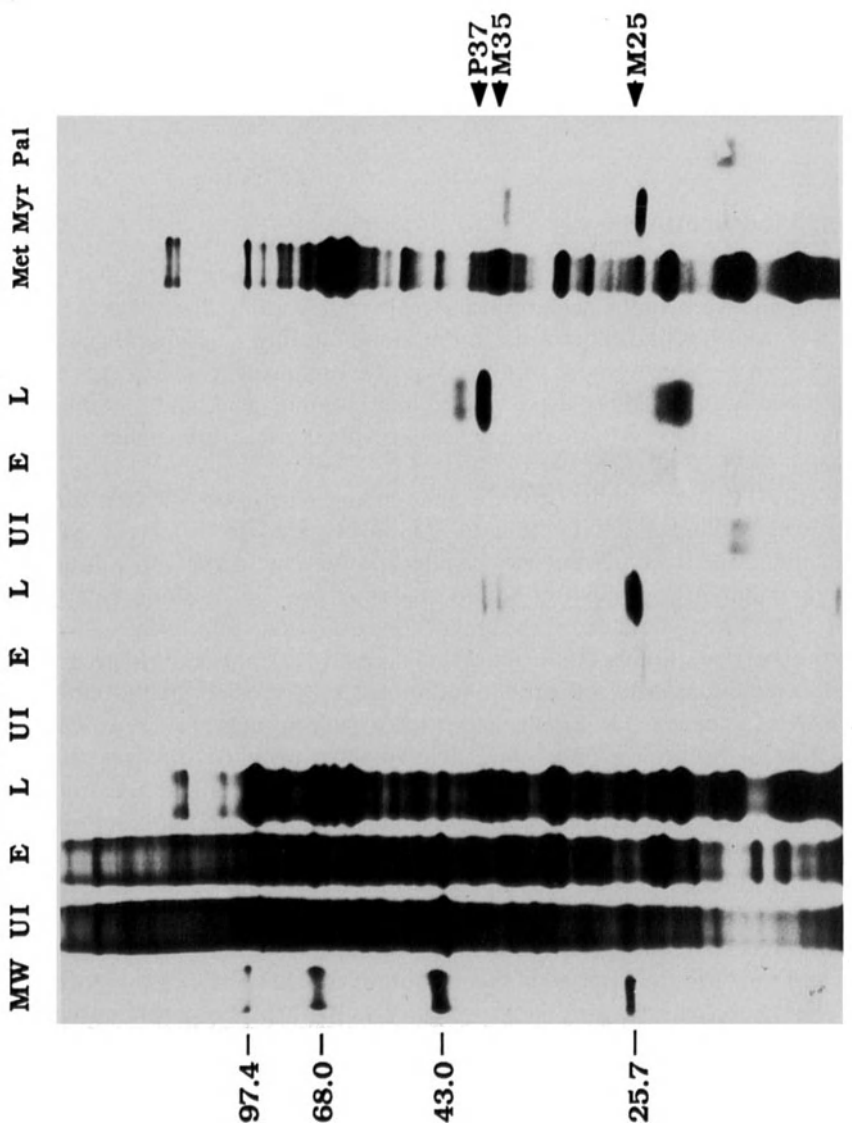


Fig. 6. Electrophoretic analysis of fatty acid acylated proteins. Proteins from uninfected (*UI*) and VV-infected cells and purified virions (*VV*) were labeled with ^{35}S-methionine (*Met*), ^{3}H-myristic acid (*Myr*), or ^{3}H-palmitic acid (*Pal*) at 0.5–2.5 h (*E* = early) and 4–8 h (*L* = late) post infection or for 24 h continuously for purified virions

to palmitate; however, there seems to be a marked preference for incorporation of the myristylated forms of M25 and M35 into mature VV virions. The biological significance of this observation and the identity of the M25 and M35 proteins are currently under investigation.

