The International Conference of Comparative Virology, which is held in Canada every four years, is in honor of Dr. André Lwoff, Nobel Prize, eminent virologist and scientist.

From right to left

(Seated)

Professors André Lwoff (Guest of Honor), Karl Maramorosch, and Edouard Kurstak (Presidents of the International Conference on Comparative Virology).

(Standing)

Professors Samuel Dales, Neville F. Stanley, M. David Hoggan, Jordi Casals, Norton Zinder, David Baltimore, and Purnell W. Choppin.

VIRUSES, EVOLUTION AND CANCER
Basic Considerations

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Preface

Cancer is a major problem confronting the medical and biological sciences. The nature of cancer is very complex, involving many different morphological types. Implicated are a host of etiological factors, such as genetic, biological, biochemical, immunological, and hormonal. The difficult problem of successfully controlling or preventing cancer has become a crusade of the civilized world.

Although some tumors can be induced by radiation and chemical carcinogens, there has been recent renewed interest in viral carcinogenesis. It has been known for quite some time that a large number of animal as well as certain plant tumors are induced by DNA or RNA viruses. Thus for many researchers, including the editors of this book, it was difficult to ignore the role of viruses in the etiology of certain types of cancer in man.

Today, the followers of this theory appear to be correct, for during the past few years several reports have indicated the presence of DNA or RNA viruses or of viral factors associated with cancers of man, such as Burkitt's lymphoma or cancers of the nasopharynx, prostate, uterus, and breast. These findings are of considerable significance, especially for the future possible production of a vaccine against certain forms of cancer. One such vaccine has already found successful application against the virus causing Marek's disease, a cancer of chickens.

In the light of this information it was desirable to proceed with an exchange of information relating to the theory that implicates viruses, evolution, and cancer. First of all one is able to detect several viruses associated with cancer in animals and in man (e.g., herpesviruses and oncornaviruses), and, on the other hand, the same viruses can be infectious or oncogenic, depending on the cellular system which they attack. Hence, the study of the evolution of viruses as pathogens and the evolution of the cell under the interacting effect of the virus could answer the difficult question of why certain DNA or RNA viruses belonging to the same family behave either as simple infectious agents or as carcinogenic agents.

Cancer seems to be closely associated with the organization of the cell, and the observation that unlike any other disease abnormal growth appears in almost all animal and even plant species suggests the impairment of some mechanism essential to life. Cellular evolution can be useful in elucidating the enigma of cancer, and the interaction between virus and cell can provide an ideal model for accomplishing this objective.

In order to discuss these ideas, about 200 experts in virology and cancer research, from 20 countries, participated in August of 1973 in the Second International Conference of Comparative Virology at Mont Gabriel near Montréal, Canada. The theme of this conference was viruses, evolution and cancer. This quadrennial conference was presided over and organized by Edouard Kurstak and Karl Maramorosch, editors of this volume. The conference was held under the auspices of the Université de Montréal and McGill University, with the financial support of the Faculty of Medicine, of the Vice-Rectorat of Research, and of the Continuing Medical Education Service of the Université de Montréal; the Faculty of Medicine and the Faculty of Graduate Studies and Research of McGill University; the Medical Research Council of Canada; the National Research Council of Canada; the Department of the Secretary of State of the Federal Government of Canada; the Ministère des Affaires Intergouvernementales; and the Medical Research Council of the Province of Québec; and under the patronage of the Canadian Society of Microbiology and the Virology Section of the International Association of Microbiological Societies.

"Viruses, Evolution and Cancer," the result of the Second International Conference of Comparative Virology, is the first book of its kind. It includes well-documented chapters written by internationally renowned scientists. All oncogenic viruses are treated in this volume from the point of view of comparative biology and evolutionary aspects of DNA and RNA oncogenic viruses.

The presentation of the most recent results of the authors' own research as well as that of other conference participants and scientists who were not able to participate in the conference add to the value of this volume. The interpretations and original conclusions reached and the inclusion of numerous original illustrations make the book a unique body of information, and bring into sharp focus current findings and new directions of viral oncology research.

It is our hope that "Viruses, Evolution and Cancer" will help bring unity to the science of viral oncology through the comparative and evolutionary approach and that it will stimulate further research on viral cancer. It is clear that such research will result in a better understanding of the interactions between viruses and abnormal growth and,

hopefully, in eventually finding a cure for human cancers of viral origin as might exist. In this respect this book will contribute both to the scientific and medical problems of cancer and will benefit all who are interested in virology and oncology.

The chairmen of the Second International Conference of Comparative Virology and editors of this book wish to express their sincere gratitude to the contributors for the effort and care with which they have prepared their chapters; to the Faculty of Medicine of the Université de Montréal for help during various stages of preparation of the treatise; to Professor André Lwoff (Institut Pasteur, Paris, France), the invited guest of honor, to whom the conference paid a special tribute and to Dr. Joseph L. Melnick (Baylor College of Medicine, Houston, Texas), chairmen of the International Advisory Board of the Conference; to all members of this board; to many others who gave unselfish support in organizing the conference; to Dr. Pierre Bois and Dr. R. F. Patrick Cronin, Deans of the Faculties of Medicine of the Université de Montréal and of McGill University, respectively, and honorary chairmen of the Conference Organizing Committee for their continued support; to Dr. Maurice L'Abbé, Vice Chancellor for Research of Université de Montréal, and Dr. Walter F. Hitschfeld, Dean of the Faculty of Graduate Studies and Research of McGill University; to Dr. de Guise Vaillancourt, Vice-Dean of the Faculty of Medicine and Director of Continuing Medical Education Service of the Université de Montréal for help in the edition of the program and in publicity matters; to Dr. Jean Mathieu, Vice-Dean of the Faculty of Medicine of Université de Montréal; Dr. Sorin Sonea, Dr. Angus F. Graham, Dr. Claude-Lise Richer, and Dr. Richard Morisset, members of the Organizing Committee of the Conference for their kind collaboration; and last, but not least, to the staff of Academic Press for their part in editing, indexing, proofreading, and other aspects of production of the volume.

Edouard Kurstak
Karl Maramorosch

Introduction

The origins of viruses are unknown, and speculation on their evolution is based on findings concerning various groups. It is usually assumed that RNA and DNA viruses originated through two distinct avenues of evolutionary development.

Most viruses known at present are independent genetic systems. They have an intrinsic ability to mediate their own transfer from one host to another, which certainly indicates that they possess an evolutionary history of their own (Luria and Darnell, 1967). Their genetic continuity and their ability to mutate present a parallel to that of microorganisms and of higher organisms. It is not known whether their evolution proceeded independently of their hosts or whether it was linked with specific hosts evolving over long periods of time. However, viral evolution seems to have been affected by an intracellular environment.

Bacteriophages have been shown to integrate into the chromosomes of host cells, and certain viruses of Mollicutes, particularly of *Spiroplasma citri* (Cole *et al.*, 1973), seem to have the same ability. In all groups of viruses infecting microorganisms, plants, lower or higher animals, there are known instances of helper-dependent viruses, indicating an evolutionary or retrograde development.

Viruses contain only one type of genetic material—either deoxyribonucleic (DNA) or ribonucleic (RNA) acid. If viruses originated from cellular nucleic acids, the DNA and RNA viruses were probably derived from different cell structures. Mitochondria and chloroplasts contain amounts of DNA that are comparable to those of small and large viruses, but mitochondria as well as chloroplasts resemble blue-green algae rather than viruses. It is conceivable that the replication of the DNA of these organelles resembles viral replication, whereas replicas of the virus genome serve as centers for the assembly of progeny virions as originally postulated by Luria and Darnell (1967). Chloroplasts and mitochondria, just as viruses, have genetic continuity, and they can replicate in an

autonomous system, governed in part by these organelles and in part by cellular controls.

The ability of viruses to infect several generations of cells and even to be transferred congenitally through the sexual process points to a very long evolutionary process, from single or from several components of cells by retrograde evolution or by transfer from cell to cell (Green, 1935; Burnet, 1945; Maramorosch, 1972). In the evolution of bacteriophages extrachromosomal elements (such as episomes, consisting of DNA fragments attached to bacterial chromosomes or located in the cytoplasm) may have been the predecessors.

Bacteriocins that often resemble phages morphologically but are unable to multiply in bacterial cells could represent episomes, along with temperate phages. According to Lwoff (1953) chromosome segments could have acquired the ability to code for a protein coat and a system for injecting its nucleic acid into a bacterium, thus permitting entry into the host cell.

Lysogenic bacteriophages may have evolved from killer particles (perhaps from large and small bacteriocins) that retained a part of the host genome but lost their ability to enter host cells. Their protein capsid cannot perform genetic transfer, a property still present in temperate phages at the end of the evolutionary ladder of viruses. While temperate phages are virulent for sensitive cells, those that lost the ability to lyse can no longer be discovered. The phage tails, according to Lwoff and Tournier (1971), probably came into being late during evolution from homologous sectors of bacterial DNA. The single-stranded RNA viruses could have evolved from corresponding messenger RNA and the double-stranded RNA viruses from single-stranded ones through an alteration of the replication system of the RNA.

Adverse nutritional factors, radiation, chemical mutagens, or extremes in temperature could favor the evolution of viruses, but no experimental data are available to support this speculation. Viruses have been described from practically all living organisms; they seem to be ubiquitous and possibly may play a beneficial role in evolution (Anderson, 1970; Maramorosch, 1972). Viruses cross the barriers of species (Kurstak, 1972), and some require for their proliferation in nature alternating plant and animal hosts (Maramorosch, 1970, 1972). These interesting viruses are transmitted directly from a plant to an invertebrate animal, and they persist only as long as this alternation exists. The viruses that pass through such alternating plant and animal hosts may possibly aid in the transfer of genetic determinants as do bacterial and mammalian viruses. It is not surprising that the substantially identical genetic codes are found in all living organisms, plants as well as animals, as has been pointed out by Anderson (1970).

One of the results of viral infection can be a cytopathic effect in which

viral progeny is produced within the cell, division is stopped, and the cell destroyed. Less common is the reaction that follows infection of cells by oncogenic, tumor-producing viruses. Such viruses do not destroy the cells but transform them, causing them to divide continuously and to become malignant tumor cells. In certain instances the same virus can cause a cytopathic effect in one type of cell and convert other cells into malignant ones.

The conversion into cells characterized by unrestricted growth is sometimes accompanied by alteration of cell morphology and by chromosomal abnormalities. In the absence of fossil viruses or other standard-type of evolutionary evidence, one can only speculate that this might have occurred in common infectious viruses and over millennia has changed them to oncogenic agents.

We know that a number of physical agents such as ultraviolet and X rays or chemical agents such as cyclic hydrocarbons as well as several RNA and DNA viruses may induce the formation of neoplasms in animals. Several RNA viruses can also induce tumors in plants. In animal cells chemical or viral carcinogens cannot be detected after the formation of tumors, but in virus-infected plants, and especially in viral plant tumors, the causative agents concentrate and remain present for prolonged periods (Maramorosch, 1970). They disappear when plant tumors are artificially maintained *in vitro* (Black, 1965).

In the past two decades several RNA and DNA tumor viruses have been studied at the cellular level. The results of these studies have helped in understanding the very complex oncogenic virus–cell interaction.

While it is likely that the DNA and RNA oncogenic viruses developed along different evolutionary lines, the final outcome of these evolutionary processes was the integration of the diverse types of nucleic acid into the host genomes. This might have led to a loss of cell contact inhibition as one of the first steps in the direction of full malignancy. Viruses might develop potential mechanisms to trigger cell transformation.

The evolution of oncogenic viruses can also be approached through a study of host evolution. Speculation on the evolution of herpesviruses, some of which have oncogenic properties, can be based on the evolution of species susceptible to herpesvirus infection. As major groups of animals became extinct, their specific viruses could have become adapted to other groups. Thus we may have in our bloodstreams viruses from dinosaurs. It is conceivable that viruses may have influenced the cause of evolution by bringing new bits of DNA into organisms. Groups such as mosses and ferns in the plant kingdom, almost devoid of known virus infection, seem to evolve more slowly than do certain groups of higher plants and of animals that harbor many different viruses.

One of the important elements in triggering the formation of cancer is

viral genetic material, often accompanied by other factors that play a secondary role. This hypothesis is supported by experimental evidence in animals and plants. It can also explain the "oncogene" theory of endogenous viruses and vertical transmission of viral genetic material (Todaro and Huebner, 1972). This is especially apparent in the case of RNA oncogenic viruses, the so-called oncornaviruses (May *et al.*, 1973). These viruses, which form a homogeneous group, have been isolated from many animal species or tissue culture systems, such as man, monkeys, guinea pigs, cattle, swine, rats, hamsters, mice, and snakes. Although it has been proved that viruses can induce cancer in animals, one can only speculate about similar associations between oncornaviruses and cancer in humans. For instance, oncornaviruses have been suspected as causative agents of human breast cancer.

It is difficult to understand why RNA or DNA viruses with similar physicochemical, morphological, serological, and biological properties may act either as simple infectious agents or as oncogenic agents. Pertinent questions might eventually be answered by studying at the cellular level the evolution of virus-infected species and the interspecific transfer of oncogenic viruses. By becoming transmissible, a nucleic acid or nucleoprotein in one species has the opportunity of being transferred to another. Selection in the evolutionary process has probably resulted in the production of some infective viruses as well as of other, solely oncogenic nucleoproteins. The present treatise with its theme Viruses, Evolution and Cancer focuses on these ideas.

Edouard Kurstak
Karl Maramorosch

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CHAPTER 1

Host–Virus Relationship at the Embryonic Level

DAVOR SOLTER, WIESLAWA BICZYSKO, AND HILARY KOPROWSKI

I. Introduction

In the broadest sense, the study of viruses and early mammalian embryos has considerable practical as well as theoretical importance. Viruses infecting embryos may cause either malformation and/or death of the fetus, or, particularly in the case of oncogenic viruses, presumably can be incorporated in the cellular genome of embryonic cells with

subsequent neoplasia. In addition to these more practical considerations of virus–embryo interaction, several theoretical questions concerning the nature of virus–cell interaction can be raised, e.g.:

1. Which oncogenic and nononcogenic viruses are able to infect the mammalian embryo in the early stages of development, and what is the ultimate fate of these infected embryos?
2. Do differentiating embryonic cells react differently to viral infection than differentiated somatic cells?
3. Is permissiveness for viral replication related to species or to stage of development?

We present here data about the existence of endogenous viral particles in early mammalian embryos and on the interaction of oncogenic and nononcogenic viruses with early mouse embryos.

II. Differentiation of Early Mammalian Embryos *in Vitro*

Studies using mammalian embryos have often been limited by the small size and limited number of the embryos and by poor accessibility and handling difficulties. In recent years, techniques have been developed for successful growth and differentiation of mammalian embryos *in vitro*. Detailed descriptions of such techniques are available (Daniel, 1971) and we will present them only briefly.

A. Growth of Preimplantation Embryos in Vitro

Preimplantation mouse embryos can be isolated from the oviduct or uterus of pregnant females at specific times after fertilization (Rugh, 1968). If a large number of embryos is needed, animals are superovulated by intraperitoneal injection of pregnant mare serum followed after 48 hours by human chorionic gonadotropin. In this way, up to 80 eggs per animal can be collected (Gates, 1971). Isolated eggs at different stages of development starting at 1-cell stage or zygotes up to blastocysts, which have about 100 cells, are used immediately or cultured *in vitro*. Metabolic requirements of mouse eggs change during cleavage but they can be cultured successfully up to the blastocyst stage in a simple, chemically defined medium (Brinster, 1965; Whitten and Biggers, 1968; Biggers *et al.*, 1971; Whitten, 1971). Blastocysts either isolated from the uterus or obtained after cleavage *in vitro* can be transferred to the uteri of pseudopregnant foster mothers using surgical (Rafferty, 1970) or nonsurgical techniques (Beatty, 1951; Marsk *et al.*, 1971). In this way,

preimplantation embryos can be exposed to various agents *in vitro*, and subsequently the effect on further development *in utero* can be investigated. Using embryos grown *in vitro* numerous biochemical parameters have been established (Ellem and Gwatkin, 1968; Woodland and Graham, 1969; Pikó, 1970; Monesi *et al.*, 1970; Daentl and Epstein, 1971, 1973; Epstein and Daentl, 1971; Knowland and Graham, 1972).

Preimplantation embryos have also been exposed to different viruses (the effect of such exposure will be described in detail later). Depending on the size of viral particles, they can penetrate zona pellucida as in the case of mengo virus (Gwatkin, 1963, 1971) or they cannot as in the case of simian virus 40 (SV40) and Moloney sarcoma virus (MSV) (Baranska *et al.*, 1971; Sawicki *et al.*, 1971). Zona pellucida can easily be removed with pronase treatment (Mintz, 1962) without affecting the viability of the embryo or its development.

B. Growth of Postimplantation Embryos in Vitro

1. Growth of Blastocysts beyond Implantation

The implantation and subsequent development of egg cylinder is the first major event in mammalian differentiation. Although during cleavage cellular differentiation, such as differentiation of inner cell mass and trophoblasts, occurs (Gardner, 1971), the morphological organization and restriction in cellular totipotency starts after implantation. First attempts to grow blastocysts *in vitro* beyond implantation stage were almost uniformly unsuccessful (Cole and Paul, 1965; Gwatkin, 1966). Resulting growth was composed mainly of trophoblastic cells spread on the bottom of culture dishes, while inner cell mass either failed to grow or grew as a cellular layer on the top of trophoblastic cells without the characteristic morphology of the developing egg cylinder (Cole and Paul, 1965; Gwatkin, 1966). The necessity for a hypothetical uterine factor (Kirby, 1962) or for some kind of tridimensional support where blastocysts could gain a better "hold" for development were usually evoked to explain such failures.

By using bovine lens fiber material, Jenkinson and Wilson (1970) tried to develop a culture system with better mechanical conditions for development. A limited but still organized growth of structures resembling egg cylinders resulted. Much better results were reported after growing the blastocysts on reconstituted rat tail collagen (Hsu, 1971, 1972, 1973) in Eagle's minimal essential medium. A considerable number (up to 50%) of blastocysts developed into comparatively normal egg cylinders that developed further into structures showing presence of neural tube, cerebral vesicles, up to 15 somites, beating heart, and rudiments of

circulation (Hsu, 1973). Thus using these techniques it is now possible to obtain the development of mouse embryos *in vitro* from the zygote up to the 9-day-old embryo, i.e., the entire first half of the gestation period.

Since we planned, by using immunofluorescent methods, to detect the presence of oncogenic viruses in developing embryos, we decided to investigate growth of embryos in collagen-free medium to avoid possible interference of collagen with the test. Blastocysts grown in 5% CO_2 in air in Falcon plastic dishes in Eagle's MEM supplemented with 10% fetal calf serum developed into the egg cylinders in more than 50% of the cases (Pienkowski *et al.*, 1974). Several factors were found to influence successful development of the blastocysts.

a. The Age of the Embryo. Late blastocysts with a well-developed cavity developed much better than earlier ones.

b. The Presence of Zona Pellucida. Blastocysts with the zona pellucida intact hatched spontaneously in the medium. However, the percentage of completely hatched blastocysts was low (40%). Blastocysts with zona pellucida failed to attach and degenerated, floating in the medium. Partially hatched blastocysts developed only the layer of trophoblastic cells, while inner cell mass did not differentiate. Blastocysts treated with pronase for 2 minutes (Mintz, 1962) attached more readily and their development into egg cylinders was more successful (Table I). The better development of blastocysts treated with pronase is probably due to the shortening of the time interval between their release into the medium and attachment to the dish.

c. Treatment with Proteolytic Enzymes. The removal of zona pellucida by hatching or with pronase and attachment to the plastic were found necessary for development. However, treatment with proteolytic enzymes (0.2% pronase or 0.05% trypsin for 30 minutes) greatly increased the number of blastocysts which developed into egg cylinders (Table I). Trypsin stimulates the division of somatic cells *in vitro* (Burger, 1973), and it is possible that it also stimulates the division of blastomeres. In addition, implantation initiating factor (IIF), which is probably necessary for implantation and development of mouse blastocysts *in vivo*, might be proteolytic in nature (Mintz, 1971).

d. The Effect of pH. Using Eagle's MEM buffered with organic buffers (Eagle, 1971) at different pH's, we tested the development of blastocysts (with zona pellucida removed by pronase and additional trypsin treatment for 30 minutes) (Table II). Approximately the same number of blastocysts developed into egg cylinders at pH 7.6 and 8.0, while at pH 7.0 they developed only trophoblastic cells.

TABLE I

Development of ICR Mouse Blastocysts *in Vitro*

	Time of cultivation														
	24 hours			48 hours			72 hours			96 hours			120 hours		
Developing structures	I[a]	II[b]	III[c]	I	II	III	I	II	III	I	II	III	I	II	III
Growing blastocysts[d]	24	145	240	54	156	218	54	128	165	29	46	72	12	31	20
Egg cylinders[e]	0	0	0	0	0	0	18	36	52	30	75	141	36	72	182
Trophoblastic layer[f]	41	38	12	47	49	21	52	57	32	73	91	36	92	118	47
Floating in medium	117	45	0	96	18	0	71	0	0	50	0	0	42	0	0
Lost	36	32	40	41	37	43	43	39	43	56	48	43	56	39	43
Total	238	260	292	238	260	292	238	260	292	238	260	292	238	260	292

[a] Blastocysts isolated from the uterus; zona pellucida intact.
[b] Blastocysts treated with pronase (0.2% pronase for 2 minutes).
[c] Blastocysts treated with pronase as above and with trypsin (0.05% for 30 minutes).
[d] Blastocysts attached with trophoblastic layer and some growth of inner cell mass.
[e] Egg cylinder with embryonic and extraembryonic parts and visible germ layers.
[f] Only layer of trophoblastic cells, inner cell mass no longer visible.

TABLE II

Development of ICR Mouse Blastocysts *in Vitro* after 120 Hours in Media with Different pH

	pH		
Developing structures[a]	7.0	7.6	8.0
Growing blastocysts	19	17	21
Egg cylinder	4	103	81
Trophoblastic layer	94	31	36
Lost	56	30	21
Total	173	181	169

[a] Blastocysts were treated with pronase and trypsin as in Table I, group III and grown in MEM buffered with organic buffer (hepes, Sigma).

e. The Presence of Serum. Simple chemically defined medium supplemented with bovine serum albumin supported the growth of mouse embryos from zygote to blastocysts (Biggers *et al.*, 1971), but it did not support further development of blastocysts. They, however, grew successfully in media containing fetal calf serum. Different batches of fetal calf serum differed greatly in their ability to support differentiation of blastocysts, some being almost ineffective. Hsu (1972) reported similar findings, and the differences in hormone content in different batches of serum (Esber *et al.*, 1973) could account for such observations.

2. *Growth of Egg Cylinders*

Rodent egg cylinders transferred to extrauterine sites (anterior chamber of the eye, testis, kidney) developed into teratoma and/or teratocarcinoma (Levak-Svajger and Skreb, 1965; Solter *et al.*, 1970a; Stevens, 1970; Damjanov *et al.*, 1971; Skreb *et al.*, 1972). Nine-day-old rat egg cylinders when grown on lens paper in air/medium interphase can differentiate *in vitro* forming small teratomas with numerous differentiated tissues (Skreb and Svajger, 1973). Normal development of rodent egg cylinders with extraembryonic membranes intact in circulating medium was mostly dependent on the age of the embryo when explanted (New, 1971). We observed the successful development of 7-day-old mouse egg cylinders in Eagle's MEM supplemented with 10% fetal calf serum into structures with well-developed neural tube, cerebral vesicles, 10–15 somites, beating heart, and blood islands in yolk sac (Table III) (Pienkowski *et al.*, 1974). When the extraembryonic part of the egg cylinder was removed, embryos attached to the plastic more readily, but developed only a neural tube, somites, and beating heart but not blood islands. This confirmed the results of Moore and Metcalf

TABLE III

Development[a] of ICR Mouse Egg Cylinder *in Vitro*

Structures	Time of cultivation (hours)				
	24	48	72	96	120
Neural tube	31	118	201	207	182
Heart beating	43	156	183	149	146
Blood islands[b]	0	74	76	73	62
Somites	0	11	37	41	43

[a] In terms of the number of embryos showing given structure. Total number of egg cylinders examined was 96 whole egg cylinders and 141 egg cylinders whose extraembryonic part was removed.

[b] The development of blood islands was observed only in whole egg cylinders grown *in vitro* and almost never in egg cylinders whose extraembryonic part was removed.

(1970) that hematopoietic cells develop from extraembryonic parts of the embryo. Development of egg cylinders usually stopped after 5 days in culture. By this time, the resulting structures were equivalent to 9-day-old embryos developing *in utero*. Some embryos persisted in the culture as large, fluid-filled vesicles or as a layer of fibroblast-like cells, but they all eventually degenerated. It is still possible, however, to use this model for observing short-term effect of viruses in development and differentiation.