7 Other Modifications

Cross-linking between amino acids appears to play a role in stabilizing a protein, as it does with polypeptides involved in the blood clotting reaction. Disulfide bonds can form between cysteine residues, but other kinds of cross linkages are found between lysine residues, aspartic acid and glutamic acid, and glutamine and lysine. Disulfide linkage formation depends on the spatial arrangement of the two cysteine residues (CREIGHTON 1984).

ICHIHASHI (1981) has studied the disulfide-linkages between VV structural proteins using sodium dodecyl sulphate (SDS) as an anionic surfactant. SDS dissolves noncovalent bonds but not covalent bonds, including those found between the sulfhydryl groups on amino acids involved in disulfide bridges. Through a "sulfhydryl-disulfide exchange" reaction that employed various combinations of denaturants that react with different covalent bonds (hydrogen, disulfide), interactions between protein subunits as they occur in the virion structure were analyzed. The results indicated a varying degree of reactivity, meaning that some proteins are more able to form disulfide linkages than others. The characteristics of each of the numerous proteins investigated and the complexes they formed were intimately related to protein topology and conformation in the vaccinia virion. Using the protein nomenclature designated by OIE and ICHIHASHI (1981), a complex [VP54K (surface tubular element) + VP16] and VP37K (a glycoprotein) were found associated with the virion's lipid membrane. Likewise, VP25K can form homopolymers through disulfide linkages and can be found inserted in the membrane of virus particles along with VP29K. These proteins are also basic, which is thought to play a role in their association with phospholipids. VP57K and VP22K also form disulfide-linked polymers and are found in the core of virus particles along with polypeptides VP61K and VP27K. VP61K and VP27K tend to complex with proteins located on the surface of the virion, suggesting that there is an interaction between core and membrane polypeptides. The relationship between these structural proteins and those reported by other workers is unclear.

A recent report (RODRIGUEZ et al. 1987) on a 14 kDa fusion protein indicated that it existed as a disulfide-linked trimer on the surface of VV lipid membranes (of INV). The amino acid sequence of this protein contains two cysteine residues adjacent to each other that are thought to donate the sulfhydryl groups responsible for this bridge formation (RODRIGUZ and ESTEBAN 1987). This protein also appears to be important in cell fusion and virus penetration, potentially explaining its topological location.

It is highly likely that many other forms of posttranslational modifications which have yet to be described occur in VV proteins. Since this virus regulates such a vast array of proteins in a tightly controlled fashion, one would predict that it would take advantage of most, if not all, of the posttranslational regulatory mechanisms which operate in mammalian cells.

8 Conclusions

Vaccinia replicates in the cytoplasm of infected host cells in a very highly controlled manner, regulating the expression of its numerous genes at the level of genomic organization, transcription, translation, and posttranslational modifications. For the virus to successfully complete a replication cycle, certain gene products must appear at specific times and in specific quantities during infection. The ability to carry out all the necessary replicative events in the same cellular compartment undoubtedly requires the use of a variety of regulatory mechanisms. The posttranslational modifications reported thus far in the vaccinia literature presumbly help accomplish this task. Proteolytic processing, glycosylation, phosphorylation, ADP-ribosylation, acylation, and disulfide cross-linking have been reported to be involved in the expression of VV structural and nonstructural proteins. The viral core and membrane polypeptides appear to be most affected by these modifications. This is not surprising when one considers that the virion is thought to contain approximately 100 proteins (ESSANI and DALES 1979), accounting for about 50% of the viral-encoded genes. As more of the VV proteins, whatever their function, are studied and detailed information becomes available, it is likely that many of these factors will also be subject to posttranslational events and novel forms of modification.

In summary, our understanding of this aspect of regulation of gene expression is still fairly underdeveloped. The information presented here likely represents a small portion of the posttranslational modifications that exist in vivo. Also limited is our understanding of the extent to which these reactions are involved in controlling the appearance of functional polypeptides. Because VV is estimated to encode approximately 200 proteins, it is not surprising that the virus employs this form of regulation for effective viral replication. As approaches in research become more sophisticated, the wealth of knowledge about posttranslational modification(s) and the effects it can have on viral gene expression should increase.

Acknowledgments. We would like to thank Stephanie J. Child and Christine A. Franke for their scientific assistance. These studies were supported by a Research Career Development Award (A1-00666) and a Research Grant (AI-21335) to D.E.H. from the National Institutes of Health. J.K.V. was funded by a N. L. Tartar fellowship. This is Ag. Exp. Station Technical paper #8861.

References

Althaus FR, Richter C (1987) The bond. In: Solioz M (ed) ADP-Ribosylation of Proteins: Enzymology and Biological Significance. Springer-Verlag, Berlin Heidelberg, pp 216–226

Arzoglou P, Drillien R, Kirn A (1979) Evidence for an alkaline protease in vaccinia virus. Virology 95: 211–214

Baroudy BM, Venkatesan S, Moss B (1982) Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. Cell 28: 315–324

Bertholet C, Drillien R, Wittek R (1985) One hundred base pairs of 5′ flanking sequence of a vaccinia virus late gene are sufficient to temporally regulate late transcription. Proc Natl Acad Sci USA 82: 2096–2100

Bertholet C, Stocco P, Van Meir E, Wittek R (1986) Functional analysis of the 5′ flanking sequence of a vaccinia virus late gene. EMBO J 5: 1951–1957

Broyles SS, Yuen L, Shuman S, Moss B (1988) Purification of a factor required for transcription of vaccinia virus early genes. J Biol Chem 263: 10754–10760

Chang W, Lim JG, Hellstrom I, Gentry LE (1988) Characterization of vaccinia virus growth factor biosynthetic pathway with an antipeptide antiserum. J Virol 62: 1080–1083

Child SJ, Franke CA, Hruby DE (1988) Inhibition of vaccinia virus replication by nicotinamide: evidence for ADP-ribosylation of viral proteins. Virus Res 9: 119–132

Cochran MA, Mackett M, Moss B (1985) Eukaryotic transient expression system dependent on transcription factors and regulatory DNA sequences of vaccinia virus. Proc Natl Acad Sci USA 82: 19–23

Condit RC, Motyczka A (1981) Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. Virology 113: 224–241

Creighton TE (1984) Covalent modification of polypeptides. In: Proteins—structures and molecular properties. WH Freeman & Co. New York, pp 70–87

Essani K, Dales S (1979) Biogenesis of vaccinia: evidence for more than 100 polypeptides in the virion. Virology 95: 385–394

Ferro AM, Olivera BM (1984) Poly(ADP-ribosylation) of DNA topoisomerase I from calf thymus. J Biol Chem 259: 547–554

Franke CA, Reynolds PL, Hruby DE (1989) Fatty acid acylation of vaccinia virus proteins. J Virol 63: 4285–4291

Garon CF, Moss B (1971) Glycoprotein synthesis in cells infected with vaccinia virus. II. A glycoprotein component of the virions. Virology 46: 233–246

Hanggi M, Bannwarth W, Stunneberg HG (1986) Conserved TAAAT motif in vaccinia virus late promoters: overlapping TATA box and site of transcription initiation. EMBO J 5: 1071–1076

Hiller G, Weber K (1982) A phosphorylated basic vaccinia virion polypeptide of molecular weight 11,000 is exposed on the surface of mature particles and interacts with actin-containing cytoskeletal elements. J Virol 44: 647–657

Hiller G, Weber K (1985) Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopement. J Virol 55: 651–659

Holowczak JA, Joklik WK (1967) Studies on the structural proteins of vaccinia virus. Structural proteins of virions and cores. Virology 33: 717–725

Hruby DE (1985) Inhibition of vaccinia virus thymidine kinase by the distal products of its own metabolic pathway. Virus Research 2: 151–156

Hruby DE, Ball LA (1981) Control of expression of the vaccinia virus thymidine kinase gene. J Virol 40: 456–464

Hruby DE, Maki RA, Miller DB, Ball LA (1983) Fine structure analysis and nucleotide sequence of the vaccinia virus thymidine kinase gene. Proc Natl Acad Sci USA 80: 3411–3415