III. Endogenous Viral Particles in Mouse Embryos

Ultrastructural examination of preimplantation stages of mouse embryos isolated from uterus revealed the presence of endogenous viral particles. These particles were classified as type A virus particles (Dalton *et al.*, 1966) and were present in embryos of all examined mouse strains. In addition immature C-type particles were found in blastocysts of AKR mouse strain.

A. Type A Viral Particles in Preimplantation and Postimplantation Mouse Embryos

Unfertilized eggs; zygotes; 2-, 4-, and 8-cell stage embryos; blastocysts; and egg cylinders of several mouse strains were examined for the presence of type A virus particles as shown in Table IV (Biczysko *et al.*, 1973a). Viral particles were never observed in unfertilized eggs and

TABLE IV

Presence of Virus Particles in Eggs and Embryos from Various Mouse Strains[a]

Strain	Characteristic[b]	Virus particles in cytoplasm of[b]			
		Unfertilized eggs	2- to 4-Cell embryos	Blastocysts	Egg cylinders
ICR/Ha	Low leukemia, low MT	−	+++	+	+
AKR	High leukemia, low MT	−	+++	+++[c]	++[c]
Balb/cfC3H	Low leukemia, high MT	−	+++	+	NT
GR/Cam	Low leukemia, high MT	−	+++	NT	NT
C57BLf/He/Cam	Low leukemia, low MT	−	+++	NT	NT
RIII/Cam	Low leukemia, high MT	−	+++	NT	NT
Balb/c	Low leukemia, low MT	−	+++	+	NT
ICR/Ha X C57BL/6	Low leukemia, low MT	−	+++	NT	NT

[a] This table is reproduced from a paper by Biczysko *et al.* (1973a) with permission from *J. Nat. Cancer Inst.*

[b] MT, mammary tumor; NT, not tested; (−), no viruslike particles observed; (+++), numerous; (+), single viruslike particles.

[c] Both A and C particles observed.

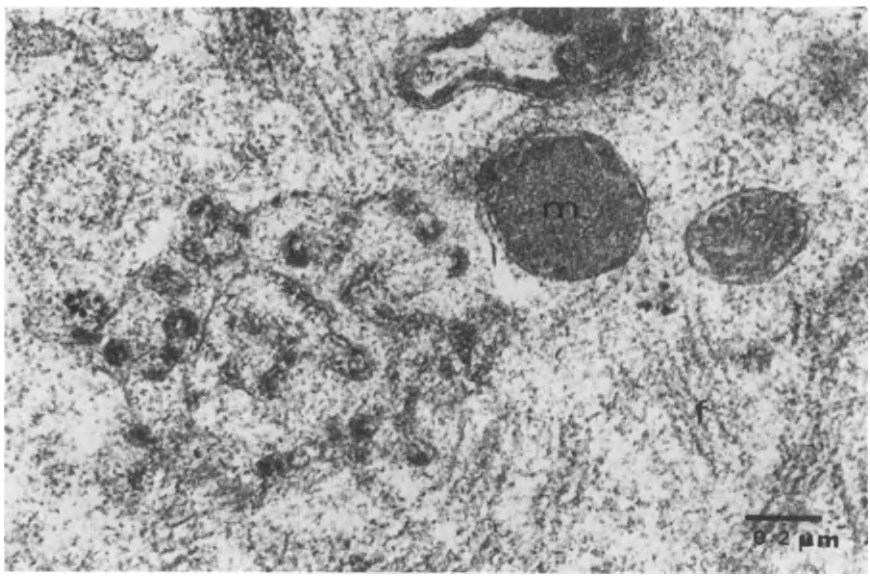

FIG. 1. Cytoplasm of 4-celled ICR mouse embryo. Group of intracisternal type A particles budding into cisterna of endoplasmic reticulum. Cytoplasm is filled with protein fibers (f) and mitochondria (m) with dense matrix.

zygotes but were found in considerable number in late 2-cell stage embryos. Particles were seen either budding from membranes of endoplasmic reticulum or within the cisternae of endoplasmic reticulum (Fig. 1). Mature particles were doughnut-shaped or oval and 80–90 nm in diameter. They possessed two electron-dense shells and an electron-lucent center approximately 40–50 nm in diameter (Fig. 2), and on the basis of this appearance were classified as intracisternal type "A" virus particles (Dalton *et al.*, 1966). Virus particles were occasionally observed between the leaflets of nuclear membrane but never intracytoplasmically or outside the cells. The number of type A particles in 2-, 4-, and 8-cell stage embryos appeared to be about equal. However, in blastocysts and egg cylinders, the number of particles decreased considerably, and only mature intracisternal particles were visible (Fig. 3). The

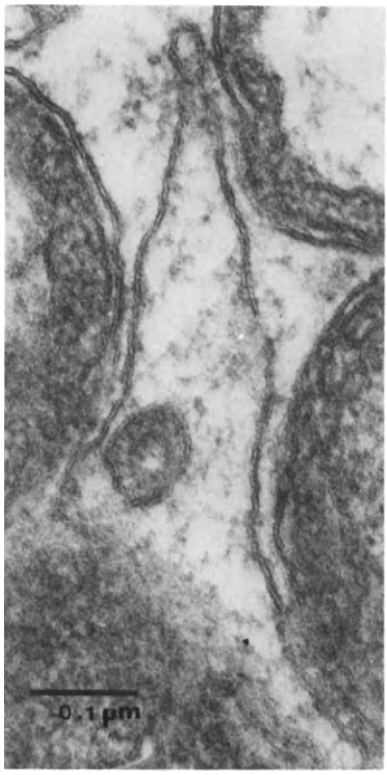

FIG. 2. Two-celled ICR embryo. Single intracisternal A particle in cisterna of endoplasmic reticulum. Notice the two concentric shells and electron-lucent center. From Biczysko *et al.* (1973a). Courtesy of *J. Nat. Cancer Inst.*

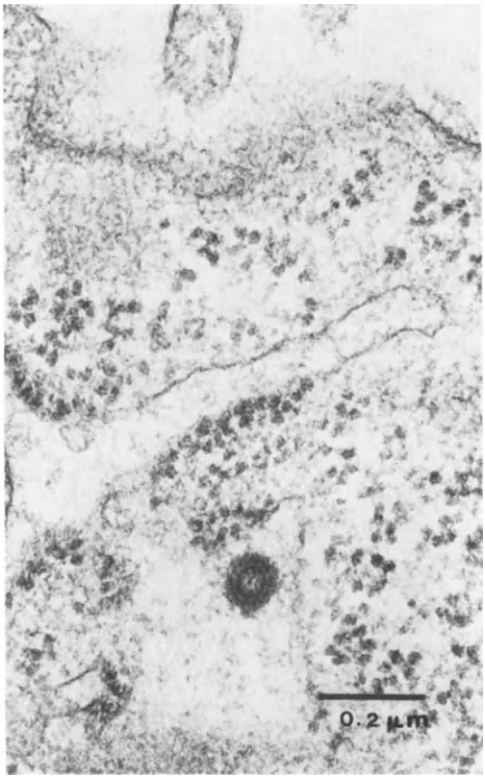

FIG. 3. Endodermal cell of ICR egg-cylinder. Single type A in enlarged cisterna of endoplasmic reticulum. Translucent center is large, and projections from outer shell of particle are visible. Ribosomes partially attached to membranes of endoplasmic reticulum. From Biczysko *et al.* (1973a), courtesy of *J. Nat. Cancer Inst.*

number and appearance of type A viral particles in blastocysts grown *in vitro* from 2-cell stage was the same as in blastocysts isolated from the uterus.

Enders and Schlafke (1965) found doughnut-shaped particles in guinea pig blastocysts, and similar structures were described by Calarco and Brown (1969) in preimplantation embryos of the Balb/c mouse strain. Recently, Calarco and Szollosi (1973) reported results very similar to ours (Biczysko *et al.*, 1973a). They found intracisternal type A viral particles in preimplantation embryos of Balb/c, CF-1, Swiss Webster, and New Zealand Black strains. Chase and Pikó (1973) have described three types of viral particles budding from endoplasmic reticulum in preimplantation embryos of outbred Swiss albino mice. Two of them are type

A viral particles, which differ in size and shape and also in the time of the appearance. All this data suggests the ubiquitous nature of type A particles in mouse embryos and possibly in the embryos of other species. Detailed analysis of the preimplantation stages of other mammalian species should furnish the evidence to test this hypothesis.

The biological activity and origin of type A virus particles are unknown. They have been described in numerous mouse tumors, i.e., plasmocytoma (Dalton *et al.*, 1961; Kuff *et al.*, 1968), leukemia (de Harven, 1962), chemically induced sarcoma (Granboulan *et al.*, 1960) and carcinoma (Kakefuda *et al.*, 1970), embryo-derived yolk sac carcinoma (Damjanov and Solter, 1973), and neuroblastoma (Schubert *et al.*, 1969). Wivel and Smith (1971) observed intracisternal type A particles in mouse tumors originating from all three germ layers and in several normal mouse cells and tissues, such as mammary gland, ovary, seminal vesicle, fibroblasts, lymphocytes, spleen, thymocytes, skeletal muscle, etc. M. L. Vernon (personal communication) examined different organs of mouse embryos in the second half of the gestation period from several inbred and random bred mice. She found intracisternal type A particles in about one-half of the examined specimens. Despite such widespread distribution of type A particles, attempts to demonstrate their biological activity have so far been unsuccessful (Kuff *et al.*, 1968).

Mature type A virus particles have some distinct biochemical characteristics and can be distinguished from RNA tumor viruses. They possess high molecular weight RNA of 70 S (Yang and Wivel, 1973), and a major structural protein of 70,000 molecular weight is associated with the inner shell of the particles (Wivel *et al.*, 1973). Antiserum against this protein reacts specifically with type A particles and cytoplasmic membrane fractions from tumors containing type A particles, but not with leukemia or mammary tumor virus, or cells infected with Rauscher leukemia virus, or normal mouse cells (Kuff *et al.*, 1972). These results would suggest that intracisternal type A particles are not related to C or B type viral particles, although they can be found together in the same cells (Wivel and Smith, 1971; Biczysko *et al.*, 1973a; Grimley *et al.*, 1973). The activity of poly(dT) polymerase requiring RNA or DNA primer was also found in association with type A particles (Wilson and Kuff, 1972). This activity was clearly different from RNA-directed DNA polymerase of murine leukemia virus (MLV), which also suggested the difference between those two types of viral particles.

At the present time, it is impossible to know if type A particles are an expression of a definite viral genome without biological activity, if they represent incomplete replication of some viral genome that would normally express itself as C or B particles, or if they are some aberration of

a normal cellular process, such as membrane synthesis. It is obviously difficult to establish how type A particles affect cells in a case such as mouse embryo cells, since mouse embryo cells without particles have not been found. As far as mouse embryos are concerned, we can only say that despite, or perhaps because of, the presence of type A particles in all embryos, they develop and differentiate successfully.

Type A viral particles are found almost exclusively within rough endoplasmic reticulum (RER), and it seems that their number depends on the amount of RER in the cells (Wivel and Smith, 1971; Damjanov and Solter, 1973). However in mouse embryos they are present in nongranular endoplasmic reticulum, so their presence is probably somehow connected with membrane formation and not directly with protein synthesis as suggested by Wivel and Smith (1971).

A vertical mode of transmission of type A particles was suggested by the findings of type A particles in mouse gonadal tissues (Wivel and Smith, 1971), in guinea pig oogonia and oocytes (Anderson and Jeppesen, 1972), and in immature mouse oocytes (Calarco and Szollosi, 1973). Since we were not able to demonstrate type A particles in mouse spermatozoa, we have decided to determine whether the presence of both a paternal and a maternal genome is necessary for the appearance of type A particles or if cell division following fertilization was sufficient stimulus for their appearance.

B. Type A Viral Particles in Parthenogenetically Stimulated Mouse Eggs

Superovulated ICR and AKR mouse eggs were stimulated to divide by using hyaluronidase and osmotic shock (Graham, 1970, 1971) and examined under the electron microscope as 2-, 4-, and 8-celled parthenogenones. [Parthenogenone is an embryo produced from a female gamete without the concurrence of a male gamete (Graham, 1971).]

Type A viral particles were first observed in 3-celled parthenogenones and were seen in all later stages (Biczysko *et al.*, 1974). Localization of particles was identical to that in fertilized eggs, i.e., in cisternae of endoplasmic reticulum (Fig. 5). The number of particles was considerably large, and as far as can be judged from electron microscope specimens, there were more than in normally fertilized eggs. The ultrastructure of parthenogenones (Solter *et al.*, 1974a) was somewhat different from that of normal mouse cleavage stages (Calarco and Brown, 1969; Hillman and Tasca, 1969), the most important difference being the persistence of primary nucleoli (Fig. 4). During normal mouse development primary nucleoli, composed of a dense fibrillar core surrounded by a narrow

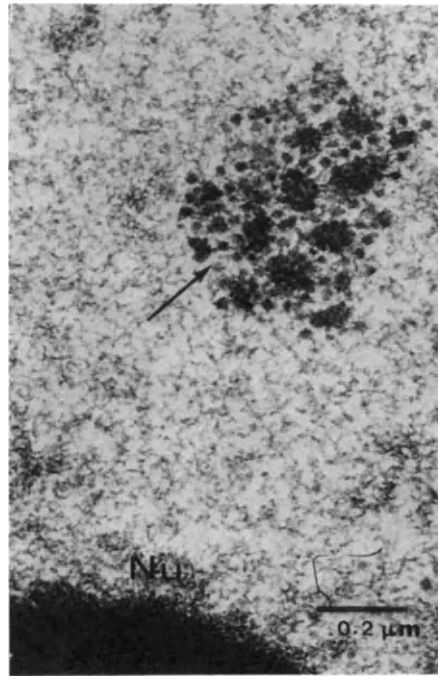

FIG. 4. Part of the nucleus of 4-celled ICR parthenogenone. Primary nucleolus (Nu) and cluster of dense, round, granular structures of various sizes (arrow). From Biczysko *et al.* (1974), courtesy of *J. Nat. Cancer Inst.*

granular area, are present in 2-cell embryos. Later they start to differentiate, and, in 4-cell and later embryos, nucleoli are well differentiated with large nucleolar mesh composed of fibrillar and granular parts (Hillman and Tasca, 1969). This development was not observed in parthenogenones.

In parthenogenones, we were able to follow what we consider a sequence of events leading to development of type A particles. Granular material, similar to the granular area of the nucleolus, was scattered through the nucleoplasma and attached to the inner leaflet of the nuclear membrane. In the nucleoplasma and in the duplications of the inner leaflet of the nuclear membrane, we also observed round, dense, granular structures about 60–70 nm in diameter composed of several granules 15 nm in diameter (Fig. 4). Recognizable type A viral particles were first seen budding from the outer leaflet of the nuclear membrane into the space between two leaflets of the nuclear membrane (Fig. 5). The outer leaflet of the nuclear membrane formed large protrusions with numerous

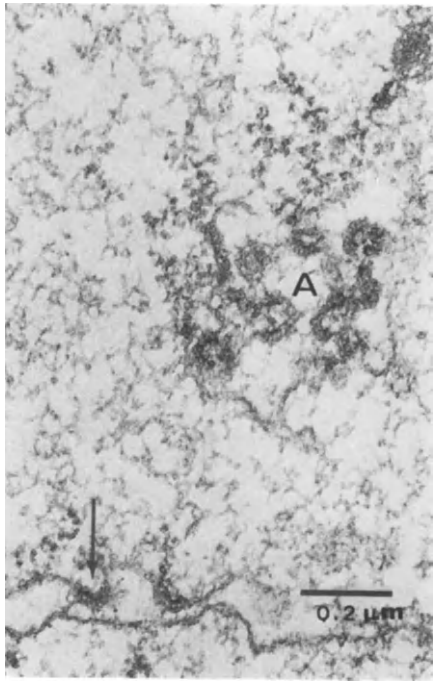

FIG. 5. Cytoplasm of 4-celled AKR parthenogenone. Two type A particles budding from the outer leaflet of nuclear membrane (arrow). Cisternae of endoplasmic reticulum with budding A particles (A). From Biczysko *et al.* (1974), courtesy of *J. Nat. Cancer Inst.*

enfoldings into the cytoplasm. Inside such enfoldings we found a large number of budding and mature type A particles (Fig. 6). Granular material mentioned earlier was often found near protrusions of nuclear membrane between the leaflets of nuclear membrane.

So far it is impossible to determine whether the dense, round structures in the nucleoplasma or in the duplication of the inner leaflet of the nuclear membrane are related in some way with type A particles, a possibility that should be explored further. It is certain, however, that information for the synthesis of type A virus particles exists in mouse ova and can be expressed even after parthenogenetic stimulation. Some indirect evidence suggests that the appearance of type A particles is related to RNA synthesis. Type A particles are present in immature oocytes in the dictyate stage but rarely thereafter (Calcarco and Szollosi, 1973). In amphibian oogenesis RNA synthesis is greatly reduced after the dictyate stage (Davidson, 1969), and the same might be true for mammalian oogenesis. The reappearance of type A particles in late 2-cell stage

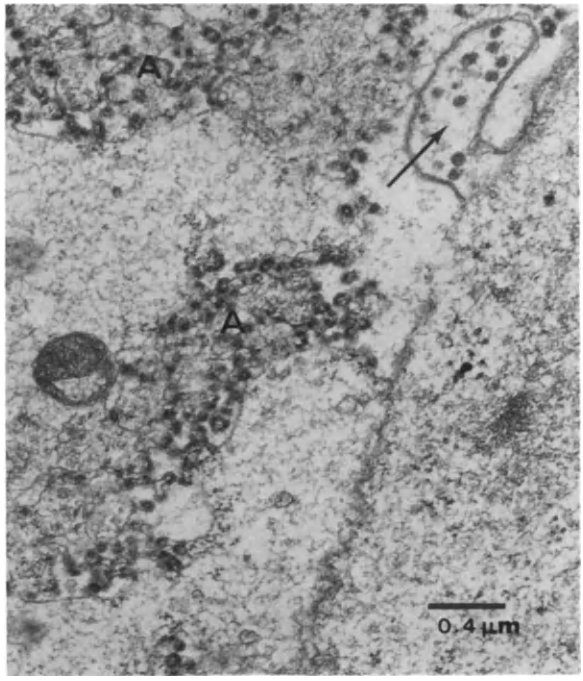

FIG. 6. Cytoplasm of 6-celled ICR parthenogenone. Numerous type A particles budding from large protrusions of outer leaflet of nuclear membrane (A). Round, dense, granular structures within protrusion of inner leaflet of nuclear membrane (arrow).

embryos coincides with the beginning of RNA synthesis (Knowland and Graham, 1972). We observed type A particles in 3- to 4-celled parthenogenones, which (at least in the case of immediate cleavage) correspond to the 2-cell stage in fertilized embryos. All these data and the fact that type A particles possess RNA (Yang and Wivel, 1973) suggest that synthesis of type A particles is somehow connected with synthesis of cellular RNA. Further studies using RNA metabolic inhibitors would probably clarify this problem.

C. Type C Viral Particles in Preimplantation and Postimplantation Mouse Embryos

As presented in Table IV, we found C type particles only in AKR blastocysts and egg cylinders. They were seen either as budding from the cell membrane or extracellularly and were approximately 90–110 nm

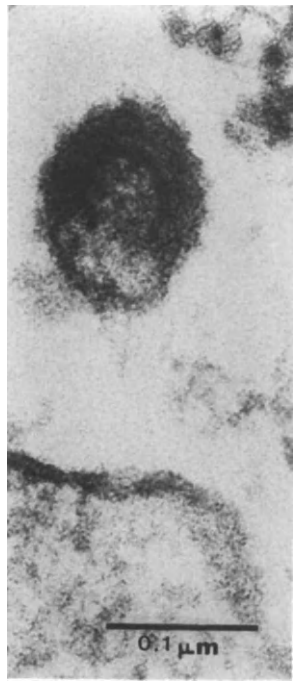

FIG. 7. Single, immature C type viral particle in intercellular space of AKR blastocyst. Biczysko *et al.* (1963a), courtesy of *J. Nat. Cancer Inst.*

in diameter with dense cores (Fig. 7). The number of C type viral particles was considerably smaller than the number of type A particles. Chase and Pikó (1973) also described the presence of C type viral particles in blastocysts of outbred Swiss albino mice and mentioned that they were also found in AKR, C3H, and C57BL blastocysts. The small number of particles is probably the reason why we have not observed them in ICR and C57BL mice (Biczysko *et al.*, 1973a). With the use of 5-bromodeoxyuridine the number of C type particles can be greatly increased (L. Pikó, personal communication), which would make the study of these particles during early embryogenesis much easier. Vernon *et al.* (1973) reported the presence of C type particles in about 85% of the embryonic or newborn mice after examination of liver, spleen, or thymus. Huebner *et al.* (1970, 1971) reported the finding of group-specific (gs) antigen coded by C type virus in visceral tissues of mouse embryos from several mouse strains. C type particles were also observed in normal human (Kalter *et al.*, 1973a) and primate (Kalter *et al.*,

1973b) placentas. It seems now that C type viral particles are present in overt or cryptic form in most, if not all, vertebrate cells (Todaro and Huebner, 1972; Gelb *et al.*, 1973), and such findings lead to the suggestion that they might represent the common cause of cancer (Todaro and Huebner, 1972). Infectious virus was isolated from embryos of highly leukemogenic AKR strains, while embryos from other strains were negative for infectious virus (Hartley *et al.*, 1969). The finding of C type viruses present in early embryonal life suggests that the information must be present in gametic cells or, much less likely, that all embryos become infected very early in gestation. Such virus is probably subinfectious and in most cases not carcinogenic, and its complete phenotypic expression, particularly that of carcinogenic potential, would depend on various genetic or external factors acting on the host. As with type A particles, we do not know how important is the information contained in the viral genome for the normal development of the embryo.

IV. Interaction of Oncogenic Viruses with Mouse Embryos

Our investigations have been limited to the ultrastructural events following exposure of mouse embryos at the 2-cell, blastocyst, and egg cylinder stages to SV40 and polyoma viruses. All embryos were examined immediately, 24, and 96–120 hours after exposure (Biczysko *et al.*, 1973b). Embryos were grown *in vitro* using methods described in Chapter 2.

A. Simian Virus 40 (SV40)

1. Two-Cell Stage Embryos

Embryos were fixed after 4 hours of viral exposure. Numerous viral particles were found attached to the surface of cells and within the cytoplasm near the cell membrane (Fig. 8). The probable mechanism of uptake is by viropexis (Dales, 1973), i.e., endocytosis of one or more virus particles. This mechanism was described in detail for permissive green monkey kidney cells (Hummeler *et al.*, 1970). Free virus particles were not found in the cytoplasm of 2-cell stage embryos. Blastomeres were destroyed after exposure to virus, so their further development could not have been observed. It is difficult at present to know whether destruction was caused by the virus itself or by some toxic substances present in crude viral preparation; this problem needs elucidating.

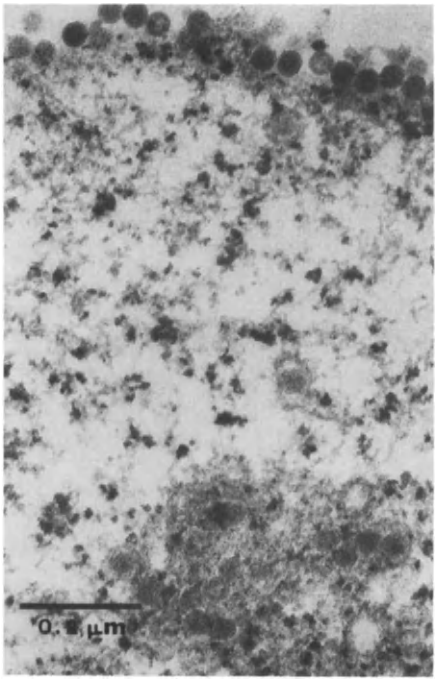

FIG. 8. Two-cell ICR embryo 4 hours after exposure to SV40. Virus particles attached to the cell surface and within endocytotic vacuoles. From Biczysko *et al.* (1973b), courtesy of *J. Nat. Cancer Inst.*

2. *Blastocysts*

The blastocysts were exposed to the same viral concentration as 2-cell stage (10^7 PFU in 0.1 ml of media), but the number of attached particles was considerably smaller. The lesser number of receptor sites for SV40 or a much quicker uptake could explain this difference. The mechanism of virus uptake was the same as in 2-cell embryos. Multiple or single virus particles were present in endocytotic vacuoles throughout the cytoplasm. Solitary viral particles often without enveloping membrane were visible in the perinuclear region often in the vicinity of RER (Fig. 9). Only a few viral particles were observed in the nucleus. Virus particles were found only in trophoblastic cells and none in the inner cell mass.

After 24 hours *in vitro* we could not detect viral particles in the embryonic cells, although the nucleoli of some cells were considerably enlarged with large holes in the nucleolar mesh. Numerous horseshoe-

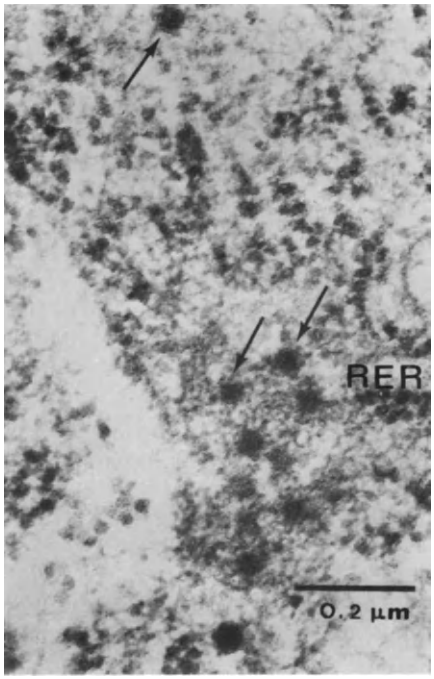

FIG. 9. ICR blastocyst 4 hours after exposure to SV40. Several viral particles (arrows) free in the cytoplasm in the vicinity of rough endoplasmic reticulum (RER). From Biczysko *et al.* (1973b), courtesy of *J. Nat. Cancer Inst.*

or doughnut-shaped dense inclusions were visible in the nucleolonema. Such nucleolar changes were considered early signs of viral replication when they occurred in monkey cells (Granboulan *et al.*, 1963).

Blastocysts grown *in vitro* for 96 hours developed into egg cylinderlike structures (Pienkowski *et al.*, 1974). The ultrastructure of such *in vitro* developed egg cylinders (Solter *et al.*, 1974b) was comparable to that of egg cylinders isolated from the uterus (Solter *et al.*, 1970b). Signs of viral replication were visible exclusively in endodermal cells. In some cells nucleolar changes similar to those seen 24 hours after exposure were observed. Other cells had homogeneous, finely granular nucleoli with clusters of viral particles within or attached to the nucleoli. The chromatin of virus-producing cells was condensed and either marginated or distributed in small clumps through nucleoplasma. Numerous viral particles were attached to such chromatin clumps. Some cells showed degenerative changes with extreme condensation of chromatin and enlargement and breaks in nuclear membrane. Virus particles were not

TABLE V

The Effect of SV40 and Polyoma on Growth of ICR Mouse Blastocysts *in Vitro*

Developing structures[a]	Control	SV40	Polyoma
Growing blastocysts	32	24	40
Egg cylinders	75	17	81
Trophoblast	16	68	19
Lost	15	32	21
Total	138	141	161

[a] Blastocysts were treated with pronase and trypsin as before, and then exposed to SV40 (10^7 PFU in 0.1 ml of medium for 4 hours) or polyoma (10^8 PFU in 0.1 ml of medium for 2 hours). After careful washing embryos were transferred to MEM plus 10% FCS and observed for 96 hours.

found in the cytoplasm. Blastocysts exposed to SV40 do not grow *in vitro* as well as noninfected controls; most developed only a monolayer of trophoblastic cells (Table V).

3. Egg Cylinders

In embryos exposed to SV40 for 4 hours, numerous viral particles were attached on the cell surface of endodermal cells on or between microvilli. Uptake by endocytosis, passage through the cytoplasm toward the nucleus, and liberation from endocytotic vacuoles in the perinuclear regions were as described for blastocysts.

Egg cylinders grown *in vitro* for 24 hours after exposure to SV40 did not possess viral particles in the cytoplasma or in the nucleus, but nucleolar changes similar to those described for blastocysts were noticed. After 96–120 hours *in vitro*, the embryos developed as did controls, showing such signs of differentiation as beating heart, somites, neural tube, and blood islands. Signs of viral replication were visible in cells of the yolk sac, hematopoietic cells, and cells of the neural tube. Numerous viral particles were seen in the nuclei either in clumps or attached to condensed chromatin. Viral replication was accompanied by disruption of the nucleus, and numerous viral particles were found in the cytoplasm attached to plasma membranes (Fig. 10). We also observed uptake of newly synthesized virus in cells of the neural tube that were in close contact with virus-producing cells. The number of cells that produced virus both in blastocysts and egg cylinders grown *in vitro* was comparatively small. Most of those cells showed signs of degeneration with broken nuclear membranes, extreme condensation of chromatin, vacuoles in the cytoplasm, and mitochondria with broken and swollen cristae. However, cells with similar degenerative changes were also occasionally observed in noninfected embryos grown *in utero*.

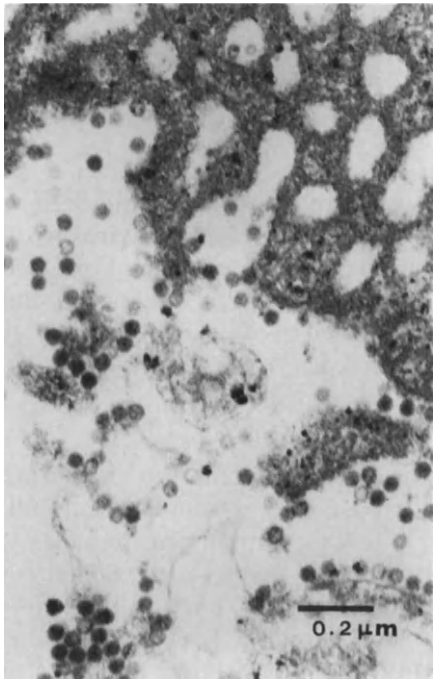

FIG. 10. ICR egg-cylinder 110 hours after exposure to SV40. Cytoplasm of a dead cell filled with numerous viral particles attached to the membranes of endoplasmic reticulum. From Biczysko *et al.* (1973b), courtesy of *J. Nat. Cancer Inst.*

Our results suggested that the behavior of SV40 in mouse embryos is similar to that reported for permissive systems (Granboulan *et al.*, 1963; Oshiro *et al.*, 1967; Hummeler *et al.*, 1970). So far mouse cells have been considered nonpermissive for lytic infection with SV40 (Knowles *et al.*, 1971), but no evidence has been obtained for a specific repressor for SV40 replication (Jensen and Koprowski, 1969). Results reported by Baranska *et al.* (1971) and Sawicki *et al.* (1971) indicated that replication of SV40 takes place in mouse embryos. Perhaps some mechanism operates in mouse somatic cells that inhibits the expression of late function of the viral genome present in the transformed mouse cells. This mechanism might be inoperative in mouse embryos during the early stages of development, suggesting that permissiveness or nonpermissiveness is not species-dependent, but dependent instead on the developmental stage of the animal. Obviously at the moment we cannot say if the control involved in permissiveness is positive or negative. To test the hypothesis that permissiveness is dependent on the developmental

stage of the animal, other models, comparable to mouse–SV40, need to be tested.

B. Polyoma

1. Two-Cell Stage Embryos

Two hours after exposure to polyoma (and even when exposure was prolonged for 4 hours) we did not find any virus particles attached to or penetrating the cells. The same percentage of exposed 2-cell embryos *in vitro* reached the blastocyst stage as did control embryos. When blastocysts grown from exposed 2-cell stage embryos were examined viral particles were not observed.

2. Blastocysts

Viral particles were visible attached to the surface of trophoblastic cells 2 hours after exposure to polyoma. Small and large endocytotic vacuoles containing a few to several virus particles were present in the cytoplasm beneath the cell membranes. Solitary viral particles associated with Golgi regions were found in the perinuclear region. The nuclei were free of viral particles. Except for viral particles in trophoblastic cells, the ultrastructure of exposed blastocysts was comparable to controls. Viral particles were not present in the blastocoel or in the cells of the inner cell mass.

Blastocysts exposed to polyoma and grown *in vitro* for 96–120 hours developed as did controls (Table V). Signs of viral replication were found exclusively in endodermal cells. In some endodermal cells nucleoli were enlarged with large holes in nucleolar mesh. Dark horseshoe- or doughnut-shaped condensations of nucleolonema and agglomerates of viral particles within the nucleus were observed (Fig. 11). The cytoplasm of cells whose nuclei were filled with viral particles was also filled with virus (Fig. 11). The plasma membrane of such cells were disrupted, and the neighboring cells showed uptake of newly synthetized virus.

Indirect immunofluorescence revealed a positive reaction for V antigen of polyoma in the endodermal cells.

3. Egg Cylinders

Adsorption and uptake of polyoma was restricted to endodermal cells. Particles were attached to or between the microvilli, and there were numerous endocytotic vacuoles containing virus particles in the cytoplasm. Polyoma-exposed embryos grown *in vitro* for 96–120 hours

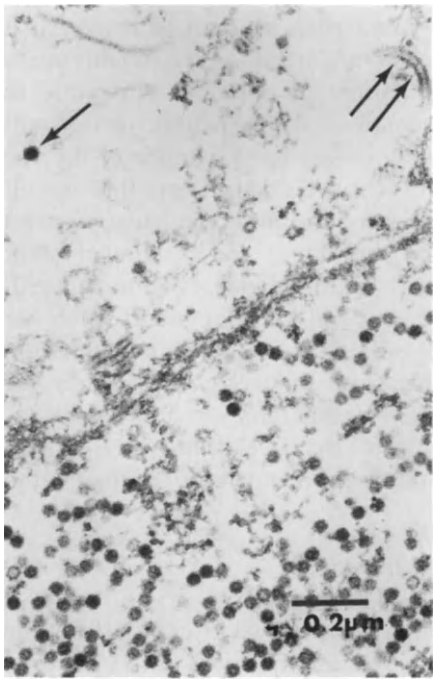

FIG. 11. ICR blastocyst 96 hours after exposure to polyoma. Nucleus filled with viral particles. Tubular form (double arrow) and single viral particles (arrow) in the cytoplasm. From Biczysko *et al.* (1973b), courtesy of *J. Nat. Cancer Inst.*

developed as did controls. Viral replication was visible in endodermal, yolk sac, and hematopoietic cells. Virus particles were present in the nuclei associated with the small, dense chromatin area. The cytoplasm of such cells was filled with viral particles. Signs of degeneration, i.e., vacuolation of cytoplasma, broken membrane of endoplasmic reticulum, and swollen mitochondria, were observed in cells containing virus. Uptake of newly synthetized virus was noticed in the cells of the neural tube. In these cells, viral particles attached to broken plasma membranes were usually found within large endocytotic vacuoles. Cells of such embryos were positive for V antigen in indirect immunofluorescence testing.

The ultrastructure of polyoma uptake and replication in mouse embryos was similar to that described for permissive mouse cells (Dourmashkin, 1962; Mattern *et al.*, 1966). It is difficult to find a reasonable explanation for the fact that 2-cell embryos are resistant to polyoma infection and susceptible to SV40 infection.

Mouse cells are permissive for polyoma replication in contrast to SV40, and the polyoma virus was found to spread among wild mouse populations (Rowe *et al.*, 1961). One could postulate that if 2-cell embryos were susceptible to polyoma virus infection, the replicating virus might cause death of the cells and, if transmitted *in utero* at the start of the pregnancy, affect the existence of the species. The "protective mechanism" of the 2-cell embryo may thus be related to the absence of cell surface receptors for polyoma virus attachment, even though these receptors are present at the blastocyst stage. Since the virus cannot penetrate *in vitro* through intact zona pellucida, one may wonder whether techniques involved in the removal of the zona may damage the "cell receptors" for polyoma virus. This is highly unlikely in light of the susceptibility of the 2-cell embryos to infection with SV40, an agent which usually displays characteristics very similar to polyoma virus.

Thus, the resistance of the 2-cell embryos to polyoma virus cannot be easily explained, and the phenomenon requires further study.

C. Oncogenic RNA Viruses

Baranska *et al.* (1971) and Sawicki *et al.* (1971) reported evidence for replication of Moloney sarcoma virus (MSV) after infection of mouse embryos at the 2-cell stage and the morula. The presence of such viral replication, however, did not disturb normal development of the mouse embryos *in vitro*.

Our preliminary data showed adsorption of murine mammary tumor virus (MuMTV) on mouse blastocysts after 4 hours exposure but not penetration.

D. Other Viruses

Gwatkin (1963, 1971) made a detailed study of 2-cell mouse embryos infected with mengo virus. After penetration through zona pellucida, the virus replicated within the cells and caused a complete arrest of development. Similar results were observed using western equine encephalitis (Gwatkin, 1971). West Nile, herpes simplex, rubella, vaccinia, cytomegalic inclusion disease, and polyoma viruses did not have any effect on mouse development *in vitro* (Gwatkin, 1971). Mouse embryos exposed to human adenovirus type 5 (AD-5) and type 12 at the 8-cell stage were arrested in development after one cell cycle, and signs of viral replication were observed under the electron microscope (Chase *et al.*, 1972).

V. Summary and Conclusions

The ease with which mammalian embryos can be maintained in tissue culture during the first half of gestation made possible the study of cellular events associated with the presence of endogenous virus and the response of embryonic cells and tissues to infection with exogenous oncogenic viruses.

The results of such studies indicated that type A virus particles become visible in large concentrations following the first mitotic division of the egg, either after fertilization or after parthenogenetic stimulation. The concentration of type A particles in embryonic cells decreased markedly at the blastocyst stage, when C type particles are first seen by electron microscopy.

The granular, heterogenous nucleolar RNA may be shown to be the site of synthesis of type A particles, particularly if it could be demonstrated that 30 S–40 S RNA is a precursor of 70 S RNA found in mature type A particles in nonembryonic cells.

The response of embryonic cells to infection with the DNA oncogenic viruses indicates that behavior of such viruses in undifferentiated cells and in cells at various stages of differentiation varies from that observed in fully differentiated somatic cells.

Permissiveness of mouse embryonic cells for replication of SV40 and resistance of 2-cell mouse embryos to attachment of polyoma virus are examples of such differences. Infection of blastocysts and egg cylinders with both polyoma virus and SV40 and the impediment of differentiation of the inner cell mass by SV40 may indicate that viruses for which adult cells are not permissive may, at the embryonic level, participate in the process of teratogenesis or even oncogenesis.

Although the study of endogenous viruses in the embryo and the interaction of early embryonic cells with exogenous viruses is still in its preliminary stages, we feel that the model presented in this chapter may play an important role in exploring and dealing with the mechanisms involved in differentiation and oncogenesis.

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CHAPTER 2

DNA Transfer and Virus–Cell Relationships

JOSEPH L. MELNICK, ANN LEWIS BOYD, AND JANET S. BUTEL

I. Introduction

There is mounting interest in the general area of gene transfer in mammalian cell systems. Diverse experimental approaches have established that foreign deoxyribonucleic acid (DNA) can penetrate animal cells under certain conditions and will persist for varying periods of time (Bhargava and Shanmugan, 1971). The greatest difficulty encountered in gene transfer experiments with mammalian cells to date has been the inability to detect expression of biological activity by the foreign DNA.

In this report, we will discuss three different experimental studies that were predicated on the "DNA transfer" method for introducing foreign mammalian cell DNA into heterologous recipient cells and which may contribute to the formulation of gene transfer concepts. The first study involved the rescue of papovavirus SV40 by the passage of transformed cell DNA in permissive simian cells; the second project revealed the ability of simian cell DNA to alter recipient hamster cells such that they could adsorb poliovirions; and the third series of experiments demonstrated that foreign DNA introduced into host cells actively supporting the replication of SV40 could be encapsidated, resulting in the formation of "heterologous" pseudovirions.

II. Rescue of SV40 from Transformed Cells

A. General Properties of SV40-Transformed Cells

1. Viral-Specific Nucleic Acids in Transformed Cells

Probably the best characterized tumor cells resulting from transformation events mediated by DNA-containing oncogenic viruses are those induced by the papovaviruses SV40 and polyoma (Black, 1968; Butel *et al.*, 1972; Butel and Melnick, 1972; Sambrook, 1972). SV40-transformed cells produce infectious virus only very rarely, if at all. This fact led to attempts to determine the state of the viral genome in such cells, particularly since assays of nucleic acid isolated from SV40-transformed cells failed to reveal the presence of any free, infectious viral DNA (Sabin and Koch, 1963b; Kit *et al.*, 1968; Boyd and Butel, 1972).

It was found that SV40-transformed hamster cells contained DNA with homology to SV40 mRNA prepared *in vitro* (Reich *et al.*, 1966; Westphal and Dulbecco, 1968). Estimations of the number of viral DNA equivalents per transformed cell ranged from as high as 58 (Westphal and Dulbecco, 1968) to only 1–9 (Levine *et al.*, 1970; Gelb *et al.*, 1971), depending on the hybridization technique used. Such estimates, of course, do not indicate whether or not some of the "equivalents" might be defective (incomplete) viral genomes.

Attempts to determine the state of the multiple copies of viral nucleic acid within the transformed cells were made using the DNA–RNA hybridization technique and polyoma- or SV40-transformed mouse cells (Sambrook *et al.*, 1968; Westphal and Dulbecco, 1968). It was concluded from such studies that the viral DNA is covalently bound to the

chromosomal DNA of the transformed cell. The site or sites of insertion of the multiple copies of the genome is still unknown.

Transcription of the integrated viral genes does occur in transformed cells. Benjamin (1966) first reported that a small fraction of pulse-labeled RNA from polyoma- or SV40-transformed cells was able to hybridize with the DNA of the corresponding virus. The mRNA formed during lytic infection prior to viral DNA synthesis ("early" RNA) is different from the mRNA present after the onset of viral DNA replication ("late" RNA) in infected cells (Aloni *et al.*, 1968; Oda and Dulbecco, 1968; Sauer and Kidwai, 1968). Approximately one-third of the SV40 genome was represented in the early RNA, whereas at least 75% of the genome was represented in late RNA. It appears that there is a strand-switch during *in vivo* transcription in infected cells. Early RNA is transcribed off one DNA strand, while "late" message is transcribed off the other strand (Khoury and Martin, 1972; Lindstrom and Dulbecco, 1972; Sambrook *et al.*, 1972). *E. coli* DNA polymerase copies only the "early," or minus, strand, and that in its entirety.

Transformed cells contain primarily early sequences copied from the minus strand. Using separated strands of ^{32}P-labeled SV40 DNA, both Ozanne *et al.* (1973) and Khoury *et al.* (1973) observed that although not more than 30% of the sequences of the early strand appear in stable species of RNA in productively infected cells, the percentages range from 35–80% in transformed mouse cells. They interpreted this as evidence that some of the viral sequences in transformed cells are "anti-late." Although presumably noninformational, the biological significance of such "anti-late" sequences is unknown. Most lines of transformed cells also contained RNA complementary to a small ($<20\%$) segment of the late strand of SV40 DNA. Controls at the transcriptional and post-transcriptional levels during productive infection by SV40 and their relevance to transcription of viral information in transformed cells have not yet been elucidated.

High molecular weight heterogeneous RNA that contains viral-specific RNA has been detected in the nucleus of transformed mouse cells (Lindberg and Darnell, 1970). Polysomal mRNA of lower molecular weight also contained viral-specific RNA. The authors suggested that the large nuclear molecules may be precursors of the cytoplasmic mRNA. Of particular interest was the fact that the largest molecules containing SV40 sequences were longer than one SV40 genome. These molecules were subsequently shown to carry both viral and cellular base sequences (Wall and Darnell, 1971). Polycistronic "viral–cell" hybrid RNA molecules have also been detected in adenovirus-2-transformed rat embryo cells and in adenovirus-7-induced hamster tumor cells (Tsuei

et al., 1972). The presence of cellular mRNA regions adjacent to viral-specific sequences is very formidable evidence in support of the concept of integration of the viral genome into that of the host cell.

2. *Viral-Specific Antigens in Transformed Cells*

The viral mRNA is translated in the transformed cells as evidenced by the synthesis of virus-specific antigens. One such antigen is the tumor (T) antigen. Using the immunofluorescence test, it normally appears as particulate granules distributed throughout the nucleus and is detected using sera from SV40 tumor-bearing hamsters. T antigen is detected in both transformed cells (Black *et al.*, 1963; Rapp *et al.*, 1964a; Pope and Rowe, 1964) and productively infected cells (Rapp *et al.*, 1964b; Sabin and Koch, 1964). In the replicative cycle, it is synthesized prior to viral DNA (Rapp *et al.*, 1965) and is called an early antigen. The function of T antigen is not known.

Mutants of defective SV40 (PARA) have been isolated (Butel *et al.*, 1969) which are distinguished by the fact that the induced SV40 T antigen is localized in the cytoplasm of infected cells, rather than in the nucleus. It was hoped that the mutants would be tools with which to gain insight into the biological function of T antigen. However, after extensive characterization of the variant PARA's (Butel *et al.*, 1969; Guentzel and Butel, 1973), there were no observable differences in their properties from those of the parental PARA. The cytoplasmic mutants were also able to transform cells (Duff *et al.*, 1970), and the majority of the transformed cells contained SV40 T antigen in the cytoplasm. The properties of several cytoplasmic T-positive cell lines were then examined to determine if the altered localization of T antigen influenced any properties of the transformed cells. There were no observable differences between the nuclear T-positive and cytoplasmic T-positive cells, other than the localization of the T antigen (Richardson and Butel, 1971). Therefore, neither the mutant viruses themselves nor the cells transformed by the viruses yielded any clues as to the biological function of SV40 T antigen.

In addition to the T antigen in the nucleus, virus-specified changes occur at the cell surface. One such change is the transplantation antigen (TSTA). The TSTA is detected using *in vivo* tests—adult animals which are immunized with the virus will reject a transplant of transformed cells. The TSTA is virus-specific; SV40-immunized animals reject SV40-transformed cells, but not cells transformed by any other virus (Deichman, 1969; Butel *et al.*, 1972).

A change at the cell surface, designated the surface (S) antigen, can be demonstrated *in vitro* by immunofluorescence (Tevethia *et al.*, 1965).

Using unfixed tumor cells and sera from animals that have rejected a tumor transplant, a reaction at the membrane can be observed. Other changes also occur at the cell surface, including the appearance of plant lectin agglutination sites, embryonic antigens, and normal cell antigens. The relationship between the TSTA and antigens detected at the cell surface by various *in vitro* tests is unclear at this time (see Butel *et al.*, 1972, for detailed discussion).

However, without exception, SV40-transformed cells do not contain viral capsid antigens. The viral replicative cycle is blocked in the transformed cells prior to the synthesis of capsid antigen. That is, early functions of the virus are expressed in the transformed cells, while late functions are not.

3. Rescue of the Transforming Viral Genome

Occasionally, SV40-induced tumors and transformed cell lines spontaneously release small amounts of infectious virus (Gerber and Kirschstein, 1962; Ashkenazi and Melnick, 1963; Black and Rowe, 1963; Sabin and Koch, 1963a,b). Induction attempts using procedures known to induce lysogenic bacteria to produce infectious bacteriophages, such as exposure to mitomycin C, proflavine, hydrogen peroxide, and X irradiation, were only minimally effective when applied to SV40 tumor cells (Sabin and Koch, 1963b; Gerber, 1964; Rothschild and Black, 1970). Combinations of inducing agents were not more effective at induction of infectious SV40 than were the individual agents alone. Only the very rare tumor cell was induced to release infectious virus in those experiments (Rothschild and Black, 1970).

Gerber (1966) discovered that recovery of infectious SV40 was more efficient if the tumor cells were placed in direct contact with susceptible indicator cells, such as primary African green monkey kidney (GMK) cells. The sensitivity of the indicator cell system was increased by the use of inactivated Sendai virus to form heterokaryons (Harris and Watkins, 1965) of tumor and indicator cells (Gerber, 1966; Koprowski *et al.*, 1967; Watkins and Dulbecco, 1967). The fusion technique increased the yield of virus about 1000-fold over simple cocultivation, but the vast majority of the heterokaryons were nonproductive (Koprowski *et al.*, 1967; Watkins and Dulbecco, 1967). Although one of the most sensitive means of virus rescue currently available, it succeeds in inducing only a rare tumor cell to produce virus. The underlying mechanism that allows the fusion technique to facilitate the rescue of SV40 is not known, although it has been reported (Croce and Koprowski, 1973) that enucleated cell cytoplasms are also active in such fusion experiments.

B. "DNA Transfer" Method of Virus Rescue

1. General Methodology

The above accumulation of knowledge about SV40-transformed cells prompted an investigation of the possibility of virus rescue mediated by transformed cell DNA. Chromosomal DNA was extracted by three different procedures from a series of SV40-transformed cell lines. In initial experiments, preparations of cellular DNA with a concentration of 150–200 μg/ml were mixed with an equal volume of diethylaminoethyl (DEAE)-dextran (1 mg/ml). About 0.2 ml of the mixture was then inoculated per culture containing 10^6 cells of a permissive cell type (GMK or CV-1). After 30–45 minutes incubation, the monolayers were washed gently and media added. The cultures were reincubated for 12–14 days before being harvested and assayed for the presence of SV40.