Ichihashi Y (1981) Unit complex of vaccinia polypeptides linked by disulfied bridges. Virology 113: 277–284

Kao S, Bauer WR (1987) Biosynthesis and phosphorylation of vaccinia virus structural protein VP11. Virology 159: 399–407

Kao S, Ressner E, Kates J, Bauer WR (1981) Purification and characterization of a superhelix binding protein from vaccinia virus. Virology 111: 500–508

Katz E, Moss B (1970a) Formation of a vaccinia virus structural polypeptide from a higher molecular weight precursor: inhibition by rifampicin. Proc Natl Acad Sci USA 66: 677–684

Katz E, Moss B (1970b) Vaccinia virus structural polypeptide derived from a high-molecular-weight precursor: formation and integration into virus particles. J Virol 6: 717–726
Kleiman JH, Moss B (1975a) Purification of a protein kinase and two phosphate acceptor proteins from vaccinia virions. J Biol Chem 250: 2420–2429
Kleiman JH, Moss B (1975b) Characterization of a protein kinase and two phosphate acceptor proteins from vaccinia virions. J Biol Chem 250: 2430–2437
Kotwal GJ, Moss B (1988) Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. Nature 335: 176–178
Krausslich HG, Wimmer E (1988) Viral proteinases. Ann Rev Biochem 57: 701–754
Lefkowitz RJ, Stadel JM, Caron MG (1983) Adenylate cyclase-coupled beta-adrenergic receptors: structure and mechanisms of activation and desensitization. Ann Rev Biochem 52: 159–186
Mars M, Beaud G (1987) Characterization of vaccinia virus early promoters and evaluation of their informational content. J Mol Biol 198: 619–631
Miner JN, Hruby DE (1989) DNA sequences that regulate the expression of a vaccinia virus late gene (L65) and interact with a DNA-binding protein from infected cells. J Virol 63: 2726–2736
Moss B (1974) Reproduction of poxviruses. In: Fraenkel-Conrat H, Wagner RR (ed) Comprehensive Virology. Plenum, New York, pp 405–474
Moss B (1985) Replication of poxviruses. In: Fields BN et al. (ed) Virology. Raven, New York, pp 685–703
Moss B, Rosenblum EN (1973) Protein cleavage and poxvirus morphogenesis: tryptic peptide analysis of core precursors accumulated by blocking assembly with rifampicin. J Mol Biol 81: 267–269
Moss B, Rosenblum EN, Garon CF (1971) Glycoprotein synthesis in cells infected with vaccinia virus. I. Non-virion glycoproteins. Virology 46: 221–232
Nakano E, Panicali D, Paoletti E (1982) Molecular genetics of vaccinia virus: demonstration of marker rescue. Proc Natl Acad Sci USA 79: 1593–1596
Oie M, Ichihashi Y (1981) Characterization of vaccinia polypeptides. Virology 113: 263–276
Payne LG (1978) Polypeptide composition of extracellular enveloped vaccinia virus. J Virol 27: 28–37
Payne LG (1979) Identification of the vaccinia hemagglutinin polypeptide from a cell system yielding large amounts of extracellular enveloped virus. J Virol 31: 147–155
Payne LG (1980) Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. J Gen Virol 50: 89–100
Pedley CB, Cooper RJ (1987) The assay, purification and properties of vaccinia virus-induced uncoating protein. J Gen Virol 68: 1021–1028
Pennington TH (1974) Vaccinia virus polypeptide synthesis: sequential appearance and stability of pre- and post-replicative polypeptides. J Gen Virol 25: 433–444
Pennington TH, Follet EAC (1974) Vaccinia virus replication in enucleate BSC-1 cells: particle production and synthesis of viral DNA and proteins. J Virol 13: 488–493
Person-Fernandez A, Beaud G (1986) Purification and characterization of a protein synthesis inhibitor associated with vaccinia virus. J Biol Chem 261: 8283–8289
Pogo BG, Katz JR, Dales S (1975) Biogenesis of poxviruses: synthesis and phosphorylation of basic protein associated with the DNA. Virology 64: 531–543
Rastl E, Swetly P (1978) Expression of poly(adenosine diphosphate-ribose) polymerase activity in erythroleukemic mouse cells during cell cycle and erythropoietic differentiation. J Biol Chem 253: 4333–4340
Reddy MK, Bauer WR (1989) Activation of the vaccinia virus nicking-joining enzyme by trypsinization. J Biol Chem 264: 443–449
Rodriguez JF, Esteban M (1987) Mapping and nucleotide sequence of the vaccinia virus gene that encodes a 14-kilodalton fusion protein. J Virol 61: 3550–3554
Rodriguez JF, Paez E, Esteban M (1987) A 14,000-M_r envelope protein of vaccinia virus is involved in cell fusion and forms covalently linked trimers. J Virol 61: 395–404
Rohrmann G, Yuen L, Moss B (1986) Transcription of vaccinia virus early genes by enzymes isolated from vaccinia virions terminates downstream of a regulatory sequence. Cell 46: 1029–1035
Rosel JL, Moss B (1985) Transcriptional and translational mapping and nucleotide sequence analysis of a vaccinia virus gene encoding the precursor of the major core polypeptide 4b. J Virol 56: 830–838
Rosel JL, Earl PL, Weir JP, Moss B (1986) Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the *Hin*dIII H genome fragment. J Virol 60: 436–449

Rosemond H, Moss B (1973) Phosphoprotein component of vaccinia virions. J Virol 11: 961–970
Sagot J, Beaud G (1979) Phosphorylation in vivo of a vaccinia virus structural protein found associated with the ribosomes from infected cells. Eur J Biochem 98: 131–140
Sarov I, Joklik WK (1972) Studies on the nature and location of the capside polypeptides of vaccinia virions. Virology 50: 579–592
Schultz AM, Henderson LE, Oroszlan S (1988) Fatty acylation of proteins. Ann Rev Cell Biol 4: 611–647
Schwer B, Visca P, Vos JC, Stunnenberg HG (1987) Discontinuous transcription or RNA processing of vaccinia virus late messengers results in a 5′ poly(A) leader. Cell 50: 163–169
Shaffer R, Traktman P (1987) Vaccinia virus encapsidates a novel topoisomerase with the properties of a eucaryotic type I enzyme. J Biol Chem 262: 9309–9315
Shida H (1986) Nucleotide sequence of the vaccinia virus hemagglutinin gene. Virology 150: 451–462
Shida H, Dales S (1981) Biogenesis of vaccinia: carbohydrate of the hemagglutinin molecule. Virology 111: 56–72
Silver M, Dales S (1982) Biogenesis of vaccinia: interrelationship between post-translational cleavage, virus assembly, and maturation. Virology 117: 341–356
Stroobant P, Rice AP, Gullick WJ, Cheng DJ, Kerr IM, Waterfield MD (1985) Purification and characterization of vaccinia virus growth factor. Cell 42: 383–393
Stryer L (1983) Transducin and the cyclic GMP phosphodiesterase: amplifier proteins in vision. Cold Spring Harbor Sym Quant Biol 48: 841–852
Van Meir E, Wittek R (1988) Fine structure of the vaccinia virus gene encoding the precursor of the major core protein 4a. Arch Virol 102: 19–27
Vaughan M, Moss J (1983) ADP-Ribosylation of proteins: an overview. In: Johnson BC (ed) Posttranslational covalent modifications of proteins. Academic, New York, pp 321–342
Venkatesan S, Gershowitz A, Moss B (1982) Complete nucleotide sequences of two adjacent early vaccinia virus genes located within the inverted terminal repetition. J Virol 44: 637–646
Villarreal EC, Roseman NA, Hruby DE (1984) Isolation of vaccinia virus mutants capable of replicating independently of the host cell nucleus. J Virol 51: 359–366
Von Heijne G (1983) Patterns of amino acids near signal-sequence cleavage sites. Eur J Biochem 133: 17–21
Weintraub S, Stern W, Dales S (1977) Biogenesis of vaccinia: effects of inhibitors of glycosylation on virus-mediated activities. Virology 78: 315–322
Weir JP, Moss B (1984) Regulation of expression and nucleotide sequence of a late vaccinia virus gene. J Virol 51: 662–669
Weir JP, Moss B (1985) Use of a bacterial expression vector to identify the gene encoding a major core protein of vaccinia virus. J Virol 56: 534–540
Wellink J, van Kammen A (1988) Proteases involved in the processing of viral polyproteins. Arch Virol 98: 1–26
Wilson EM, Edbauer C, Hruby DE (1988) Characterization of a binding factor that interacts with the sequences upstream of the vaccinia virus thymidine kinase gene. Virus Genes 2: 31–48
Wilson EM, Franke CA, Black ME, Hruby DE (1989) Expression vector pT7:TKII for the synthesis of authentic biologically active RNA encoding vaccinia virus thymidine kinase. Gene 77: 69–78
Wittek R (1982) Organization and expression of the poxvirus genome. Experientia 38: 285–297
Wittek R, Hanggi M, Hiller G (1984) Mapping of a gene coding for a major late structural polypeptide on the vaccinia virus genome. J Virol 49: 371–378
Wold F (1983) Posttranslational protein modifications: perspectives and prospectives. In: Johnson BC (ed) Posttranslational covalent modifications of proteins. Academic, New York, pp 1–17
Yang W, Kao S, Bauer WR (1988) Biosynthesis and post-translational cleavage of vaccinia virus structural protein VP8. Virology 167: 585–590
Yuen L, Davison AJ, Moss B (1987) Early promoter-binding factor from vaccinia virions. Proc Natl Acad Sci USA 84: 6069–6073