The procedure employed for the extraction of the nucleic acid did not seem to affect the efficacy of virus rescue, but relatively large amounts of transformed cell DNA (> 10 μg/culture of 10^6 cells) were necessary to detect rescue of SV40 by passage in monkey cells. Preparations with relatively high concentrations of DNA form a visible precipitate after mixture with the DEAE-dextran. Sometimes such a precipitate is difficult to pipet, and the nucleic acid probably fails to get distributed evenly over a series of replicate cultures. Therefore, the procedure has been modified slightly. The host cells are pretreated briefly with DEAE-dextran, then the DNA solution is inoculated and the DNA and DEAE-dextran mixed in a corner of the up-ended flask of cells. The cultures are then incubated as detailed above. If ≥ 20 μg of DNA is to be inoculated per culture, it is added sequentially in two or three smaller aliquots with 15-minute incubation periods between each addition. The cultures are not washed until after the final incubation.

2. Recovery of SV40 from Yielder and Nonyielder Cell Lines

Following the protocol described in the previous section, virus was recovered from three different species of SV40-transformed cells (Boyd and Butel, 1972). Representative results are presented in Table I. The efficiency of virus rescue varied from about 50 to 80% of the trials using various cell lines as the source of the donor DNA. It was noteworthy that the DNA transfer method succeeded in rescuing virus from a number of cell lines that were nonyielders in fusion experiments (e.g., SRHH, H-50, and VLM).

The virus that was rescued from the transformed cell lines was iden-

TABLE I

Recovery of Virus from Simian Cells Inoculated with DNA from SV40-Transformed Cells

Source of cellular DNA	Species of origin	Transforming agent	Yield SV40 by fusion	No. positive experiments/ No. trials	Yield of virus (PFU[a]/10^6 cells) First GMK cell passage of cellular DNA extracted by[b] Method 1	Method 2	Method 3
TSV-5	Hamster	SV40	+	9/11	1.3×10^4[c]	4.5×10^3	3.0×10^3
$RIBS_{11}$	Hamster	SV40	+	10/13	6.0×10^3	3.3×10^3	3.0×10^3
SRHH	Hamster	SV40	0	12/15	4.6×10^4	5.5×10^3	6.0×10^4
H-50	Hamster	SV40	0	5/10	9.0×10^2	2.4×10^3	1.1×10^3
SV-3T3	Mouse	SV40	+	4/5	8.5×10^2	ND[d]	ND
VLM	Mouse	SV40	0	6/10	1.0×10^3	1.1×10^3	1.1×10^3
BSC-1-S	Monkey	SV40	0	2/4	8.0×10^2	1.1×10^3	ND
T-22	Monkey	SV40 (T fraction, irradiated)	0	0/3	$< 10^1$	$< 10^1$	ND
DMBA	Hamster	Dimethylbenzanthracene	ND	0/4	$< 10^1$	$< 10^1$	$< 10^1$
Ad 31 ts	Hamster	Adenovirus 31 ts	ND	0/4	$< 10^1$	$< 10^1$	$< 10^1$
SHL-7 (39)	Hamster	PARA (39nT)-adeno 7	ND	0/4	$< 10^1$	$< 10^1$	$< 10^1$
GMK	Monkey	–	ND	0/6	$< 10^1$	$< 10^1$	$< 10^1$
SV40-infected GMK (Hirt supernatant fluid)	–	–	–	6/6	4.1×10^6	3.5×10^7	3.0×10^6

[a] PFU, plaque-forming units.

[b] See Boyd and Butel (1972) for description of extraction methods.

[c] With each type of cellular DNA, no virus was recovered if the DNA were treated with deoxyribonuclease prior to passage or if the DEAE-dextran were omitted at the time the DNA was applied to the simian cells.

[d] ND, not done.

TABLE II

Identification of Virus Recovered from Transformed Cells as SV40

Source of cellular DNA[a]	First GMK cell passage			Plaques induced by first GMK cell passage: induction of SV40 tumor and viral antigens
	Plaque formation neutralized by SV40 antiserum	Papovavirus particles by EM[b]	Induction of SV40 tumor and viral antigens	
TSV-5	+ (3/3)[c]	+	+ (3/4)	+ (3/4)
$RIBS_{11}$	+ (3/3)	+	+ (4/5)	+ (5/5)
SRHH	+ (3/3)	+	+ (5/5)	+ (4/4)
H-50	+ (1/1)	+	+ (5/5)	+ (4/4)
SV-3T3	+ (1/1)	+	+ (2/2)	+ (5/5)
VLM	+ (1/1)	+[d]	+ (4/4)	+ (3/4)
BSC-1-S	+ (1/1)	+	+ (3/3)	+ (2/3)
T-22	ND[b]	0	0 (0/3)	ND
DMBA	ND	ND	0 (0/1)	ND
Ad 31 ts	ND	ND	0 (0/1)	ND
SHL-7 (39)	ND	0	0 (0/2)	ND
SV40 virus stock	+	+	+ (6/6)	ND

[a] The data shown in this table were obtained using DNA prepared by a modification of the Hirt procedure. See Table I for list of transforming agents responsible for initiation of cell lines.

[b] EM, electron microscope; ND, not done.

[c] Numbers in parentheses: numerator, number of samples positive; denominator, number of samples tested.

[d] Results obtained with second GMK cell passage material.

tified as SV40 as shown in Table II. Plaque formation was inhibited by pretreatment with SV40-specific antiserum; the samples contained typical papovavirus-like particles when viewed by electron microscopy; and both SV40 T and viral (V) antigens were detected by immunofluorescence when GMK cells were inoculated with the rescued agents. The absence of T and V antigen induction by material from cells inoculated with DNA from cells transformed by a chemical carcinogen (DMBA), by adenovirus type 31 (Ad 31 ts), or by a defective SV40–adenovirus 7 hybrid [SHL-7(39)] substantiates the conclusion that the virus was rescued from the SV40-transformed cells and was not introduced as a contaminant during the experiments.

Attempts were then made to ascertain the state of the infectious DNA in the transformed cell. Results are summarized in Table III. It appeared that the DNA transfer method was recovering the integrated viral genome because (1) the infectious DNA from transformed cells was found in the Hirt pellet (1967) of precipitated cellular DNA rather

TABLE III

State of the Infectious DNA in SV40-Transformed Cells

Sample tested	Sample should contain the following materials if present in the transformed or infected cells	Recovery of infectious virus
Transformed cells[a]		
Cell lysate	Complete virus	0
Hirt[b] supernate	Free SV40 DNA	0
Hirt pellet	Cellular DNA	+
Marmur[c]-extracted DNA	Total DNA	+
Marmur-extracted DNA, 100°C for 15 min	Denatured linear DNA, intact circular SV40 DNA	0
SV40-infected GMK cells		
Hirt supernate	Free SV40 DNA	+
Hirt pellet	Cellular DNA, trapped SV40 DNA	0[d]
Hirt supernate mixed with Hirt pellet from control GMK cells, 100°C for 15 min	Denatured linear DNA, intact circular SV40 DNA	+

[a] Identical results obtained with 3 different transformed hamster cell lines.

[b] Salt precipitation method of Hirt (1967).

[c] Method described by Marmur (1961).

[d] If higher concentrations of infected GMK cells are extracted ($> 5 \times 10^7$), some trapped SV40 DNA will be detected in the Hirt pellet. The infectivity of the trapped DNA is not abolished by heating.

than the supernate where free SV40 DNA appears and (2) the infectivity was inactivated by boiling which does not destroy free SV40 circular DNA. This technique, therefore, provided additional evidence that the resident viral genome is associated with the cellular DNA.

C. Significance

The evidence is strong that in SV40-transformed cells the viral genome is integrated into the chromosomal DNA of the host cell. Furthermore, it is probable that the complete viral genome is present in most transformed cell lines, although the absolute number of copies of that genome present per cell is still the subject of some controversy.

One of the most intriguing of the unresolved areas pertinent to SV40-transformed cells is the mechanism by which the integration of the viral genome is maintained. Four hypotheses can be proposed: (1) A virus-specific "repressor" is synthesized and the presence of this material blocks the replicative cycle. Cassingena and colleagues (1969; Cas-

singena and Tournier, 1968) reported on the presence of a specific "repressor," protein in nature, in cells of different species transformed by SV40, as well as in cells productively or abortively infected by SV40. However, other investigators using a variety of cell lines and experimental approaches have failed to obtain results compatible with the existence of a cytoplasmic virus-specific repressor (Jensen and Koprowski, 1969; Swetly *et al.*, 1969; Barbanti-Brodano *et al.*, 1970; Butel *et al.*, 1971; Kit *et al.*, 1971). (2) The transformed cells lack some essential factor required by the virus for replication. Several of the above studies showed that the majority of transformed monkey and human cell lines were susceptible to superinfection by SV40 DNA, even though some were resistant to challenge with complete SV40. Therefore, it appears that transformed cells can possess all the necessary SV40 replication factors. (3) Defective viral genomes that are inherently unable to replicate because of a lack of certain genetic information are the transforming agents. Some SV40-transformed monkey kidney cell lines, as well as many SV40-transformed human cell lines, will yield infectious virus under appropriate rescue conditions (Ashkenazi and Melnick, 1963; Koprowski *et al.*, 1967; Kit *et al.*, 1970; Boyd and Butel, 1972), so one cannot conclude that permissive cells can be transformed only by defective particles. (4) An excision system required to release the viral genome from integration is absent.

Recent results by other investigators are encouraging in that they suggest the DNA transfer method may be applicable to other transformed cell systems, in addition to SV40. A similar experimental approach recovered infectious Rous sarcoma virus (Hill and Hillova, 1972; Montagnier and Vigier, 1972) and avian myeloblastosis virus (Lacour *et al.*, 1972) from virus-free, transformed cells.

III. Alteration of Hamster Cells by Exposure to Simian Cell DNA

A. Selection of System

It was described in the preceding section that the transfer of SV40-transformed cell DNA into susceptible monkey cells resulted in the recovery of infectious SV40, even from some transformed cell lines that had been nonyielders in fusion experiments. Since the bulk of the DNA taken up by the permissive cells in such rescue experiments represents cellular genetic information rather than viral sequences, the possibility was considered that donor cellular genes might be expressed in the recipient cells. As mentioned before, in previous gene transfer experi-

ments with mammalian cells it has been extremely difficult to detect expression of biological activity by the foreign DNA.

Therefore, a virus-related marker, susceptibility to poliovirus, was selected to monitor for biological activity in such a gene transfer study. Nonprimate cells, such as those of hamster or mouse origin, do not possess poliovirus receptor sites, while primate cells (e.g., those of monkey or human origin) readily adsorb and eclipse poliovirions (McLaren *et al.*, 1959; Holland and McLaren, 1959; Holland *et al.*, 1959). It was found that after exposure to DNA extracted from monkey kidney cells, hamster cells developed the capacity to attach poliovirions (Boyd *et al.*, 1974a).

B. Adsorption of Poliovirus by DNA-Treated Hamster Cells

Cultures of hamster (BHK-21) cells were exposed to cellular DNA as described above. At various time intervals, some of the treated cultures were inoculated with poliovirus, while replicate cultures were trypsinized and passed before being challenged with poliovirus at different times after seeding. Cultures were harvested 24 hours after inoculation with virus and assayed to determine the amount of recoverable virus (Fig. 1). In contrast to cultures of BHK-21 cells treated only with DEAE-dextran or with mouse 3T3 cellular DNA, BHK-21 cells that had been exposed to monkey BSC-1 cell DNA and subcultured ap-

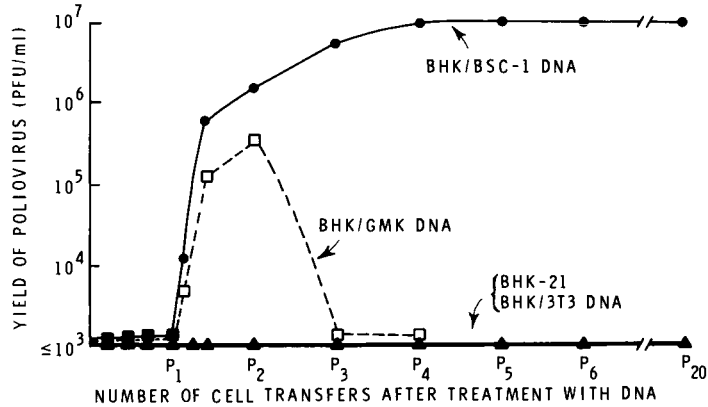

FIG. 1. Response of hamster (BHK-21) cells to poliovirus after treatment with 20–30 μg of simian (BSC-1, GMK) cell or mouse (3T3) cell DNA in the presence of DEAE-dextran. Replicate cultures were inoculated with virus at intervals after treatment with DNA before and after subculturing. Cultures were harvested 24 hours after infection, and the amount of recoverable virus determined by plaque assay in BSC-1 cells.

peared to yield increased amounts of poliovirus. Only background levels of virus were recovered from treated cells that had not been passed. Cells exposed to BSC-1 DNA that had been digested with DNase responded similar to the normal BHK-21 cells when challenged with virus (not shown on graph), indicating that the alteration was mediated by DNA.

In some instances, the affinity of the treated cells for poliovirus was a "stable" phenomenon and was expressed for about 20 passages. The cells then reverted and resumed their original characteristic of resistance to poliovirus. In other experiments, the increased susceptibility to poliovirus was more transient and was observed for only a few passages (e.g., BHK/GMK DNA in Fig. 1). Experiments utilizing GMK cell DNA were only performed two times, so it would be premature to interpret the curves in Fig. 1 as a reflection of different transforming activities by DNA's derived from GMK and BSC-1 cells.

The reproducibility of the phenomenon is summarized in Table IV. BHK-21 cells exposed to BSC-1 cellular DNA exhibited increased susceptibility to poliovirus 8 times in 14 separate experiments. Three times the alteration was a "stable" phenomenon, lasting about 20 passages before the cells reverted to normal. In both experiments in which GMK cell DNA was employed as the donor DNA, recipient BHK-21 cells exhibited a transient susceptibility to poliovirus, while no change was observed in the virus susceptibility of BHK-21 cells treated with mouse 3T3 cell DNA.

TABLE IV

Reproducibility of Phenomenon of Alteration of BHK-21 Cells by Monkey Cell DNA[a]

Host cell	Donor cellular DNA	Increased susceptibility to poliovirus type 1		
		No. of trials	No. of positive experiments[b]	No. of times alterations stable[c]
BHK-21	BSC-1	14	8	3
BHK-21	GMK	2	2	0
BHK-21	3T3	2	0	0

[a] Cultures containing 10^6 BHK cells were exposed to 20–30 μg of cellular DNA in the presence of 1 mg/ml DEAE-dextran. The cells were passed and challenged with poliovirus type 1 at various intervals after seeding. Twenty-four hours after inoculation the cultures were harvested and the yield of poliovirus determined by plaque assay in BSC-1 cells.

[b] An experiment was designated positive if the yield of poliovirus from treated cultures was at least 10-fold greater than that from normal BHK-21 cells.

[c] The alteration was considered to be "stable" if it persisted for at least 20 passages. In all 3 cases, the altered BHK-21 cells eventually reverted to normal.

The relative percentage of cells in a population which were altered by the DNA transfer procedure was investigated using an infectious center assay. Cultures were inoculated with poliovirus, incubated, and then treated with poliovirus antiserum. Known cell densities were plated on monolayers of BSC-1 cells, and the frequency of infectious center formation was calculated from the number of plaques that developed relative to the number of cells plated (Table V). With normal BHK-21 cells and BHK-21 cells treated with 3T3 cell DNA, not more than 0.2% of the virus-exposed cells initiated plaque formation. In contrast, BHK-21 cells exposed to BSC-1 cell DNA exhibited increased frequencies of plaque formation, ranging from 1.2% in one experiment (B/B-II) to 8% with cells from a second experiment (B/B-I). Clonal lines derived from the B/B-II culture exhibited much higher levels of infectious center formation (14–90%).

To explain the increased susceptibility to poliovirus by the BSC-1 DNA-treated BHK-21 cells, it was possible either (a) that the inoculum virus was tightly adsorbed to the hamster cells and was recovered 24 hours later, or (b) that it was undergoing a complete cycle of replication. Two of the derivative clonal cell lines (B/B-II clone 5 and B/B-II clone 7) were selected to serve as hosts for poliovirus growth cycle studies (Fig. 2). A typical growth cycle was obtained in BSC-1 cells, while no virus growth was observed in normal BHK-21 cells. No evidence for eclipse and replication of poliovirus in either clone derived from the BSC-1 DNA-treated BHK-21 cells was obtained. These results suggest

TABLE V

Frequency of Infectious Center Formation by DNA-Treated BHK-21 Cells after Exposure to Poliovirus Type I[a]

Cell line	No. of experiments	Average percent plaque-forming cells
BHK-21	3	0.1
BHK-21/3T3 DNA	2	0.2
BHK-21/BSC-1 DNA (B/B-I)	2	8.0
BHK-21/BSC-1 DNA (B/B-II)	2	1.2
B/B-II clone 5	1	14.0
B/B-II clone 7	1	90.0
B/B-II clone 8	2	75.0

[a] Cultures of each cell line were inoculated with 1–5 PFU/cell of poliovirus type I, were incubated at 37° for 1 hour, and were washed with Tris buffer. Poliovirus antiserum was added, the cultures were reincubated at 37°C for 30 minutes, and were washed with Tris buffer. The cells were trypsinized, counted, plated on monolayers of BSC-1 cells, and overlaid with agar after an adsorption period of 1 hour at 37°C. The percentage of plaque-forming cells was determined from the number of plaques which developed.

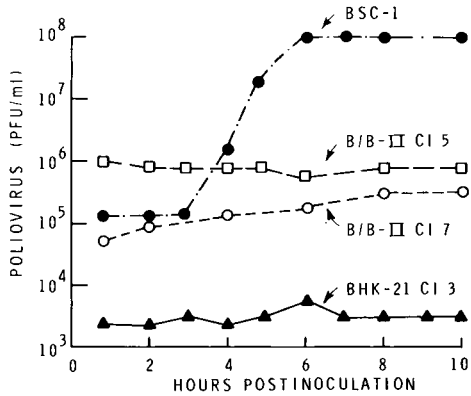

FIG. 2. Kinetics of replication of poliovirus type I in monkey (BSC-1) cells, hamster (BHK-21) cells, and two clonal lines derived from BHK-21 cells exposed to BSC-1 cell DNA (B/B-II clone 5, B/B-II clone 7). Replicate cultures containing 10^6 cells were inoculated with virus and were harvested hourly over a 10-hour period. The amount of virus recovered was determined by plaque assay in BSC-1 cells.

that the increased "yields" shown in Fig. 1 are actually due to an increased attachment of poliovirus to the cells. Although the attached virions do not appear to be eclipsed (Fig. 2), the data in Table V suggest that they are in an antibody-resistant state.

Since virus receptor sites are a membrane phenomenon, the altered hamster cells were analyzed using cytotoxic antibody prepared in rabbits against hamster and monkey cells and kindly provided by Dr. S. S. Tevethia (Table VI). The two anti-cell sera killed only 5 and 7% of the

TABLE VI

Demonstration of Cell Surface Antigens on DNA-Treated BHK-21 Cells by Cytotoxicity Tests[a]

Cell line	Percent cells killed by cytotoxic antibody		
	Normal rabbit serum	Anti-hamster cell serum	Anti-monkey cell serum
BHK-21	0	95	5
BSC-1	0	7	87
BHK-21/BSC-1 DNA (B/B-I)	6	80	90
BHK-21/BSC-1 DNA (B/B-II)	0	67	17
B/B-II clone 5	0	64	29
B/B-II clone 7	0	70	50
B/B-II clone 8	0	70	50

[a] Cells were trypsinized and treated with antiserum and complement. The number of viable cells was determined by staining with trypan blue.

cells obtained from the heterologous species (BHK-21 and BSC-1 cells for the anti-monkey and anti-hamster cell sera, respectively). However, the anti-monkey cell serum did show significant levels of cytotoxicity against all the BSC-1 DNA-treated BHK-21 cell lines tested. That the cell lines were of hamster origin was indicated by the high percentage of cells killed by the anti-hamster cell sera, as well as by the fact that all the cells eventually reverted to their original hamster cell property of resistance to poliovirus infection.

C. Significance

This study demonstrated that it is possible to transfer functional DNA from monkey cells to hamster cells in the presence of DEAE-dextran. The marker used to detect biological activity by the foreign DNA was the ability of hamster cells to adsorb poliovirus type I. The cells altered by the transforming monkey BSC-1 cell DNA appeared to have uncoupled the phenomenon of attachment (and possibly penetration) from that of uncoating of poliovirus, since the adsorbed virus did not become eclipsed. Such cells would be useful for a detailed study of the one phenomenon (attachment) in the absence of the second (uncoating). Currently, we have no knowledge of the intracellular state of the foreign DNA in the recipient cells. The fact that the membrane alterations that allowed attachment of poliovirions did not prove to be permanent would suggest that the simian DNA was probably not incorporated into the hamster cell chromosomes. The factors that influence the stability of expression of biological functions coded for the transferred DNA remain to be determined.

There is a burgeoning interest in the general area of gene transfer in mammalian cell systems. A variety of approaches have been attempted (Bhargava and Shanmugan, 1971). Some recent studies have accomplished the transduction of galactose-1-phosphate uridyltransferase in galactosemic fibroblasts by bacteriophage λ (Merril *et al.*, 1971), the introduction of a thymidine kinase gene into L cells lacking the enzyme by irradiated herpesvirus (Munyon *et al.*, 1971), the synthesis after infection by Shope rabbit papilloma virus of arginase in human fibroblasts from a patient with the enzyme deficiency (Rogers *et al.*, 1973), the synthesis of melanin in skin cells from albino mice after exposure to nucleoprotein from pigmented mouse cells (Ottolenghi-Nightingale, 1969), and the appearance of hypoxanthine-guanine phosphoribosyltransferase in mouse cells after exposure to metaphase chromosomes isolated from Chinese hamster cells (McBride and Ozer, 1973). The present study illustrates (a) the feasibility of using the DNA transfer method for ac-

complishing gene-transfer between mammalian cells, as well as (b) the usefulness of virus-related markers for the detection of biological activity by the transferred DNA.

IV. Formation of "Heterologous" SV40 Pseudovirions

A. Description of Pseudovirions

Pseudovirions are viral particles which contain cellular DNA rather than the normal viral genome. They were first detected in preparations of polyoma virus by Michel *et al.* (1967) and subsequently in SV40 stocks (Levine and Teresky, 1970; Trilling and Axelrod, 1970). Pseudovirions have attracted attention because of their inherent potential for acting as transducing particles in mammalian cells. The possibility that pseudovirions might transduce genetic information from one host cell to another is strengthened by the following observations: (1) Pseudovirions of polyoma virus are adsorbed to, and uncoated by, mouse embryo cells as evidenced by the susceptibility of the pseudovirion DNA to digestion by pancreatic DNase (Osterman *et al.*, 1970). (2) Monkey cell DNA carried by SV40 pseudovirions was detected in the nuclei of mouse cells after infection with the pseudovirions and was still physically intact (Grady *et al.*, 1970). (3) Mouse DNA was introduced into the nuclear fraction of both human and mouse cells following exposure to labeled, purified polyoma pseudovirions (Qasba and Aposhian, 1971). However, a major problem of the system with respect to transduction capabilities is a lack of specificity, since cellular DNA sequences are apparently incorporated randomly into pseudovirions (Grady *et al.*, 1970).

As one approach to this problem, we hypothesized that mammalian cell DNA introduced exogenously during a viral replicative cycle might be encapsidated during viral maturation. Experiments designed to test the hypothesis utilized the "DNA transfer" methodology shown to effect the recovery of SV40 from transformed cell DNA and to alter hamster cells so that they adsorbed poliovirions (Sections II and III). In this study, total cellular DNA from a heterologous species (hamster) was used rather than the ideal of a specific DNA sequence. Evidence was obtained that the production of such "heterologous" pseudovirions is both biologically and technically feasible (Boyd *et al.*, 1974b).