Subject Index

Current Topics in Microbiology and Immunology

Volumes published since 1983 (and still available)

Vol. 109: **Doerfler, Walter (Ed.):** The Molecular Biology of Adenoviruses 1. 30 Years of Adenovirus Research 1953–1983. 1983. 69 figs. XII, 232 pp. ISBN 3-540-13034-9

Vol. 110: **Doerfler, Walter (Ed.):** The Molecular Biology of Adenoviruses 2. 30 Years of Adenovirus Research 1953–1983. 1984. 49 figs. VIII, 265 pp. ISBN 3-540-13127-2

Vol. 112: **Vogt, Peter K.; Koprowski, Hilary (Ed.):** Retroviruses 3. 1984. 19 figs. VII, 115 pp. ISBN 3-540-13307-0

Vol. 113: **Potter, Michael; Melchers, Fritz; Weigert, Martin (Ed.):** Oncogenes in B-Cell Neoplasia. Workshop at the National Cancer Institute, National Institutes of Health, Bethesda, MD March 5–7, 1984. 1984. 65 figs. XIII, 268 pp. ISBN 3-540-13597-9

Vol. 115: **Vogt, Peter K. (Ed.):** Human T-Cell Leukemia Virus. 1985. 74 figs. IX, 266 pp. ISBN 3-540-13963-X

Vol. 116: **Willis, Dawn B. (Ed.):** Iridoviridae. 1985. 65 figs. X, 173 pp. ISBN 3-540-15172-9

Vol. 122: **Potter, Michael (Ed.):** The BALB/c Mouse. Genetics and Immunology. 1985. 85 figs. XVI, 254 pp. ISBN 3-540-15834-0

Vol. 124: **Briles, David E. (Ed.):** Genetic Control of the Susceptibility to Bacterial Infection. 1986. 19 figs. XII, 175 pp. ISBN 3-540-16238-0

Vol. 125: **Wu, Henry C.; Tai, Phang C. (Ed.):** Protein Secretion and Export in Bacteria. 1986. 34 figs. X, 211 pp. ISBN 3-540-16593-2