B. Incorporation of Hamster Cell DNA into SV40 Particles

GMK cells infected with SV40 and incubated in the presence of [^{3}H]thymidine (TdR) were inoculated at 16 hours postinoculation (p.i.)

with 20 μg/culture of ^{14}C-labeled adenovirus-transformed hamster cell (Ad 12 Cl_4) DNA mixed with 1 mg/ml DEAE-dextran. Virus was harvested at 48 hours p.i., purified, and centrifuged to equilibrium in a gradient of cesium chloride (CsCl) (Fig. 3). A ^{3}H-labeled peak was observed at 1.330 gm/ml, while ^{14}C label was detected in a peak that was almost coincident but was slightly lighter, nearer a density of 1.329 gm/ml. The peaks of radioactivity were collected, dialyzed, incubated with 100 μg/ml of DNase for 30 minutes at 37°C, and then treated with 10% trichloroacetic acid (TCA) or anti-SV40 monkey serum. As can be seen in Table VII, the majority of both the ^{3}H- and ^{14}C-labeled DNA remained acid-precipitable (75% and 69%, respectively). Ninety percent of the ^{3}H label and 80% of the ^{14}C label was precipitated by SV40-specific antibody. These data indicate that the ^{14}C-labeled DNA was enclosed in a deoxyribonuclease-resistant state in particles having the antigenicity of SV40 and a density near that of complete SV40 virions. One interpretation of these results is that SV40 pseudovirions carrying DNA from a heterologous species had been formed.

The physical state of the ^{14}C-labeled DNA carried by the pseudovirions was then characterized. DNA was extracted from purified preparations of pseudovirion-containing virus grown in GMK and CV-1 cells, and the DNA was analyzed by alkaline sucrose gradient centrifugation (Fig. 4). In samples from both GMK (Fig. 4A) and CV-1 (Fig. 4B) cells, the ^{3}H-labeled DNA separated into two bands. The faster sedimenting band was in the region of 53 S, the sedimentation coefficient for SV40

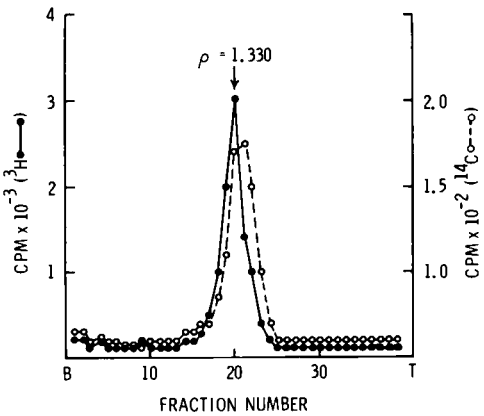

FIG. 3. CsCl equilibrium gradient centrifugation of purified virus from GMK cells. Infected cultures were incubated in the presence of [^{3}H]TdR for 16 hours, the [^{3}H]TdR-containing medium was removed, and 20 μg/culture of [^{14}C]TdR-labeled Ad 12 Cl_4 hamster cell DNA added in the presence of 1 mg/ml DEAE-dextran. Virus was harvested at 48 hours p.i., purified, and centrifuged in CsCl.

TABLE VII

Precipitation of Radioactive Label in Purified Virus Obtained from SV40-Infected Cells Exposed to Adenovirus-Transformed Hamster Cell DNA[a]

Reagent used for precipitation of purified virus	Distribution of counts/minute	Radioisotope	
		^{3}H	^{14}C
Trichloroacetic acid	Total	20,540	1747
	Precipitate	15,410	1210
	Supernate	4,100	257
	% in precipitate	75	69
SV40-specific antiserum	Total	15,430	1057
	Precipitate	13,800	845
	Supernate	1,590	207
	% in precipitate	90	80

[a] GMK cells were infected with SV40 in the presence of 3 μCi/ml of [^{3}H]TdR. At 16 hours p.i., the [^{3}H]TdR was removed and 20 μg/culture of ^{14}C-labeled Ad 12 Cl_4 DNA added in the presence of 1 mg/ml DEAE-dextran. Virus was harvested 48 hours p.i., treated with DNase and RNase, and centrifuged to equilibrium in gradients of CsCl. The peak of radioactivity was collected, dialyzed, and divided into two samples. One sample was treated with DNase and then precipitated with cold 10% TCA. The other sample was exposed to SV40-specific antiserum and the precipitate removed by centrifugation. The percentage of each radioisotope which was precipitable under the above conditions was then determined.

component I DNA under alkaline conditions (Vinograd *et al.*, 1965). The second ^{3}H-labeled band was slower sedimenting (16 S–18 S). Under alkaline conditions, the nicked form II SV40 DNA separates into single-stranded circles (18 S) and single-stranded linear molecules (16 S). Monkey cellular DNA labeled during the 16-hour exposure to the [^{3}H]TdR and incorporated into the pseudovirions would also be expected to appear in this region of the gradient (~ 14 S). All the ^{14}C-labeled DNA from the pseudovirions in the virus preparations sedimented in the 15 S–18 S region of the gradient, indicating it had a linear configuration. It can be seen by comparing Figs. 4A and 4B that much less ^{14}C-labeled DNA was recovered from virus grown in CV-1 cells than from virus harvested from primary GMK cells.

An aliquot of the DNA preparation extracted from purified virus was also centrifuged in CsCl gradients containing 250 μg/ml ethidium bromide, while a second aliquot of the DNA was denatured and chromatographed on a nitrocellulose column. Both these techniques confirmed that the ^{14}C-labeled DNA incorporated in pseudovirions was linear.

The effect of the relative amount of inoculum of heterologous DNA

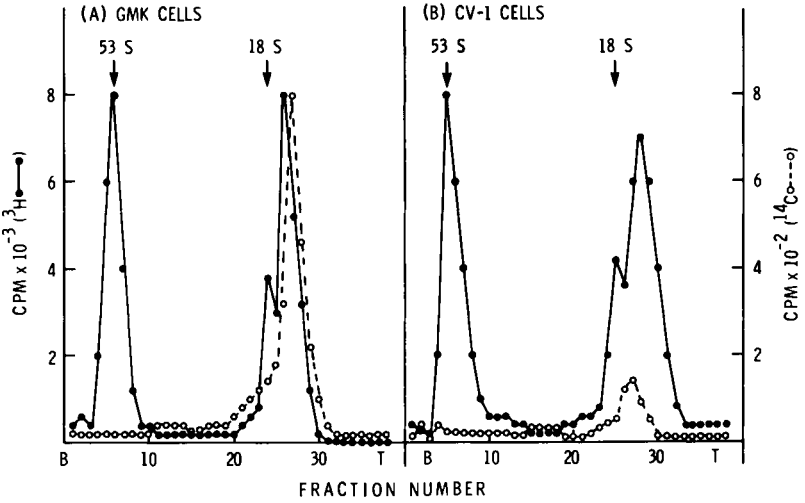

FIG. 4. Sedimentation analysis of DNA extracted from "heterologous" pseudovirion-containing purified virus in 5–30% linear gradients of alkaline sucrose. Gradients were centrifuged at 21°C for 2 hours at 35,000 rpm using an SW 50.1 rotor. Virus was grown as described in Fig. 3. (A) Virus from GMK cells. (B) Virus from CV-1 cells.

on the size of the pseudovirion population produced was then determined. The experiment was carried out as before, except that varying amounts of ^{32}P-labeled Ad 12 Cl_4 DNA were added to SV40-infected GMK cells. DNA from purified progeny virus was analyzed by alkaline sucrose sedimentation (Fig. 5). Negligible counts of ^{32}P were recovered from SV40-infected GMK cultures exposed to 1 μg Ad 12 Cl_4 DNA (Fig. 5A). Figure 5B shows results with DNA extracted from virus recovered from cells exposed to 10 μg Ad 12 Cl_4 DNA. A ^{32}P-labeled peak was observed in the 15 S–18 S region of the gradient. The results obtained when cells were inoculated with 50 μg of hamster cell DNA are shown in Fig. 5C, where a larger ^{32}P-labeled peak was observed at 15 S–18 S. The relative amount of hamster cell DNA in the pseudovirions produced was directly related to the amount of heterologous cellular DNA added to the infected cells.

C. Significance

Pseudovirions produced as "by-products" of a normal viral replicative cycle resemble "generalized transducing" bacteriophage in that no specific region of the cellular genome appears to be preferentially incorpo-

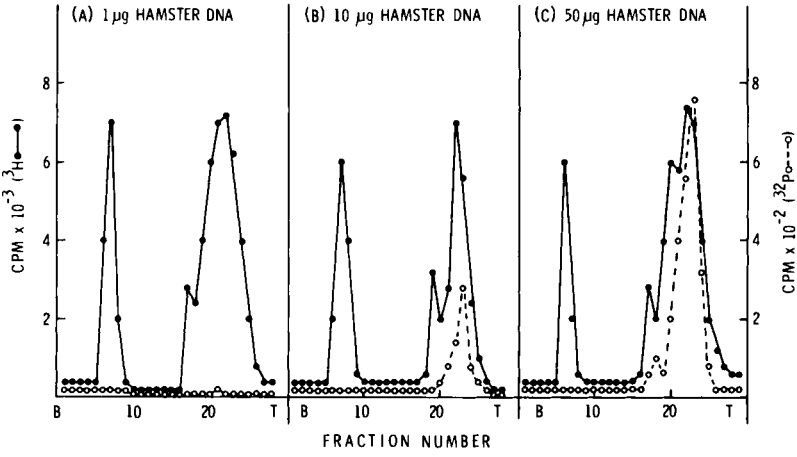

FIG. 5. Effect of amount of inoculum of heterologous DNA on production of "heterologous" pseudovirions. Varying amounts of ^{32}P-labeled Ad 12 Cl_4 hamster cell DNA (specific activity, 4×10^3 cpm/μg) were added per infected GMK cell culture following the protocol outlined in Fig. 3. Three replicate cultures were pooled for each sample. DNA was extracted from purified virus and analyzed by alkaline sucrose sedimentation. Samples were centrifuged at 21°C for 4 hours at 25,000 rpm using an SW 25.1 rotor. (A) 1 μg Ad 12 Cl_4 DNA added per SV40-infected GMK cell culture. (B) 10 μg Ad 12 Cl_4 DNA added per culture. (C) 50 μg Ad 12 Cl_4 DNA added per culture.

rated within the papova viral capsids (Winocour, 1968; Grady *et al.*, 1970). To fulfill the ultimate goal of using pseudovirions for the treatment of human genetic defects, it is necessary that a "gene-enrichment" technique be devised that would facilitate the preparation of pseudovirions containing the desired host chromosome fragment. Although the mouse–hamster hybrid cell line described by Basilico and Burstin (1971) accomplishes an enrichment in the proportion of pseudovirions in the polyoma virus yield, there is no evidence to suggest that any concomitant enrichment in the incorporation of a specific host cell gene into the pseudovirions occurs as well.

The results described above reveal that exogenously added DNA can be encapsidated within SV40 particles, indicating that the production and detection of heterologous pseudovirions can be accomplished. To add specificity, one must use a defined sequence of donor DNA rather than total cellular DNA employed in the current study. Biological activity has not yet been demonstrated for the heterologous pseudovirions, an obvious prerequisite before denoting them successful transducing agents. However, this problem should be minimized and probably surmounted by the use of a specific donor gene or genes.

V. Discussion and Forecast

The three studies described here serve to illustrate the promise that the DNA transfer method holds for the future with respect to the transfer of genetic information between mammalian cells. In all three studies, total cellular DNA from one species was inoculated into tissue cultures of cells derived from another species, and, in all three cases, it was possible to show that something happened to the foreign DNA once it was engulfed by the recipient cells. First, the SV40 genome was excised and replicated, resulting in the formation of progeny virus. Second, the foreign (simian) DNA was at least transiently functional because a small fraction of the recipient hamster cells developed the capacity to attach poliovirions. Third, the inoculated foreign DNA was cleaved in cells infected with SV40 and encapsidated, producing what were designated "heterologous" pseudovirions.

It is apparent that more questions were raised by these observations than were answered. In none of the studies was evidence available to indicate the underlying mechanism of action. At this time, there is no need to speculate that a single basic mechanism is operative in all three cases. Nevertheless, the usefulness of the DNA transfer method as a tool for transmitting genetic information between somatic cells is probably limited primarily by a technical inability to monitor for numerous biological markers in such systems.

It would be premature to speculate at this time as to the relative merits of the three general approaches described in this report or to attempt to predict which might ultimately be of more value. Exhaustive quantitative studies need to be performed to determine the amount of foreign DNA that can be encapsidated and ultimately delivered to a new recipient cell by the heterologous pseudovirions and contrast that with the amount of naked DNA taken up directly by a cell.

Data obtained to date (Kelly and Butel, 1973; Boyd *et al.*, 1974b) suggest that a prime difficulty with the DNA transfer method is the inefficient uptake of DNA by mammalian cells. Using DEAE-dextran as a facilitator, the most efficient compound currently available, only a small fraction of the DNA inoculated into a culture appears to remain bound to the recipient cells. Generally, cultures containing 10^6 cells will only take up 2–3 μg of DNA, regardless of the quantity of nucleic acid inoculated. It is apparent, then, that to increase the extent of biological alterations in any type of transfer experiment methodology needs to be developed to increase the amount of foreign DNA introduced into the recipient cells. This should not be an insurmountable problem, however.

A more complex problem that must be considered is the need for

greater specificity in the transfer studies. Known nucleic acid sequences coding for delineated gene products must ultimately be employed in order to make the technique practical as a means of efficiently adding new genetic information to deficient cells in the absence of the specter of adding, at the same time, vast numbers of foreign genes with unforeseeable consequences.

Although the problems outlined above are many, it is not inconceivable that gene transfer studies are the wave of the future. Indeed, they may well be one of the more prominent legacies of the sophisticated virology practiced throughout the last decade.

ACKNOWLEDGMENTS

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CHAPTER 3

Viruses with Separately Encapsidated Complementary DNA Strands

H. D. MAYOR AND E. KURSTAK

I. Introduction and Nomenclature

Since their first isolation over a decade ago there has been a rapid growth in the number of small viruses that contain single-stranded DNA (parvoviruses, picodnaviruses). Recent isolates now include agents that infect various rodents, dogs, pigs, cats, cattle, mink, chickens, quail, geese, monkeys, and man. Because of this rapid burst of potential parvovirus candidates, the details of their system of classification have not been finalized; however, many of their biological and biochemical properties are very similar. These viruses are all small (20–25 nm in diameter), contain single-stranded DNA, and display icosahedral symmetry.

They are resistant to high temperatures (e.g., 56°C for 30 minutes) and contain no essential lipids, since their infectivity is resistant to treatment with chloroform or ether. They all show a relatively high density in cesium chloride (1.38–1.46 gm/cm^3). The known agents are currently divided into two subgroups. Subgroup A includes the autonomously replicating parvoviruses, with the prototype virus being the Kilham rat virus, RV (Kilham and Olivier, 1959). Other examples are the hamster osteolytic viruses H_1, H_3 (Toolan, 1960a,b), the X_{14} virus of rats (Payne *et al.*, 1963), minute virus of mice, MVM (Crawford, 1966), and feline panleukopenia virus (Johnson *et al.*, 1967). Subgroup B comprises the adeno-associated satellite viruses ASV, AAV (Mayor *et al.*, 1965; Archetti *et al.*, 1966; Atchison *et al.*, 1966; Smith *et al.*, 1966), parvoviruses which are defective and cannot multiply unless adenoviruses are present as helper viruses (Atchison *et al.*, 1965; Parks *et al.*, 1967a). Herpesviruses may act as partial helper viruses. However, in their presence, satellite structural antigens are made (Atchison, 1970; Blacklow *et al.*, 1970, 1971) and infectious DNA is replicated (Boucher *et al.*, 1971). No infectious particles are produced.

Although some satellite virus serotypes are indigenous to man, these agents have not been associated with any disease process. DNA extracted from satellite virus by conventional methods acts as a double-stranded helix (Parks *et al.*, 1967b). However inside the virion the DNA reacts with formaldehyde and stains with acridine orange as a single-stranded species (Mayor *et al.*, 1969a). Either a "plus" or a "minus" strand of complementary DNA is encapsidated in each virion. After extraction at high ionic strength (2 × SSC), plus and minus strands anneal rapidly to yield double-stranded DNA (Mayor *et al.*, 1969b; Rose *et al.*, 1969).

Viruses with properties similar to the parvoviruses described above also infect insects, plants and bacteria. One of these is the densonucleosis virus (DNV) of the insect *Galleria mellonella* L (Meynadier *et al.*, 1964). Many biophysical properties of this virus make it a candidate for inclusion among the parvoviruses. It is, however, unique in that although like the adeno-associated satellite viruses, DNV contains separately encapsidated "plus" and "minus" single strands of DNA in individual virions, the agent can replicate autonomously without participation of a helper virus. In addition a detailed electron microscopic examination of the capsid structure of DNV (Kurstak and Côté, 1969) indicates that the number of capsomeres may be 42—a figure in excess of the largest count obtained for other parvoviruses (32 capsomeres). It was, therefore, suggested that DNV might be more properly isolated in a separate group of its own, Densoviridae. This point has not been

settled and must await further scrutiny and comparison with the numerous parvovirus candidates that are currently appearing (see review by Hoggan, 1971). However, whatever the outcome there are still only two distinct groups of small DNA viruses in which the population of virus particles contains separately encapsidated, complementary single DNA strands. These are the adeno-associated satellite viruses (ASV, AAV) of man and animals and the densonucleosis virus (DNV) of the insect *Galleria mellonella* L. These viruses have a number of properties in common and a number of intriguing differences which we will pursue in the subsequent sections.

II. Adeno-associated Satellite Viruses (ASV, AAV) and Densonucleosis Virus (DNV)

A. Biological and Biophysical Properties

Because of their ubiquity, physical stability, and defective nature, adeno-associated satellite viruses have proved excellent prototype viruses, and a wide range of reliable biological and biophysical data has been assembled on the various serotypes. Type 4 satellite virus (Ito and Mayor, 1968) and the bovine satellite virus (Luchsinger *et al.*, 1970)

TABLE I

Properties of Satellite Virus and Densonucleosis Virus

Property	Adeno satellite virus (ASV)	Densonucleosis virus (DNV)
Physical characteristics	~20 nm diameter, icosahedral symmetry, 12 to 32 capsomeres, 1.38–1.43 gm/cm^3 in CsCl, heat stable, ether resistant	~20 nm diameter, icosahedral symmetry, 42 capsomeres ?, 1.40–1.44 gm/cm^3 in CsCl, heat stable, ether resistant
Immunological characteristics	Types 1, 2, 3 human origin, type 4 simian origin (HA +) avian, canine, bovine types	?
Chemical characteristics	Protein 74%, single-stranded DNA 26%, + and − strands separately encapsidated	Protein 63% single-stranded DNA 37% + and − strands separately encapsidated
Replication characteristics	Nonconditionally defective	Autonomous

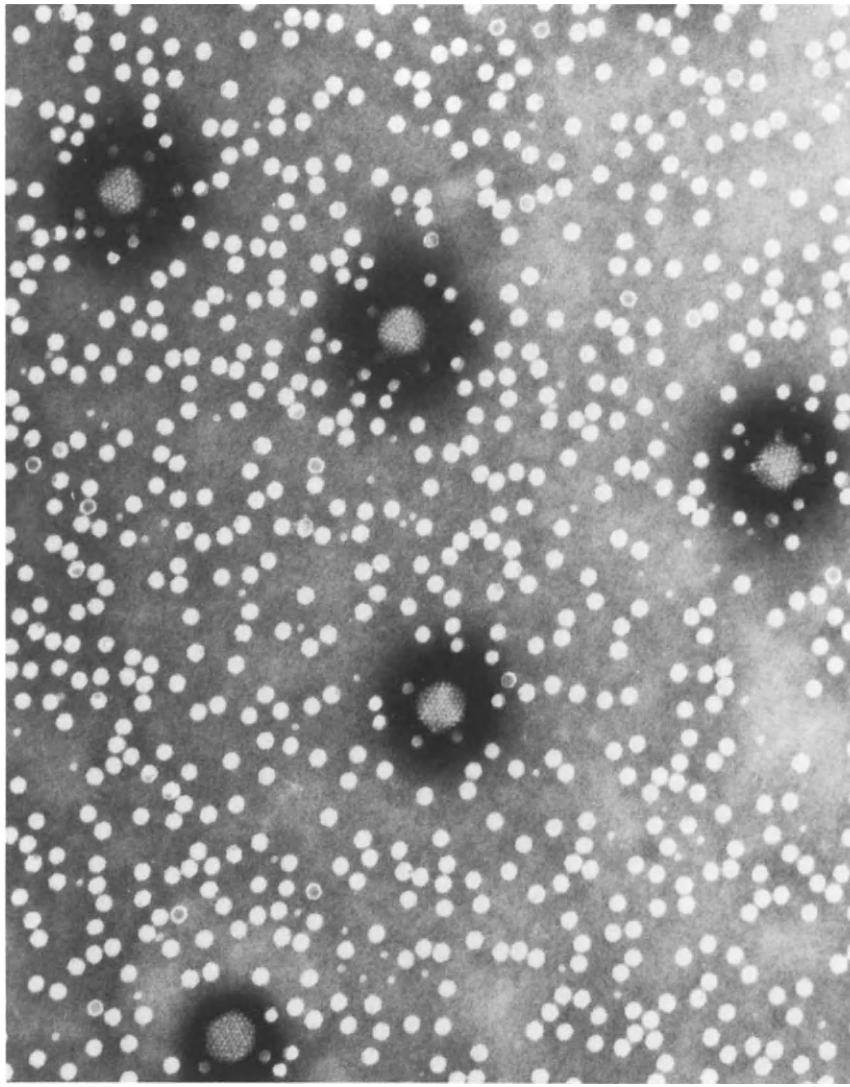

FIG. 1. Human adenovirus type 31 (larger particle 70 nm diameter) and type 1 satellite virus (smaller particle 20 nm diameter). ×127,500.

have been found to have an associated hemagglutinin, while types 1, 2, and 3 and densonucleosis virus have not demonstrated any hemagglutinating activity using a wide range of red blood cells (Kurstak, 1972).

Table I compares and contrasts some of the physical and biological properties of satellite viruses and DNA.

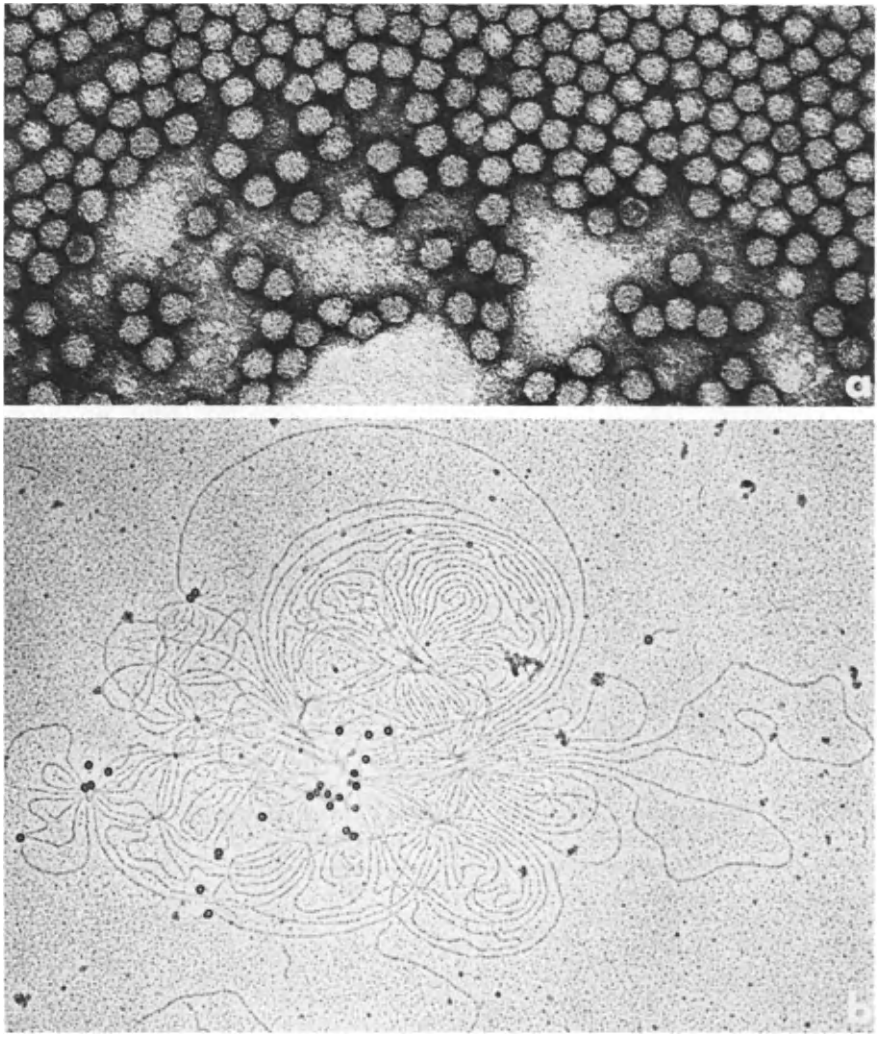

FIG. 2. (a) DNV 20 nm diameter purified by zonal centrifugation in sucrose gradients. Stained with uranyl acetate 1%. ×200,000. (b) Molecules of tangled viral DNA obtained after extraction at high ionic strength and moderate temperature. Spread and shadowed. ×44,000.

Satellite viruses are spherical and 20–25 nm in diameter (Fig. 1). They display cubic symmetry of the icosahedral pattern (Mayor and Melnick, 1966), but because of their small size and uneven penetration by negative stains used in electron microscopy it is difficult to determine the actual number of capsomeres making up the virus capsid. Currently cap-

somere counts of 12, 20, and 32 have been reported and all are possible solutions. Their significance has been discussed by Mayor in a recent review (Mayor, 1973). The DNV capsid has been examined by negative staining by Kurstak and Côté (1969). Particles were approximately 20 nm in diameter and exhibited icosahedral symmetry (Fig. 2a). Again it was difficult to discern the actual number of capsomeres, but even casual scrutiny indicated that in favorable images there were more capsomeres than in the case of satellite viruses. Kurstak and Côté considered 42 to be the most likely solution. Empty capsid components void of DNA were also observed, but there did not appear to be any incomplete particles with a partial complement of DNA similar to those reported by Torikai *et al.* (1970).

Both satellite viruses and DNV have a relatively high buoyant density in CsCl (1.38–1.44 gm/cm^3), with the type 4 satellite being the most dense and type 2 the lightest. DNV has a density equivalent to that of type 4, but, unlike type 4 virus, has no hemagglutinin activity. Type 4 satellite agglutinates human, monkey, guinea pig, and sheep cells well at 4°C but not at 37°C, while the other ASV types 1, 2, and 3 do not appear to agglutinate red cells either at 4°C or 37°C (Ito and Mayor, 1968). Kurstak (1972) has shown that DNV does not agglutinate erythrocytes of monkey, horse, cow, duck, goat, hamster, sheep, pig, mouse, or rooster. Comparative hemagglutination patterns for some selected small DNA viruses are shown in Table II.

In comparative serological studies Hoggan (1971) has shown that

TABLE II

Hemagglutination Patterns of Selected Picodnaviruses at 4°C with Various Red Blood Cells[a]

Virus	Guinea pig	Human	Rat	Monkey	Sheep
H-1	++	++	+	+	−
H-T	++	−	−	−	−
H-3	++	+	++	++	−
RV	++	++	+	++	−
X14	++	−	N.D.[b]	++	−
HB	++	−	+	−	−
MVM	++	−	+	N.D.	−
ASV 1	−	−	−	−	−
ASV 2	−	−	−	−	−
ASV 3	−	−	−	−	−
ASV 4	+	++	N.D.	+	+
DNV	−	−	−	−	−

[a] These data have been selected from a number of sources: Ito and Mayor (1968), Mirkovic *et al.* (1971), Toolan (1968), Hoggan (1970), Crawford (1966), and Kurstak (1972).

[b] Not determined.

there is some immunological cross reactivity between satellites type 2 and 3. This reactivity is demonstrated by both complement fixation (CF) and fluorescent antibody (FA). No cross reactions were observed between DNV and satellite viruses types 1, 2, 3, and 4.

B. Replication Cycles and Helper Viruses

In the absence of viable adenovirus, morphological observations on infected cells coupled with quantitative fluorescent antibody studies show that adeno-associated satellite virus does not synthesize its own structural antigens, indicating a nonconditional dependence on the helper for full expression of satellite virus genes (Ito *et al.*, 1967; Blacklow *et al.*, 1967). Although the exact nature of satellite defectiveness is not currently understood, it is clear that events in the adenovirus replication cycle requiring approximately 10 hours must take place before satellite virus synthesis can proceed to completion. This represents a fairly late step in adenovirus replication, certainly later than T antigen production or synthesis of adenovirus DNA. Multiplication of satellite virus appears to take place in the nuclei of infected cells (Atchison *et al.*, 1965; Archetti *et al.*, 1966; Mayor *et al.*, 1967) but in discrete regions that are not usually in close juxtaposition (Fig. 3). How-

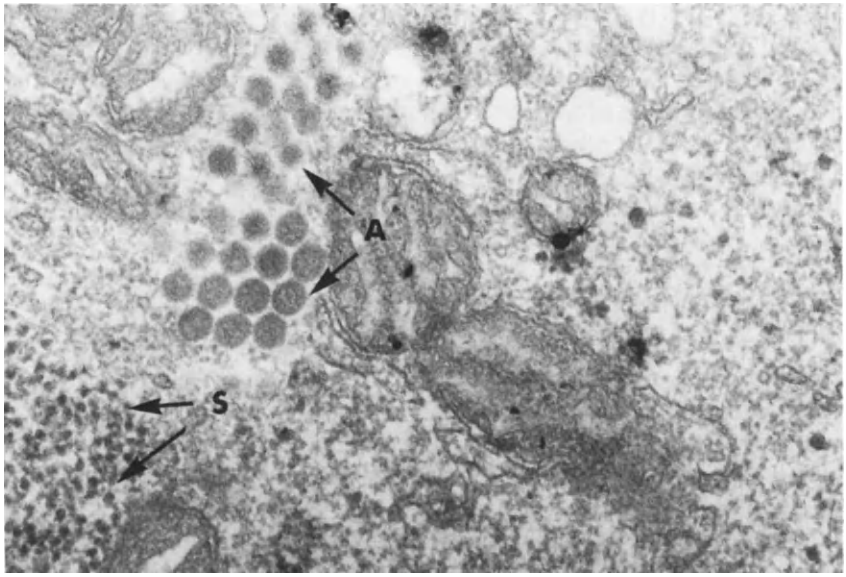

FIG. 3. Monkey kidney tissue culture monolayer infected with simian adenovirus (A) and adeno-associated satellite virus (S). Viruses appear to form separate inclusions. ×56,000.

ever, Henry (1973) has recently reported that large crystalline arrays of satellite virus may be observed in the same nucleus at the periphery of adenovirus crystals, suggesting coincident maturation of satellite and adenovirus. Once initiated, satellite virus synthesis interferes with adenovirus replication (Casto *et al.*, 1967a,b; Parks *et al.*, 1968). Satellite viruses also interfere with one another (Torikai and Mayor, 1969).

A recent important finding is that, in presence of herpesvirus as helper, satellite virus can synthesize its own antigens (Atchison, 1970; Blacklow *et al.*, 1971) and replicate infectious DNA (Boucher *et al.*, 1971). However no infectious virus particles or complement fixing (CF) antigens are formed. These findings suggest that there is a two-step defectiveness for satellite and that only adenovirus can trigger the maturation step.

Further dissection of the defective satellite replication cycle will depend on experiments using conditionally defective adenoviruses and herpesvirus helpers to determine the specific genes required by satellite virus to complete its replication. Ito and Suzuki (1970) have made a move in the right direction in a set of experiments using a temperature-sensitive mutant of adenovirus type 31, ts 13, a mutant that is defective in viral DNA synthesis and adenovirus capsid production, but that could still complement a cycle of satellite virus replication at the nonpermissive temperature. The ts mutant synthesizes T antigen, thymidine kinase, and DNA polymerase whose functions might presumably be required for satellite virus synthesis. However studies by Hoggan (see p. 65) indicate that presence of T antigen is not sufficient for satellite virus replication. In addition, temperature-sensitive herpesvirus mutants (Schaffer, 1973) are now available for complementation studies (Drake and Mayor, 1973).

In general conditionally defective helper activities fall into 3 broad groups.

1. Genetically Controlled Complementation

This includes all potential conditional lethal mutants of adenovirus (Ito and Suzuki, 1970; Mayor and Ratner, 1972) (see Section II,C) and herpesvirus and systems in which, by juggling the order and time of inoculation of herpesvirus and adenovirus helpers, the kinetics of the three component system satellite, herpesvirus, and adenovirus can be evaluated.

Drake and Mayor (1973) have recently demonstrated the ability of a temperature-sensitive mutant of herpes simplex virus (ts 343) (Schaffer *et al.*, 1971) to complement satellite DNA replication and transcription of structural protein in tissue culture cells of African green monkey kidney.

The mutant ts 343 does not synthesize any detectable viral DNA at the nonpermissive temperature of 40°C. It was, therefore, a relatively simple task to isolate infectious satellite virus DNA from the system at the nonpermissive temperature. Satellite virus particles were not isolated from the infected cultures, indicating failure of the maturation step. However the results showed clearly that demonstrable replication and/or assembly of the helper virus structural components was not a necessary condition for potentiation of its helper function. The system is now under extensive analysis at the molecular level.

2. Environmentally Controlled Complementation

This includes systems where the helper virus is rendered defective through a nutritional deficiency, e.g., adenovirus in arginine-free medium (Mayor and Ratner, 1972), and infections where the ambient temperature differs from optimal (Lefebvre *et al.*, 1973; Drake and Mayor, 1974).

Studies on the assembly processes during productive adenovirus infections have involved systems using tissue culture media deprived of arginine. In these conditions adenoviruses fail to mature although viral DNA is synthesized and capsid antigens are produced (Rouse and Schlesinger, 1967). Arginine-rich proteins are believed to be structural components of the adenovirus core (Russell, 1971). Recent studies have revealed that in contrast to the case of helper adenovirus there is no arginine-rich structural component in satellite virus (Rose *et al.*, 1971). These studies provided the rationale for an investigation by Mayor and Ratner (1973). Their system comprised either human embryo kidney (HEK) cells in their second or third passage inoculated with oncogenic human adenovirus type 31 and type 1 satellite virus, or BSC-1 cells inoculated with simian adenovirus SV15 and type 4 satellite and maintained in medium lacking arginine.

Tissue cultures of human embryo kidney (HEK) cells or stable green monkey cells (BSC-1) were used in stationary phase. At least 2 hours before inoculation, cultures destined for incubation in arginine-minus conditions were changed to arginine-free Eagle's synthetic medium.

In the HEK system viruses used were human adenovirus type 31 and type 1 satellite. The ASV 1 and adenovirus type 31 were pelleted at 40,000 rpm for 1 hour and resuspended in 1 ml of arginine-free medium. Individual bottles were inoculated with both viruses separately and in combination in arginine-containing and arginine-free media. During the incubation period ^{3}H-labeled thymidine (50 μCi per bottle) was added, and the cultures were harvested 48 hours after inoculation when marked adenovirus CPE was present in the arginine-positive adenovirus cul-

tures. The harvests were analyzed by density gradient centrifugation in cesium chloride (Fig. 4).

Fractions with peak activity were pooled and dialyzed against 0.1 × SSC before electron microscopic identification, particle counting, and infectivity assay. In arginine-minus conditions a peak of radioactivity was obtained at a density of about 1.36 gm/cm^3 (Fig. 4), and only satellite particles were seen in the electron microscope. In arginine-plus conditions a sharp peak of radioactivity coincided with satellite particles at 1.37 gm/cm^3 and a shoulder at 1.34 gm/cm^3 with numerous adenovirus particles. No radioactivity or physical particles were found in gradients run after inoculation with adenovirus alone and arginine-minus conditions. Similar results were obtained with BSC-1 cells, SV15 adenovirus, and type 4 satellite virus. In these experiments the major peaks of satellite virus activity coincided with densities around 1.42 gm/cm^3. The infectivity of this material was greater than 10^6 hemagglutinin-producing units (HAU) per ml, indicating that viable satellite virus was being replicated in absence of arginine in conditions where adenovirus was not isolated. These findings indicate that arginine is not necessary for satellite maturation and that there is no significant arginine-rich protein in satellite virus cores.

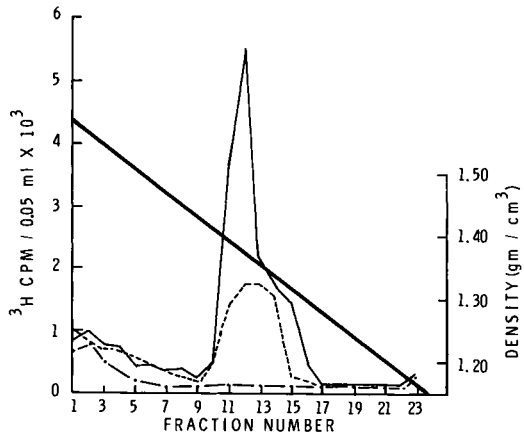

FIG. 4. Density gradient fractionation of harvests from cells infected with adenovirus type 31 maintained in arginine-free medium (—·—·), adenovirus type 31 and ASV 1 maintained in arginine-free medium (---), and adenovirus type 31 and ASC 1 maintained in medium containing arginine (—). 4 to 5 hours after inoculation ^{3}H-labeled thymidine (50 μCi per bottle) was added, and incubation continued until harvest (usually 48 hours after inoculation). Gradient fractions were collected by bottom puncture and assayed for ^{3}H radioactivity and buoyant density.

3. *Host-Controlled Complementation*

a. The Cell Is Influencing Satellite Virus. Boucher *et al.* (1969) studied the enhancement of satellite virus by human and simian adenoviruses in a variety of tissue culture cells. Satellites 1 and 3 were enhanced with human adenoviruses 7 or 12 in human kidney cells, whereas satellite 4 grew poorly in any cell that was not of simian origin. Drake and Mayor are currently studying a number of host cell systems infected with selected adenovirus helper viruses and satellite virus. Simian adenovirus SA7, which grows well in BSC-1 cells, does not enhance ASV 4. ASV 4 appears to grow well in BSC-1 cells with a variety of helper adenoviruses but is poorly enhanced in VERO cells, although both BSC-1 and VERO are cells of green monkey origin. In contrast ASV 1 is readily enhanced in VERO cells. In human embryonic kidney cells (HEK) simian adenovirus SV15 grows well, and the activity of ASV 1 is enhanced but not the activity of ASV 4. It is apparent that the host cell is related to the enhancing potential of satellite virus, and this important interaction should be further studied.

b. Cell Causes Helper Virus to Be Defective. The helper virus may itself be conditionally defective in the host cell chosen. Smith and Gehle (1967) found that a canine adenovirus ICH, which did not replicate in human cells, could be used to pass satellite virus serially if the passage was reinfected each time with helper virus. Mayor and Ratner (1972) were able to potentiate satellite virus in hamster embryo fibroblasts using human adenoviruses 2 and 1 as helpers. Adenovirus 7 does not replicate at all in stationary cultures of hamster cells, and adenovirus 2 replicates very poorly. These abortive adenovirus infections warrant closer investigation at the molecular level to determine which early virus gene products are being synthesized.

c. The Transformed Cell Is Host. In addition the reactions of cells transformed by oncogenic adenovirus to satellite viruses should be considered here. Hoggan *et al.* (1966) were unable to produce satellite particles in adenovirus 12-transformed hamster cells. These cells contain virus-specific T antigen (Huebner *et al.*, 1964; Levinthal *et al.*, 1966); this early adenovirus function is clearly not sufficient for satellite virus replication. Apparently neither of the two distinct defective steps in satellite replication can be complemented by adenovirus-transformed cells. Henry (1973) has carried out further experiments that indicate that adenovirus-transformed hamster cells are nonpermissive hosts for satellite growth even in the presence of apparently sufficient adenovirus "helper" activity. Lefebvre *et al.* (1973) have attempted a mixed infection of adenovirus-transformed hamster cells with satellite plus herpes

simplex virus and were able to detect satellite antigens, but only at 35°C and not 37°C—in nontransformed cells the complementation occurred at both 37° and 35°C. These authors suggested a herpesvirus-induced product, which was cellular, rather than viral, and thermosensitive in transformed cells, was necessary for satellite antigen synthesis.

4. Replication of Densonucleosis Virus

Densonucleosis virus (DNV) was originally isolated from larvae of *G. mellonella* (Meynadier *et al.*, 1964), and in its host of origin DNV infects practically all tissues (Kurstak *et al.*, 1968a,b). DNV has also been adapted to infect mammalian cells, such as the L cells of the mouse Kurstak *et al.*, 1967, 1969b). As in the case of satellite virus, the main lesions are also in the nucleus of infected cells, and their development has been studied by fluorescence microscopy (acridine orange, fluorescent antibody) and electron microscopy including autoradiography. Localization of DNV early antigens by immunofluorescence was first observed in the cytoplasm of cells of *G. mellonella* 4 hours after infection. This pattern was replaced by one of nuclear antigen by 8 hours after

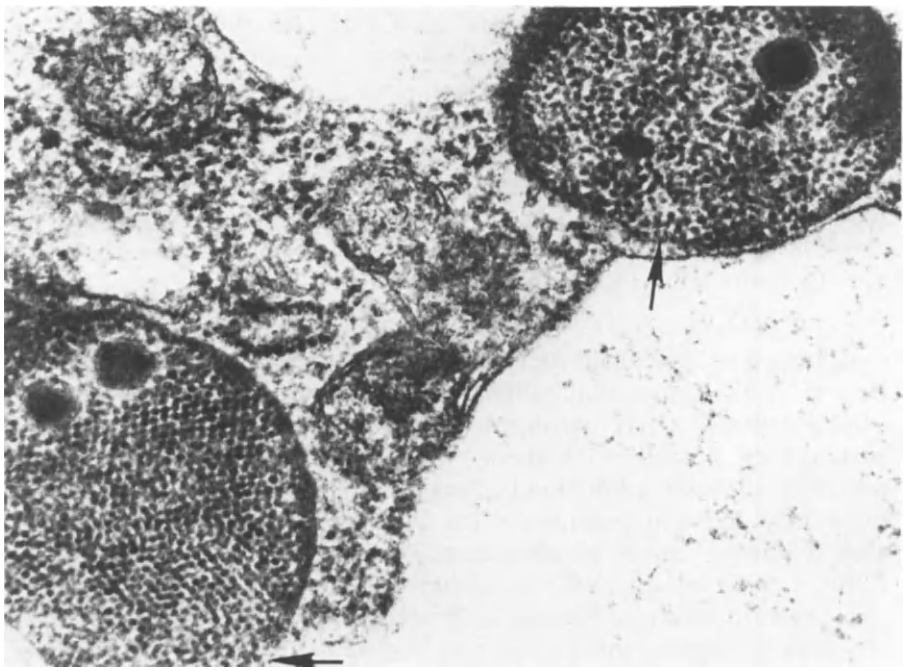

FIG. 5. Intracytoplasmic vesicle sequestering virions of TIV and DNV (arrow). ×55,250.

infection. These findings have been confirmed by the use of the immunoperoxidase technique and electron microscopy (Kurstak *et al.*, 1969a, 1970a,b).

The replication of DNV in the nucleus of *G. mellonella* cells in large quantities can also be detected in the case of double infections with DNV and a large DNA virus—*Tipula* irridescent virus (TIV) (Kurstak and Garzon, 1971; Kurstak *et al.*, 1972). The replication of TIV proceeded to completion in the cytoplasm, whereas DNV followed the pattern described above. Late in the infection both virions were located in cytoplasmic vesicles (Fig. 5). In contrast with the adeno-associated satellite virus replication cycle, there was no interference demonstrated between the two agents and no helper action. The replication cycle of DNV has not been explored at the molecular level or dissected with the use of radioactive isotopes. The genome of DNV is either a "plus" or a "minus" strand of DNA, and yet DNV can replicate autonomously. Intensive future studies at the molecular level, in the absence of the helper virus activity necessary in the satellite virus system, should yield unequivocal basic information concerning this novel type of DNA.

C. Properties of Viral Nucleic Acids

Satellite virus and densonucleosis virus are the only small DNA agents in which virions contain complementary single DNA strands. The DNA of satellite virus is defective and needs a helper for its full expression, whereas the DNA of DNV is autonomous. Comparison of these two viral genomes and their properties would appear to be of fundamental importance (Table III).

TABLE III

Properties of the DNA of Satellite Virus and Densonucleosis Virus

Property	Densonucleosis virus DNA	Satellite virus DNA
Conformation	Linear	Linear
Percent G + C	42	58
Density (gm/cm^3)		
Single stranded	1.711	1.728
Double stranded	1.701	1.718
Molecular weight (daltons)		
Single stranded	2×10^6	1.5×10^6
Double stranded	4×10^6	3×10^6
Sedimentation coefficient ($s_{20,w}$)	16	16

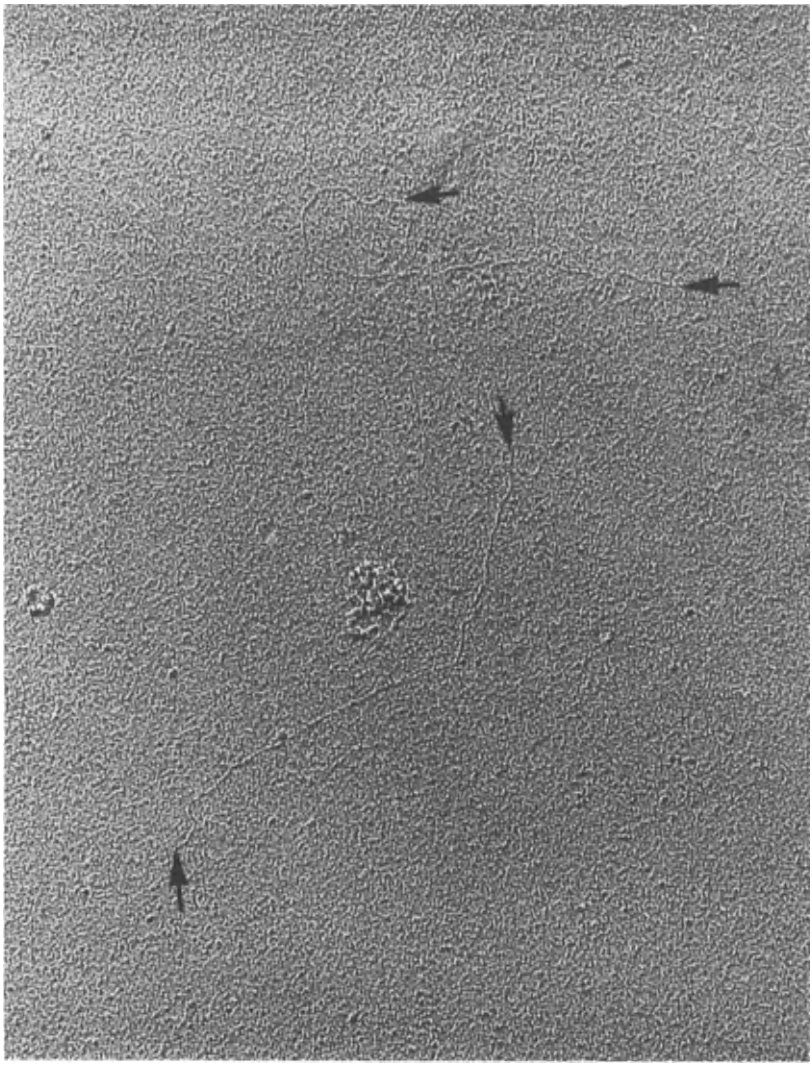

FIG. 6. Single-stranded DNA shocked from individual dense satellite virions by using 12 *M* ammonium acetate and spread in the presence of urea. ×35,700.

DNV appears to contain a greater complement of DNA (2.0×10^6 daltons) (Fig. 2b) compared with satellite virus (1.5×10^6 daltons), and it is possible that these additional nucleotide sequences could be partly responsible for the fact that DNV can replicate autonomously while satellite virus is nonconditionally defective in every host cell system that

has been investigated. Electron microscopic examination of satellite DNA released from individual virions by osmotic shock with 12 *M* ammonium acetate and spread in the presence of urea (Torikai *et al.*, 1970) has revealed single-stranded DNA of modal length 1.6 μm (Fig. 6). Even on renaturation these strands rarely remain associated with the empty capsids from which they came. By contrast DNV DNA of opposite polarity shows a marked tendency to reassociate in conjunction with two empty capsids, one at each end of the double-stranded helix (Figs. 7 and 8). The contour length of this strand is 1.95 μm, a figure fitting to its greater molecular weight compared to satellite virus DNA. In agreement with the previous findings of Mayor *et al.* (1969b), the DNA of DNV remained single stranded at low ionic strength, but when heated a high ionic strength reassociated it to double-stranded DNA (Kurstak *et al.*, 1973a). The G + C content of DNV DNA (42%) is certainly in line with that of the autonomous parvoviruses with varying molecular weights (Table IV) and significantly different from that of defective satellite virus DNA (58–62%). It might be suggested that regions involved in initiating DNA replication might be rich in AT sequences and that suitable sequences are present in DNV but lacking in satellite virus DNA. Current active work on detection of specific mutation sites for SV40 DNA replication (Danna and Nathans, 1972; Thoren *et al.*, 1972) may soon provide data of general significance on this important point.

The utility of studies using temperature-sensitive helper adenoviruses as tools in dissecting the replication cycle of satellite virus has been recently demonstrated in cells infected with human adenovirus ts 13 mutant and satellite virus type 1 at a temperature of 40°C—nonpermissive for adenovirus DNA synthesis but permissive for satellite DNA replication (Mayor and Ratner, 1973). Infectious satellite DNA was isolated and analyzed. The DNA was a linear, 16 S species approximately 1.5 μm in length. No naturally occurring circular molecules were detected either by electron microscopy or nitrocellulose chromatography.