Vol. 126: **Fleischer, Bernhard; Reimann, Jörg; Wagner, Hermann (Ed.):** Specificity and Function of Clonally Developing T-Cells. 1986. 60 figs. XV, 316 pp. ISBN 3-540-16501-0

Vol. 127: **Potter, Michael; Nadeau, Joseph H.; Cancro, Michael P. (Ed.):** The Wild Mouse in Immunology. 1986. 119 figs. XVI, 395 pp. ISBN 3-540-16657-2

Vol. 128: 1986. 12 figs. VII, 122 pp. ISBN 3-540-16621-1

Vol. 129: 1986. 43 figs., VII, 215 pp. ISBN 3-540-16834-6

Vol. 130: **Koprowski, Hilary; Melchers, Fritz (Ed.):** Peptides as Immunogens. 1986. 21 figs. X, 86 pp. ISBN 3-540-16892-3

Vol. 131: **Doerfler, Walter; Böhm, Petra (Ed.):** The Molecular Biology of Baculoviruses. 1986. 44 figs. VIII, 169 pp. ISBN 3-540-17073-1

Vol. 132: **Melchers, Fritz; Potter, Michael (Ed.):** Mechanisms in B-Cell Neoplasia. Workshop at the National Cancer Institute, National Institutes of Health, Bethesda, MD, USA, March 24–26, 1986. 1986. 156 figs. XII, 374 pp. ISBN 3-540-17048-0

Vol. 133: **Oldstone, Michael B. (Ed.):** Arenaviruses. Genes, Proteins, and Expression. 1987. 39 figs. VII, 116 pp. ISBN 3-540-17246-7

Vol. 134: **Oldstone, Michael B. (Ed.):** Arenaviruses. Biology and Immunotherapy. 1987. 33 figs. VII, 242 pp. ISBN 3-540-17322-6

Vol. 135: **Paige, Christopher J.; Gisler, Roland H. (Ed.):** Differentiation of B Lymphocytes. 1987. 25 figs. IX, 150 pp. ISBN 3-540-17470-2

Vol. 136: **Hobom, Gerd; Rott, Rudolf (Ed.):** The Molecular Biology of Bacterial Virus Systems. 1988. 20 figs. VII, 90 pp. ISBN 3-540-18513-5

Vol. 137: **Mock, Beverly; Potter, Michael (Ed.):** Genetics of Immunological Diseases. 1988. 88 figs. XI, 335 pp. ISBN 3-540-19253-0

Vol. 138: **Goebel, Werner (Ed.):** Intracellular Bacteria. 1988. 18 figs. IX, 179 pp. ISBN 3-540-50001-4

Vol. 139: **Clarke, Adrienne E.; Wilson, Ian A. (Ed.):** Carbohydrate-Protein Interaction. 1988. 35 figs. IX, 152 pp. ISBN 3-540-19378-2

Vol. 140: **Podack, Eckhard R. (Ed.):** Cytotoxic Effector Mechanisms. 1989. 24 figs. VIII, 126 pp. ISBN 3-540-50057-X

Vol. 141: **Potter, Michael; Melchers, Fritz (Ed.):** Mechanisms in B-Cell Neoplasia 1988. Workshop at the National Cancer Institute, National Institutes of Health, Bethesda, MD, USA, March 23–25, 1988. 1988. 122 figs. XIV, 340 pp. ISBN 3-540-50212-2

Vol. 142: **Schüpbach, Jörg:** Human Retrovirology. Facts and Concepts. 1989. 24 figs. 115 pp. ISBN 3-540-50455-9

Vol. 143: **Haase, Ashley T.; Oldstone, Michael B. A. (Ed.):** In Situ Hybridization. 1989. 33 figs. XII, 90 pp. ISBN 3-540-50761-2

Vol. 144: **Knippers, Rolf; Levine, A. J. (Ed.):** Transforming Proteins of DNA Tumor Viruses. 1989. 85 figs. XIV, 300 pp. ISBN 3-540-50909-7

Vol. 145: **Oldstone, Michael B. A. (Ed.):** Molecular Mimicry. Cross-Reactivity between Microbes and Host Proteins as a Cause of Autoimmunity. 1989. 28 figs. VII, 141 pp. ISBN 3-540-50929-1

Vol. 146: **Mestecky, Jiri; McGhee, Jerry (Ed.):** New Strategies for Oral Immunization. International Symposium at the University of Alabama at Birmingham and Molecular Engineering Associates, Inc. Birmingham, AL, USA, March 21–22, 1988. 1989. 22 figs. IX, 237 pp. ISBN 3-540-50841-4

Vol. 147: **Vogt, Peter K. (Ed.):** Oncogenes. Selected Reviews. 1989. 8 figs. VII, 172 pp. ISBN 3-540-51050-8

Vol. 148: **Vogt, Peter K. (Ed.):** Oncogenes and Retroviruses. Selected Reviews. 1989. XII, 134 pp. ISBN 3-540-51051-6

Vol. 149: **Shen-Ong, Grace L. C.; Potter, Michael; Copeland, Neal G. (Ed.):** Mechanisms in Myeloid Tumorigenesis. Workshop at the National Cancer Institute, National Institutes of Health, Bethesda, MD, USA, March 22, 1988. 1989. 42 figs. X, 172 pp. ISBN 3-540-50968-2

Vol. 150: **Jann, Klaus; Jann, Barbara (Ed.):** Bacterial Capsules. 1989. 33 figs. XII, 176 pp. ISBN 3-540-51049-4

Vol. 151: **Jann, Klaus; Jann, Barbara (Ed.):** Bacterial Adhesins. 1990. 23 figs. XII, 192 pp. ISBN 3-540-51052-4

Vol. 152: **Bosma, Melvin J.; Phillips, Robert A.; Schuler, Walter (Ed.):** The Scid Mouse. Characterization and Potential Uses. EMBO Workshop held at the Basel Institute for Immunology, Basel, Switzerland, February 20–22, 1989. 1989. 72 figs. XII, 263 pp. ISBN 3-540-51512-7

Vol. 153: **Lambris, John D. (Ed.):** The Third Component of Complement. Chemistry and Biology. 1989. 38 figs. X, 251 pp. ISBN 3-540-51513-5

Vol. 154: **McDougall, James K. (Ed.):** Cytomegaloviruses. 1990. 58 figs. IX, 286 pp. ISBN 3-540-51514-3

Vol. 155: **Kaufmann, Stefan H. E. (Ed.):** T-Cell Paradigms in Parasitic and Bacterial Infections. 1990. 24 figs. IX, 162 pp. ISBN 3-540-51515-1

Vol. 156: **Dyrberg, Thomas (Ed.):** The Role of Viruses and the Immune System in Diabetes Mellitus. 1990. 15 figs. XI, 142 pp. ISBN 3-540-51918-1

Vol. 157: **Swanstrom, Ronald; Vogt, Peter K. (Ed.):** Retroviruses. Strategies of Replication. 1990. 40 figs. XII, 260 pp. ISBN 3-540-51895-9

Vol. 158: **Muzyczka, Nicholas (Ed.):** Viral Expression Vectors. 1990. approx. 20 figs. approx. XII, 190 pp. ISBN 3-540-52431-2

Vol. 159: **Gray, David; Sprent, Jonathan (Ed.):** Immunological Memory. 1990. 38 figs. XII, 156 pp. ISBN 3-540-51921-1

Vol. 160: **Oldstone, Michael B. A.; Koprowski, Hilary (Ed.):** Retrovirus Infections of the Nervous System. 1990. 16 figs. XII, 176 pp. ISBN 3-540-51939-4

Vol. 161: **Racaniello, Vincent R. (Ed.):** Piconaviruses. 1990. 12 figs. X, 194 pp. ISBN 3-540-52429-0

Vol. 162: **Roy, Polly; Gorman, Barry M. (Ed.):** Bluetongue Viruses. 1990. 37 figs. X, 200 pp. ISBN 3-540-51922-X