Koczot *et al.* (1973) have examined purified DNA for satellite virions and have found that at least 7% of plus and minus strands of satellite virus DNA contain self-complementary sequences at or near their termini. Self-annealing of these sequences generated circular molecules that were closed by duplex hydrogen-bonded segments. The same laboratory reported previously (Garon *et al.*, 1972) that denatured adenovirus DNA could also self-anneal to form single-stranded circles. This finding is of great interest in view of the fact that satellite replication is unconditionally dependent on adenovirus as helper. Of even greater interest would be similar experimentation on the autonomous DNA of DNV to determine whether similar self-complementary sequences are found

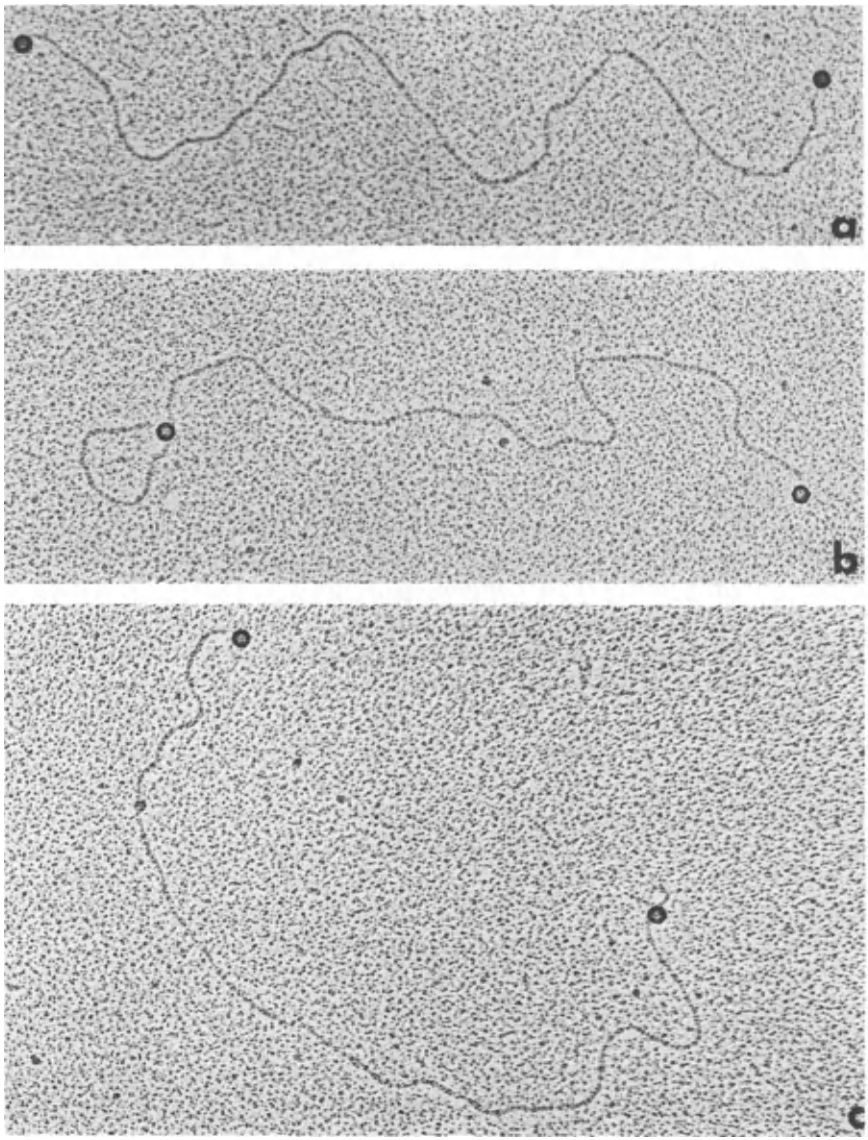

FIG. 7. DNA of densonucleosis virus (DNV). Stained with 1% uranyl acetate × 42,000. (a), (b), and (c) Molecules of DNA double stranded in appearance with empty capsids of DNV at each extremity. This suggests probably a reassociation of complementary single strands of opposite polarities (+ and −) in a solution of high ionic strength. Each strand would be encapsidated separately giving two types of virions each with either a + or − strand of DNA.

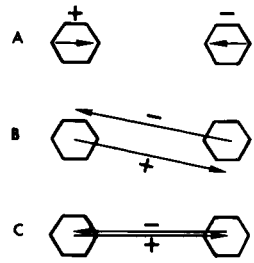

FIG. 8. Schematic representation of complementary strands of opposite polarities (+ and −). (A) As separate single strands encapsidated in virions of DNV, (B) after extraction not reassociated in a solution of low ionic strength, and (C) reassociated by complementarity in a solution of high ionic strength giving a molecule of double-stranded DNA connected to two empty virus capsids as shown in Fig. 7.

there or whether they are unique to the satellite–adenovirus replication system.

Hadidi *et al.* (1973) have shown recently that without helper virus participation defective satellite virus can induce *de novo* DNA synthesis in nongrowing BSC-1 cells. The DNA was revealed by autoradiography to be nuclear in origin, and DNA–DNA hybridization indicated that it was of cellular rather than of viral origin. In contrast, Tennant and Hand (1970) have shown that a number of autonomous parvoviruses, RV, H, MVM, require participation of a preexisting radiosensitive cellular DNA. This DNA was not required by satellite virus, which, in this respect, is less defective than the autonomous parvoviruses. Does DNV stimulate *de novo* host cell synthesis like ASV or is it, like the autonomous parvoviruses, dependent on UV-sensitive cellular DNA sites? These questions remain to be answered.

TABLE IV

Guanine and Cytocine Content of Small DNA Viruses

Virus	G + C (moles %)	Molecular weight (10^6 daltons)
H-1	45.2	1.7
RV	43.5	1.2
MVM	40.9	1.5
ϕX174	42.6	1.7
ASV 1, 2, 3	58	1.8
ASV 4	58	1.4
DNV	42	2.0

D. Properties of Viral Structural Proteins

The structural proteins of types 1, 2, and 3 adeno-associated satellite virus have been analyzed by acrylamide gel electrophoresis (Rose *et al.*, 1971; Johnson *et al.*, 1971). In each case one major (C) and two minor components (A and B) have been identified. The major component had a molecular weight of about 62,000 daltons, while the molecular weights of A and B were found to be 87,000 and 73,000, respectively. Component C appeared to be the major unit of capsid structure, accounting for 86% of the total protein. It was suggested that A and B might be internal proteins. Comparison of the three satellite types reveals a close similarity in their patterns of electrophoretic motility. The combined molecular weight of the satellite polypeptides, 222,000 daltons appears to be in excess of the estimated coding capacity of the satellite genome—170,000 daltons. This may indicate that virion structural proteins may arise from posttranslational cleavage of a large precursor protein derived from a polycistronic message in a manner analogous to that reported for poliovirus structural proteins (Summers and Maizel, 1968; Baltimore, 1971).

In a current study (Kurstak *et al.*, 1973b), DNV proteins have been examined and characterized by their molecular weights (MW). The MW was determined by analyzing the retardation of the proteins in polyacrylamide gel electrophoresis in different total polyacrylamide percentages.

The proteins were dissolved in SDS and reduced with mercaptoethanol. Following SDS electrophoresis, one minor and three major proteins were detected. This pattern did not change after carboxymethylation, cyanoethylation, or oxidation of the protein sulfhydryl groups by iodoacetamide, acrylonitrile, and performic acid, respectively.

As shown in Table V the minor protein δ has a MW twice that of the α molecule and may prove to be an α dimer. The quantity variance

TABLE V

Protein Components of DNV

MW	± moles/virion	± moles/virion if $\delta = 2\alpha$
α 48,700	49.3	50.2
β 58,500	8.9	8.9
γ 69,000	7.6	7.6
δ 98,000	0.46	
Total molecules	66.26	66.7

(0.2–7% of δ) in each sample was greater than that observed for the other three proteins. However, this may be due to the small quantity involved.

The MW of DNV DNA is 1.69×10^6 to 2×10^6 (Kurstak *et al.*, 1973a). To code for four proteins a DNA with a MW of 2.46×10^6 would be required. For a three protein system a DNA with a MW of 1.59×10^6 would be necessary. As supplementary protein coding is necessary for viral reproduction, these values are substantially higher than expected for a feasible viral system and similar to the results obtained for satellite virus structural proteins. This problem is one common to all parvoviruses so far studied.

III. Adeno-associated Satellite Viruses and Densonucleosis Virus and Disease

There is no evidence that infections with adeno-associated satellite virus lead to any clinical disease (Blacklow *et al.*, 1968; Hoggan, 1971). For current information on the ecological aspects of distribution of satellite virus and antibody in the human population the reader is referred to recent reviews by Hoggan (1970), Henry (1973), and Mayor (1973).

In contrast Meynadier *et al.* reported in 1964 that DNV was responsible for a fatal disease of larvae of the arthropod *Galleria mellonella*. Larvae were killed by DNV in 4–6 days at 28°C (Vago *et al.*, 1964). Almost all tissues of the natural host are infected by DNV, and virus can be isolated from them. DNV has been adapted to infect mammalian cells such as the L cells of the mouse (Kurstak *et al.*, 1967, 1969b; Belloncik, 1969). However, after administration of virus to newborn mice and rabbits, Giran (1966) found no evidence that DNV was virulent from these animals. DNV has, in fact, been used as a living insecticide (Lavie *et al.*, 1965; Giauffret, 1966), but the probable danger of this enterprise has been emphasized by Kurstak (1972).

IV. Adeno-associated Satellite Viruses and Densonucleosis Virus and Cancer

The inhibitory action of adeno-associated satellite virus on adenovirus oncogenesis has been demonstrated (Kirchstein *et al.*, 1968). More recently Mayor *et al.* (1973) have studied the development of tumors in newborn hamsters inoculated with highly oncogenic human adenovirus type 31. In these experiments prior inoculation of newborns with satel-

lite virus before challenge with adenovirus reduced the level of tumor incidence from 50% to 0%. When satellite virus was introduced in simple mixtures with adenovirus the reduction in oncogenesis was not as marked. No inhibitory effects were noticed when satellite virus was inoculated prior to challenge with oncogenic simian virus, SV40, indicating that the phenomenon was adenovirus-specific. Additional studies carried out by Casto (1973) have demonstrated *in vitro* inhibition of adenovirus transformation by satellite virus, and important studies have been carried out in an effort to quantitate the inhibition of transformation as compared with inhibition of infectivity in permissive systems. These studies showed that inhibition of adenovirus 12 transformation required from 2–3 times as much satellite virus as was required for inhibition of plaque formation in hamster embryo fibroblasts by adenovirus type 1. Inhibition of oncogenic simian adenovirus SA7 transformation required 7–9 times as much satellite as was needed for inhibition of

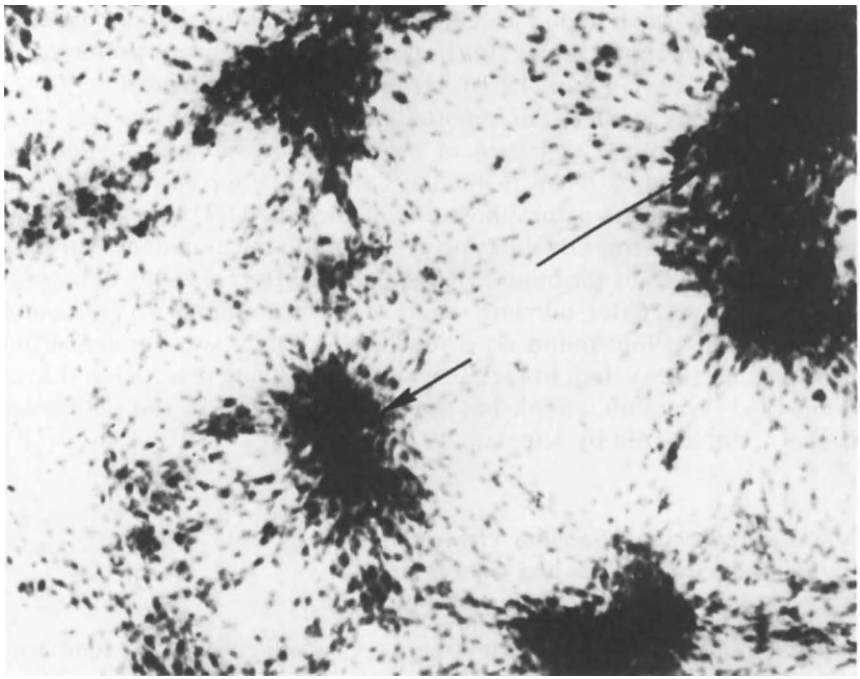

FIG. 9. Transformed rat embryo fibroblasts following DNV infection. Aspect of foci (arrows) after the fifth passage without new experimental reinfection with DNV.

infectivity. All these experiments indicate the importance of making a complete analysis of the oncogenic inhibitory effect in stationary tissue culture systems where the mechanisms of abrogation and inhibition can be studied in a quantitative way and where the phenomenon of prior inoculation with satellite virus can be completely explored.

In contrast to the anti-tumor effect of ASV, DNV appears to demonstrate a transforming ability in mouse L cells (Kurstak *et al.*, 1969b). By day 3 or 4 after infection with DNV, the regular growth pattern of L cells was disrupted, and by days 7 to 8 characteristically transformed cells were observed and DNV antigens could be detected in their nuclei by immunofluorescence. Similar results were obtained with rat embryo cells (Kurstak and Onji, 1972) (Fig. 9). In addition to DNV antigens, RNA type C particles were also evident in electron micrographs of these transformed cells. Their significance, possible latency, and activation by DNV and relation to the transformation phenomenon are not currently understood.

V. Conclusions

This review has stressed the obvious similarities between adeno-associated satellite viruses and densonucleosis virus and the less obvious, but possibly even more important, differences both in their modes of genetic expression and their fundamental architecture. The spectrum of disease, both in the natural and artificial hosts, ranges from complete absence of any clinical findings through abrogation of oncogenesis, transformation of tissue culture cells to fulminating disease with protean manifestations, and death. There is much still to be learned from these apparently contradictory states. The fact that a wealth of data has been accumulated on the satellite viruses is a bonus that should yield an abundant harvest when applied to the densonucleosis agent. We can expect to see the fruit of this harvest in the very near future.

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CHAPTER 4

The Epstein-Barr Viral Genome and Its Interactions with Human Lymphoblastoid Cells and Chromosomes

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The Epstein-Barr virus, the only virus of man that has been consistently associated with certain human malignancies, poses a classic challenge, namely, the establishment of an etiologic relation between virus and neoplasia. In this chapter I review the evidence mainly from this laboratory that sets forth the relation between the Epstein-Barr virus and human cells and tissues. One way out of the dilemma of distinguishing between association and a causal relation is by an understanding of the molecular interaction between the viral genome and the mammalian DNA. The techniques elaborated recently to detect and to quantitate the viral DNA of the Epstein-Barr virus in cells help in that they provide an insight also into mechanistic aspects of the interaction between virus and cell. In other words, the presence of the viral genome in a given tissue is significant, but the demonstration of a consistent state of the viral genome in relation to the cellular chromosome in biophysical terms establishes a second level of significance to the association of virus and cell. Work in this latter area is just beginning.

I. The Virus

The Epstein-Barr virus is the most recently discovered member of the group of human herpes-type viruses that include the herpes simplex viruses, varicella-zoster virus, and the human cytomegaloviruses (Epstein *et al.*, 1964). All are large DNA-containing viruses with an outer lipid-containing envelope and a nucleocapsid with 162 capsomeres (Hummeler *et al.*, 1966). The core of the Epstein-Barr virus is believed to contain linear double-stranded DNA of molecular weight 100×10^6 daltons, although this value has not yet been firmly established. The density of the viral DNA is 1.718 gm/cm^3, making the guanine + cytosine content of the genome 58% (Weinberg and Becker, 1969; zur Hausen and Schulte-Holthausen, 1970; Nonoyama and Pagano, 1971). There is some evidence that the viral DNA contains a number of single-stranded nicks in either strand in the native state (Nonoyama and Pagano, 1972a), but these strand interruptions may be introduced upon extraction and storage of the virus or its DNA.

The virus is obtained from virus-producing cultures of a lymphoblastoid line of cells (HR1K) derived from a Burkitt's lymphoma. These cells when maintained in continuous culture discharge virus into the extracellular fluid. The cells grow with clumping in suspension in stationary cultures, with a doubling time of approximately 24 hours despite the fact that the cells that produce virus are killed. This apparent paradox is explained by the fact that only a fraction of the cell population is producing virus at a given time, although all of the cells probably

carry the viral genome as shown by cloning experiments. Between 7% and at most 11% of the cells display viral capsid antigen by immunofluorescent staining. Approximately 7% of the cells show evidence of viral DNA replication by the *in situ* cytohybridization technique carried out by hybridization of virus-specific complementary RNA to the cellular DNA and autoradiography (Fig. 1).

It appears that viral replication involves a transition from a latent state of the genome in a majority of the cell population to the virus-producing state. There is a balance; cells in which the genome is in the repressed state are able to divide, and the culture continues despite the production of virus.

Other virus-producing lymphoblastoid cell lines are available, but none yields as much virus as do the HR1K cells which are a subline of the P_3J cells (Hollinshead *et al.*, 1971). Productive lines include some derived from the peripheral blood of patients with infectious mononucleosis as well as from other explants of Burkitt's lymphoma.

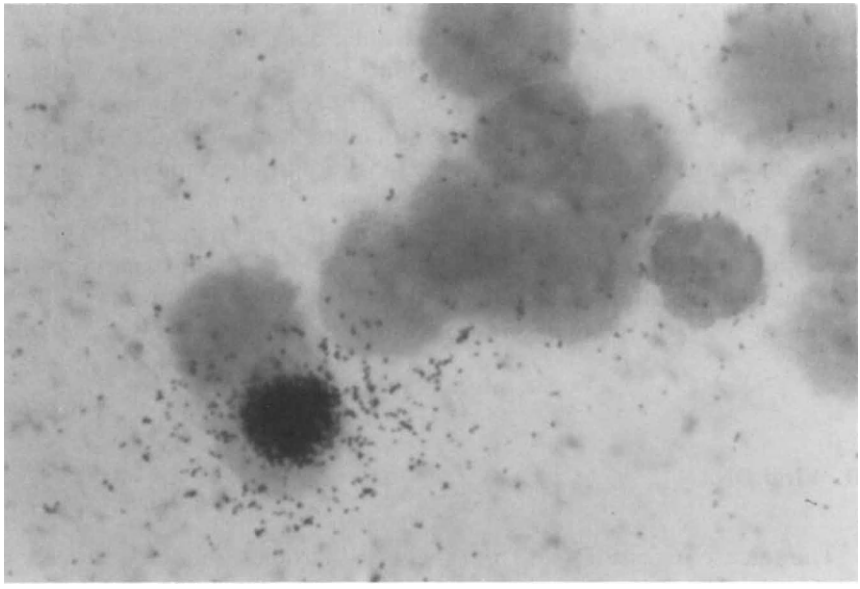

FIG. 1. Cytohybridization with Epstein-Barr viral complementary RNA in the HR1K line of Burkitt's lymphoma lymphoblastoid cells. EBV cRNA (2×10^5 cpm; specific activity, 10^7 cpm/μg) was hybridized to the DNA of the isolated nuclei for 22 hours at 66°C after the cellular DNA had been denatured *in situ* by dipping in sodium hydroxide. The preparation was washed extensively with buffered solutions and treated with RNase to remove nonspecifically bound cRNA before autoradiography (from Pagano and Huang, 1974).

Recently a line of simian leukocytes that sheds a large amount of virus has been described by Miller *et al.* (1972) and Miller and Lipman (1973). This cell line (B95-8) was established by transformation of marmoset leukocytes with extracts of a lymphocyte line from a patient with posttransfusion mononucleosis. We are studying the degree of homology between the virus shed from these cells and virus from HR1K cells because of the possibility that the virus harvested from the B95-8 cells may contain a simian agent related to EBV but indigenous to the marmoset leukocytes. Another possibility is that these cells are replicating the original infecting "mononucleosis virus" which differs from the standard HR1K virus.

With the agar pseudoreplica technique for quantitation of virus particles by electron microscopy (Sharp, 1960), we have found that there are higher yields from the marmoset line, B95-8. The yield of particles appears to be ten times greater than from a comparable volume of tissue culture fluid from the HR1K cells.

With the same counting technique we are now comparing the recovery of viral particles from HR1K cells at the earlier stages in two purification procedures. The EBV-containing fluids are harvested after a temperature shift to 32°C for 10 days and then clarified by low-speed centrifugation. In the next step, pelleting in the T19 rotor (Beckman), there is some difficulty with quantitation of virus because of clumping. If concentration by centrifugation in the continuous flow rotor (CF32) is substituted for this step there is little clumping of particles, and the recovery of virus appears to be improved. The final step of purification is centrifugation through a sucrose gradient (Nonoyama and Pagano, 1971). A newer method devised for purification of human cytomegalovirus involving centrifugation on a preformed gradient of CsCl appears to be a promising way of reducing carryover of cellular DNA (Huang *et al.*, 1973).

II. Viral DNA

The release of viral DNA from the virus particles and the purification of the viral DNA have been described (Nonoyama and Pagano, 1971). We have had little success with the use of DNase to rid the viral particles of cellular DNA before extraction; there is substantial loss of viral DNA even when apparently intact virions are exposed to enzyme, and the DNase is then inactivated with SDS before extraction of the viral DNA with pronase.

The liability of the viral DNA to shearing and its scarcity (only 6 to 10 μg of viral DNA from 20 to 40 liters of tissue culture fluid) has deterred exact characterization of the DNA.

III. Techniques of Nucleic Acid Hybridization

A. Complementary RNA–DNA Hybridization on Membrane Filters

Synthesis of virus-specific cRNA is carried out *in vitro* with DNA-dependent RNA polymerase from *E. coli* on a template of pure Epstein-Barr viral DNA. About 4–5 μg of viral DNA is needed, and we prefer synthesis times limited to 2 hours. The purified polymerase contains σ factor. The radioactive label is tritiated uridine nucleoside triphosphate; we are experimenting with the use of all four labeled nucleoside triphosphates in order to increase the specific activity of the cRNA, which usually ranges between 7 and 10×10^6 cpm/μg. We have not determined the percentage of the genome that is transcribed by the bacterial polymerase, nor do we know whether some regions of the genome are under- or over-represented in the transcripts. However this does not preclude the use of these preparations of cRNA not only as probes but also for estimation of number of genome equivalents based on reconstruction curves. The cRNA is used for hybridization with known quantities of unlabeled viral DNA mixed with unlabeled carrier DNA after denaturation and fixation on membrane filters. The estimates based on this type of reconstruction curve are in good agreement with the exact quantitation of viral DNA that is provided by DNA–DNA renaturation kinetics analyses (see Section III,C) (Nonoyama and Pagano, 1973).

The cRNA–DNA hybridization technique is convenient because it is relatively easy to perform, quite reproducible, and does not require large amounts of cellular DNA from the tissues or cells under test. The test is most useful when the average number of genome equivalents per cell is above three or four. The limits of sensitivity of the test are in the vicinity of 1 to 2 genomes per cell. At this level there is ambiguity because of background radioactivity from residual cRNA or nonspecific hybridization.

B. Cytohybridization in Situ with cRNA

In this technique adapted from Gall and Pardue (1971) and Jones (1973) and described in Huang *et al.* (1973) and Pagano and Huang

(1974) smears of cells or tissue sections that have been fixed to slides with glacial acetic acid are exposed briefly to alkali under conditions that make denatured cellular DNA available for hybridization with the virus-specific cRNA without severe disruption of the cytoarchitecture. After extensive washing and RNase treatment to remove residual nonhybridized cRNA, an autoradiogram is prepared to disclose the intracellular location of both virus and viral DNA. This technique can obviously yield unique information not possible with membrane hybridization. The power of the technique is in the localization to site and not in its sensitivity. However if the viral DNA is confined to a few cells in a population, then the technique may be relatively sensitive, since the membrane hybridization technique averages the DNA extracted from the whole population of cells, whether they contain viral DNA or not. We are experimenting with technical variations to increase the sensitivity and enable rapid screening for the presence of virus or viral DNA both in Epstein-Barr virus and cytomegalovirus systems.

C. DNA–DNA Renaturation Kinetics Analyses

This technique requires pure viral DNA of high specific radioactivity. Since viral DNA of specific activity above 5×10^4 cpm/μg is difficult to prepare in the cell cultures (radioisotopes other than tritium are impracticable because of the large volume of culture fluid), we devised a technique for labeling the viral DNA *in vitro* (Nonoyama and Pagano, 1973).

The pure viral DNA is first treated with low concentrations of pancreatic DNase I in order to introduce single-stranded nicks. The viral DNA is then labeled by repair synthesis with Kornberg's DNA polymerase I, fraction VII (Jovin *et al.*, 1969) (termed fraction IX by Richardson *et al.*, 1964) in the presence of tritiated thymidine triphosphate and the other unlabeled triphosphates. DNA replication proceeds by 3′ polymerization and 5′-exonuclease activity at the nicked points. The reaction is carried out at relatively low temperature (17°–19°C) in order to prevent branching and redundancy. Under these conditions replication is restricted to the filling in of the gap produced by DNase I and extended by the 5′-exonuclease activity of the repair enzyme. A comparison of the repaired DNA and the DNA labeled in the cell cultures did not disclose altered velocity sedimentation properties, but more precise experiments to determine the character of the DNA labeled in this way are in process. The label is assumed to be evenly distributed throughout the genome because DNase I produces random nicks. The specific activity of the labeled DNA obtained by this technique is in the range of 3 to 4×10^6 cpm/μg. The DNA is fragmented by

sonic vibrations to ~5 S (1 to 2 × 10^5 daltons) before use in the renaturation assay.

The procedure for renaturation kinetics analysis has been described for Epstein-Barr virus and also for cytomegalovirus (Nonoyama and Pagano, 1973; Kawai *et al.*, 1973; Huang *et al.*, 1973). The principle of the method is that after denaturation a precisely defined amount of tritiated viral DNA in a low concentration is allowed to renature (Kohne and Britten, 1971). The renaturation follows characteristic kinetics dependent on the concentration of homologous DNA, so that the effects of various added cellular DNA's can be analyzed by the deflection of the slope of the renaturation of the index DNA that they produce. The unreannealed and duplex molecules are separated by elution at different salt concentrations from small hydroxyapatite columns at 60°C: 0.14 *M* phosphate buffer for single-stranded and 0.4 *M* buffer for double-stranded DNA. The hydroxyapatite must be screened by production lot; some lots give much better separation.

Double- and single-stranded DNA can also be distinguished by digestion with the S1 enzyme, a single-stranded exonuclease derived from *Aspergillus oryzae,* or by the single-stranded nuclease with similar activity from *Neurospora crassa.* These enzymes not only digest unrenatured strands, but also attack the single-stranded regions of imperfect duplex molecules, so that the use of the enzyme provides a more exact measure of homology between DNA species than does hydroxyapatite chromatography. The enzyme preparation must be screened for at least 95% preference for single-stranded nucleic acid (Huang and Pagano, 1974).

The technique of DNA renaturation kinetics has great power by virtue of its sensitivity (as little as 0.1 viral genome with index DNA of sufficiently high specific activity and 2 mg of test cell DNA) and its specificity. When related or identical species of nucleic acids are compared up to 95% homology can be established, but with double-stranded DNA there is an inevitable 5% ambiguity. This is due to slight double-stranded DNase activity in the S1 enzyme, imperfect separation of single and double strands by chromatography, slowing of the reaction when the very dilute concentrations of index DNA needed for utmost sensitivity are being used, and self-annealing of the index viral DNA. There may also be small stretches of mismatching of bases between homologous sequences which would be protected from digestion even with S1 enzyme. This problem is diminished by fragmentation of the DNA to small pieces, for example, 10^5 daltons.

The analysis can also be used to determine fractional homology between DNA species, but if the degree of homology is less than 5%, heteroduplex formation is a more sensitive technique, although more cumbersome because it requires electron microscopy.

The kinetic hybridization technique also permits the determination of the presence of specific fractions of the viral genome. For this type of analysis the equation $Do/D_t = 1 + K\,Co_t$, in which Do is the original concentration of single-stranded DNA and D_t is the remaining single-stranded DNA at a point in time, is used in order to permit this second order reaction to be plotted as a straight line. $Do/D_t = 2$ indicates that 50% of the molecules have been reannealed (the Cot_{50} value in the conventional plot). The point at which the straight line of renaturation is interrupted signifies the percentage of the genome that is present.

The existence of a fraction of the viral genome can be discerned by a careful analysis of the early points in the time course of the renaturation, since the presence of any viral sequences in excess of those of the index DNA is reflected in an initial acceleration of the renaturation of the index DNA. From work with similar analyses of human cervical carcinomas for the presence of herpes simplex viral DNA type II we estimate that it is possible to detect the presence of as little as 20% of the viral genome (N. S.-T. Chen, E.-S. Huang, J. Shaw, and J. S. Pagano, unpublished).

IV. Interactions of the Viral Genome in Various Tissues

A. African Burkitt's Lymphoma

In order to determine the relation of the Epstein-Barr virus to Burkitt's lymphoma, we employed the cRNA–DNA hybridization technique to search for viral DNA directly in malignant tissues obtained at biopsy (Nonoyama *et al.*, 1973). Such tissues give no clue to the presence of virus by electron microscopy or by immunofluorescent staining for viral antigens, although if cell lines are established from the tissues they often, but not always, disclose the virus. We chose to study the original malignant tissue rather than cell lines derived from it in order to avoid disturbing the latent relation between virus and cell, since reactivation of the genome was likely to occur under cultural conditions.

Our findings to date, summarized in Table I, indicate that 24 of 26 specimens of the Burkitt's tissue, obtained from S. Singh of the Kenyatta National Hospital through a collaboration with Professor George Klein, Stockholm, disclosed the presence of Epstein-Barr virus DNA in appreciable amounts ranging from 113 to 5 genome equivalents per cell on the average. One of the two negative specimens (from the patient Fausta Mswema) was also tested by the DNA–DNA renaturation kinet-

ics analysis, which confirmed the absence of viral DNA (less than 0.4 genome) (Kawai *et al.*, 1973). Both this specimen and the other negative specimen (from the patient Josephine Adiambo) also did not contain the Epstein-Barr virus-specific complement-fixing antigen as detected by an indirect fluorescent technique (the Epstein-Barr nuclear antigen or "EBNA" test) (Reedman and Klein, 1973). The specimen from the first patient was from the ovary, and the patient had a high serum antibody titer to viral capsid antigens as did the second negative patient. It is possible that the case of F. M. was atypical in that there was extensive infiltration of the bone marrow with neoplastic cells, and there is a similar question about the second patient.

When all the specimens of African Burkitt's lymphoma, tested either in this laboratory or in zur Hausen's by cRNA–DNA hybridization (zur Hausen *et al.*, 1972) or in Klein's laboratory by the EBNA tests, were summed up only 2 of 91 failed to disclose direct evidence of the presence of the Epstein-Barr viral genome (G. Klein, personal communication).

B. African Nasopharyngeal Carcinoma

This malignancy is also associated with high Epstein-Barr virus antibody titers in patients with the disease (Henle *et al.*, 1970b). Being a carcinoma, the malignant cells are of epithelial origin, but the neoplasm is also heavily infiltrated with lymphocytes. The association of the tumor with the Epstein-Barr virus is of particular interest because the virus otherwise has an exclusive association with lymphoid tissue.

As shown in Table II the association of the viral DNA with this malignancy is as consistent as with the Burkitt's lymphoma, 32 out of 38 specimens having been shown to harbor the viral DNA in amounts ranging from 85 to 5 genome equivalents. We have retested at least one of the nasopharyngeal carcinoma specimens without Epstein-Barr viral DNA by DNA–DNA renaturation kinetics and again failed to detect viral DNA (Kawai *et al.*, 1973). On the other hand, the homologous DNA in a positive specimen of nasopharyngeal carcinoma selected at random bore at least 90% identity to the viral DNA of Burkitt's lymphoma (Nonoyama and Pagano, 1973).

It now appears that if only those specimens of nasopharyngeal carcinoma independently confirmed as meeting the histopathologic criteria for the disease are tested and the others, for example those without tumor infiltrate, are excluded, 100% of the specimens of nasopharyngeal carcinoma contain Epstein-Barr viral DNA by cRNA–DNA hybridization (H. zur Hausen, personal communication).

TABLE I

Epstein-Barr Viral DNA in African Burkitt's Lymphoma[a,b]

Patients	KCC	No. tests	cpm hybridized/ 50 μg DNA	No. of EBV genome equivalents/cell	Serum antibody titers		
					VCA	EA-D	EA-R
I.R.	1225	2	4311	113	1280	320	
P.A.	1343	2	3381	92	1280	80	
O.O.	1349	2	2148	57	180	20	320
		1	1054	23			
O.O.		2	1197	27			
K.K.	1464	2	2027	52	160	<10	
N.B.	1315	2	1818	48	$640 \sim 160^c$	<10	80
M.C.	1260	2	1877	47	$320 \sim 160$	<10	80
R.W.	1166	2	1761	45	$640 \sim 160$	$10 \sim 40$	40
A.M.		2	1490	38			
N.K.	1393	2	1482	38	40	<10	
K.S.	1364	2	1451	37	320	<10	160
M.N.		2	1413	36			

O.O.	1373	2	1415	36	640	<10	320
M.A.	1367	2	1365	34	1280	320	
		2	910	22			
A.O.	1311	2	1363	34	640	10 ~ 20	160 ~ 320
M.N.	1223	2	1160	26	640 ~ 1280	80 ~ 320	
J.C.	1305	2	1055	25	640 ~ 80	10 ~ 40	160 ~ 320
S.O.[e]	1442	2	793	18	160	<10	160
N.K.	1397	2	693	15	320	<10	160
P.N.	1413	4	242	4	160	<10	<10
F.M.[d]	1143	4	152	<2	640 ~ 2560	20 ~ 40	160 ~ 320
J.A.[d]	1450	2	102	<2	320	<10	<10
O.S.O.	1513	1	401	7	160	10	10
F.K.	1517	1	356	5	10	<10	<10
S.O.[e]	1442	1	389	6			

[a] Data from J. S. Pagano, G. Klein, and S. Singh. Published in part in Nonoyama *et al.* (1973).
[b] Tissue obtained at biopsy.
[c] Titers given as a range are from sera collected over a period of months.
[d] F.M., is Fausta Mswema; J.A., Josephine Adiambo.
[e] Same patient.

TABLE II

Epstein-Barr Viral DNA in Tissues From Nasopharyngeal Carcinoma[a,b]

Patients	KCC	No. tests	cpm hybridized/ 50 μg DNA	No. of EBV genome equivalents/cell	Serum antibody titers		
					VCA	EA-D	EA-R
R.K.	1444	2	3131	85	160	<10	40
O.R.	1452	2	1813	46			
J.A.		2	1018	24			
K.M.	1434	2	983	23	160	20	20
M.E.		2	901	20	1280	160	
W.M.		2	935	20			
D.K.		2	911	20	640	160	
K.T.	1232	2	801	17	640 ~ 1280[c]	320	
K.C.	1278	4	786	16			
K.M.	1268	2	710	14	320 ~ 160	40	320 ~ 160
N.K.	1262	2	675	13	640 ~ 320		
J.A.	1326	2	661	13	2560	1280	
K.A.		4	476	8			
M.K.	1279	4	442	7	160	20 ~ 40	
R.K.		2	358	5			
A.O.	1352	4	352	5	320	<10	
N.R.	1433	2	342	5	160	20 ~ 40	

M.P.		2	348	5	160	<10	<10
N.M.		2	178	<2			
E.W.	1385	2	158	<2	160		
B.M.		4	161	<2	80	<10	<10
J.N.		4	138	<2	40	<10	<10
C.M.		2	141	<2			
T.M.	1402	2	481	10			
S.N.	1472	2	819	18			
O.A.	46341	2	760	16	1280 ~ 2560	320	640
M.W.	1511	1	978	23			
O.J.	1515	1	672	13	640	160	
K.N.	1516	2	126	<2			
J.O.	1494	2	1238	30			
J.K.	1496	2	1036	25			
I.A.	1495	1	356	5			
C.G.	1377	1	1080	27	320	80 ~ 320	
M.M.	1497	2	714	14			
J.O.	1494	1	513	12			
J.K.	1496	1	956	22			
I.A.	1495	1	504	11			
M.M.	1372	1	348	5	640 ~ 1280	<10	80

[a] From J. S. Pagano, G. Klein, and S. Singh. Published in part in Nonoyama *et al.* (1973).

[b] Tissue obtained at biopsy.

[c] Titers given as a range are from sera collected over a period of months.

TABLE III

Epstein-Barr Viral DNA in Tissues from African Tumors[a,b]

Patients	KCC	Disease	No. tests	cpm hybridized/ 50 μg DNA	No. of EBV genome equivalents/ cell	Serum antibody titers		
						VCA	EA-D	EA-R
P.W.		Adenocarcinoma (mandible)	2	1782	45			
J.M.	1265	Melanoma (nose)	4	581	11	840	320	
N.M.		Carcinoma (antrum)	2	391	6	320	<10	<10
S.N.	1408	Reticulum cell sarcoma	2	574	11	1280	320	
T.M.	1419	Adenocarcinoma (mandible)	2	399	6			
C.S.	1237	Carcinoma (maxilla)	4	151	<2	20	<10	<10
N.M.	1423	Carcinoma (maxilla)	4	161	<2	320	<10	$80 \sim 160$
S.N.		Cervical lymph node (tuberculous)	4	143	<2	160	<10	<10
M.K.		Carcinoma (antrum)	2	158	<2	20	<10	<10
G.M.	1439	Carotid body tumor	2	131	<2	20	<10	<10
N.N.		Carcinoma (leg)	2	152	<2			
M.M.	1334	Epidermoid carcinoma (nose)	2	128	<2	20	<10	<10
N.M.		Carcinoma (maxilla)	2	148	<2			
D.N.	1240	Myosarcoma (maxilla)	2	169	<2	$10 \sim 40$		
G.M.	1104	Leukemia	2	153	<2	80	<10	<10
M.N.		Malignant lymphoma	2	171	<2	640	<10	<10
N.N.		Carcinoma (esophagus)	2	132	<2	<10	<10	<10
W.W.	1461	Lymphoblastic lymphoma (mandible)	2	145	<2	20	<10	<10
A.M.	1455	Squamous cell carcinoma (mandible)	2	167	<2	20	<10	<10

R.S.		Carcinoma (tongue)	2	147	<2	320	<10	<10
J.M.	1400	Lymphosarcoma (mandible)	2	143	<2	160	<10	<10
M.K.	1424	Carcinoma (tonsil)	4	157	<2	40	<10	<10
C.M.	1264	Lymphoblastic lymphoma	2	147	<2	40		
K.M.		Carcinoma (maxilla)	2	153	<2			
N.M.A.	767/72	Malignant sarcoma (orbit)	2	147	<2			
K.M.	1474	Adenocarcinoma (cystic)	2	118	<2			
W.J.	1484	Squamous cell carcinoma	2	148	<2			
M.L.	1489	Carcinoma (antrum)	2	123	<2			
M.N.	1433	Squamous carcinoma	2	119	<2			
M.M.	1493	Squamous carcinoma (tongue)	2	107	<2			
W.M.	47443	Squamous carcinoma	1	115	<2			
F.N.[c]	1499	Squamous carcinoma	1	146	<2	160	<10	<10
M.W.	33905	Carcinoma (tongue)	1	133	<2			
K.N.	1510	Squamous carcinoma (mandible)	1	153	<2			
F.N.[c]	1499	Squamous carcinoma	1	110	<2			
K.A.	34989	Squamous cell carcinoma	1	117	<2			
N.N.	1479	Carcinoma (tonsil)	1	125	<2			
K.T.	1482	Carcinoma (antrum)	1	171	<2	80	<10	<10
M.E.	1476	Squamous carcinoma	1	143	<2			
Raji		Burkitt lymphoma line, non-virus-producing, established *in vitro*	4	2323	60			
HEp2		Human fibroblastic cells, established *in vitro*	6	152 ± 23	–			

[a] From J. S. Pagano, G. Klein, and S. Singh. Published in part in Nonoyama *et al.* (1973).
[b] Tissue obtained at biopsy.
[c] Same patient.

Wolf *et al.* (1973) have evidence obtained by the cytohybridization technique that the viral DNA in the tumor tissue appears to be located predominantly, if not exclusively, in the epithelial (carcinomatous) elements, and these cells also appear to contain viral antigen determined by the EBNA test. This important result requires confirmation, and similar studies are under way in this laboratory.

C. Other African Tumors

We have tested a variety of other malignant tissues from sarcomas, carcinomas, and lymphomas for the presence of Epstein-Barr viral DNA by cRNA–DNA hybridization. These tissues came from patients from the same region of Kenya, and several (5 of 39) of the specimens contained Epstein-Barr viral DNA (as shown in Table III). With a single exception the amount of viral DNA tended to be somewhat lower than in the Burkitt's lymphoma or nasopharyngeal carcinoma. A determination of the degree of homology of the DNA in one of the specimens to the DNA from the HR1K virus did not disclose a difference (Kawai *et al.*, 1973). It will be important to determine the biophysical state of the viral genome in these tissues in which the Epstein-Barr virus is only occasionally, rather than consistently, detected. Also, mistaken diagnoses are a real possibility.

TABLE IV

Tests for Epstein-Barr Viral DNA in Peripheral Lymphocytes from Kenya[a,b]

Patients	Code no.	Diagnosis	No. tests	cpm hybridized/ 50 μg DNA	No. of EBV genome equivalents/ cell
J.A.	39656	Burkitt's lymphoma	1	112	<2
K.N.	2718/72	Burkitt's lymphoma	1	104	<2
P.O.[c]	KCC1459	Burkitt's lymphoma	1	137	<2
P.O.[c]	KCC1459	Burkitt's lymphoma	1	157	<2
P.O.[c]	KCC1459	Burkitt's lymphoma	1	178	<2
J.W.	KCC1484	Carcinoma, meatus	1	108	<2
N.W.	57937	Sarcoma (orbit)	1	163	<2
M.N.	29150	Carcinoma (palate)	1	139	<2
M.M.	37102	Squamous carcinoma (tongue)	1	188	<2

[a] From J. S. Pagano, G. Klein, and S. Singh (unpublished).
[b] 30–40 μg leukocyte DNA tested in each case.
[c] Same patient.

We have also examined a number of samples of leukocytes collected from the peripheral blood of African patients, but we have not detected Epstein-Barr viral DNA thus far (Table IV).

D. American Tumors

Assorted specimens from patients in the United States with various malignancies have also been tested. These tissues come from the Sloan-Kettering Institute through Dr. Yashar Hirshaut. As shown in Table V, none of the 19 specimens, whether tested by cRNA-DNA hybridization or DNA–DNA renaturation kinetics analyses, harbor detectable Epstein-Barr viral DNA. We have not yet tested the lymphocyte-depleted form of Hodgkin's disease.

TABLE V

Lack of Detectable Epstein-Barr Viral DNA in Tumors of American Origin Tested by DNA–cRNA or by DNA–DNA Renaturation Kinetics[a]

Patients	Code No.	Disease	cpm hybridized/ 50 μg DNA	No. of EBV genome equivalents/ cell
S.S.	Ti784T	Lymphoma	117	<2
S.S.	Ti767T	Lymphoma	138	<2
A.L.	Ti740T	Carcinoma, laryngeal	115	<2
E.K.	Ti678T	Mammary carcinoma, metastatic	127	<2
E.V.	Ti684T	Recto-sigmoid carcinoma	116	<2
Y.C.N.	Ti662T	Carcinoma of parotid	129	<2
E.D.	Ti754T	Carcinoma (neck)	118	<2
C. son	Ti780T	Giant cell tumor	140	<2
J.K.	Ti775T	Malignant teratoma	119	<2
L.D.	Ti794T	Carcinoma, parotid	102	<2
W.D.	Ti742T	Hodgkin's disease	–	<0.4
D.McC.	Ti730T	Hodgkin's disease	–	<0.4
L.N.	Ti760T	Hodgkin's disease	–	<0.4
R.W.	Ti817T	Hodgkin's disease	–	<0.4
J.M.	Ti802T	Hodgkin's disease	–	<0.4
W.P.	Ti801T	Hodgkin's disease	–	<0.4
K.S.	Ti690T	Malignant melanoma	–	<0.4
J.I.	Ti748T	Malignant melanoma	–	<0.4
M.DeD.	Ti805T	Malignant melanoma	–	<0.4

[a] From J. S. Pagano and Y. Hirshaut (unpublished); see also Fig. 2.

TABLE VI

Lack of Epstein-Barr Viral DNA in American Burkitt's Lymphomas Analyzed by DNA–DNA Renaturation Kinetics[a]

Patients	Code No.	Tissue	EB viral DNA
R.McC.	BRL 2613	Kidney	<0.4
R.McC.	BRL 2618	Spleen	<0.4
R.McC.	BRL 2619	Tumor	<0.4
K.W.	1649P	Omentum	<0.4
M.D.	None	Liver	<0.4
M.D.	BRL 1512	Liver	<0.4
T.O.	BRL 2879	Liver	<0.4
T.O.	BRL 2880	Spleen	<0.4

[a] From Pagano *et al.*, 1973b; see also Fig. 2.

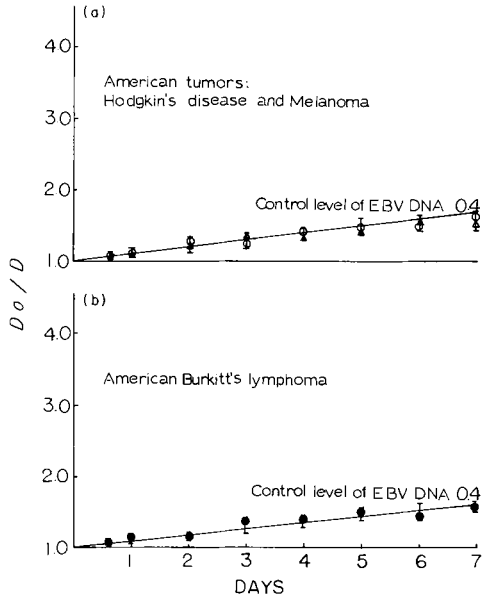

FIG. 2. (a) Analysis of the cellular DNA of American malignancies for Epstein-Barr viral DNA by DNA–DNA renaturation kinetics. The index radiolabeled DNA (0.02 μg of purified tritiated EBV DNA, equivalent to 0.4 genome) was renatured in the presence of 2 mg of cellular DNA from three tissue specimens of malignant melanoma (Δ) or six specimens of Hodgkin's disease (○). The rate of renaturation of the index DNA was unaffected. (b) The DNA (2 mg) from eight specimens of tumor-infiltrated tissue from four patients (see Table VI) with American Burkitt's lymphoma was analyzed under the same conditions as in (a). Double-stranded and single-stranded DNA were separated by hydroxyapatite chromatography. There was no detectable viral DNA (less than 0.4 genome) in the tissues. From Pagano *et al.* (1973b). Reprinted by permission from *N. Engl. J. Med.* **289,** 1395.

E. American Burkitt's Lymphoma

Since the association of Epstein-Barr virus with malignant conditions seems to be restricted to Africa thus far, the rare American specimens of Burkitt's lymphoma have a special importance. The material that we studied was from cases regarded by the American Burkitt's Lymphoma Registry Board of the National Cancer Institute, Bethesda as histologically indistinguishable from the African variety of Burkitt's lymphoma (Table VI). The disease in the United States perhaps exhibits time–space clustering, an epidemiologic feature characteristic of the tumor in East Africa and suggestive of a transmissible etiology, although the rarity of cases makes the definition of epidemiologic patterns difficult in the United States.

The eight specimens of tissue, most of them infiltrated with tumor cells, from four patients contained no detectable Epstein-Barr viral DNA (less than 0.4 and probably less than 0.2 genome per cell) when tested by DNA–DNA renaturation kinetics analyses (Fig. 2). Analyses of the renaturation of three concentrations of the radiolabeled index DNA, as well as Raji cell DNA (a non-virus-producing cell line known to contain a fixed amount of Epstein-Barr viral DNA) and examples of African Burkitt's lymphoma and nasopharyngeal carcinoma, are shown by way of contrast (Figs. 3, 4, and 5). As shown in earlier publications the addition of either HEp-2, HeLa cell, calf thymus, or *E. coli* DNA had no effect on the course of renaturation of the purified labeled viral DNA (Pagano *et al.*, 1973b; Kawai *et al.*, 1973). The total DNA concentration in the renaturation reaction mixtures is equalized by the addition of one of these inert DNA's, usually calf thymus.

The antibody titers in these 4 patients were not especially high, 1:40, 1:160, 1:160, and $<1:10$.

F. Nasopharyngeal Carcinomas from Other Regions

This tumor is widely distributed, especially among persons of Asiatic origin. In contrast to patients with Burkitt's lymphoma, patients with nasopharyngeal carcinoma in parts of the world other than Africa also have high antibody titers to viral capsid antigens of the Epstein-Barr virus. It is most important to determine whether these tissues from non-African sources contain Epstein-Barr viral DNA, and such studies are in progress. We have preliminary evidence that tissues from nasopharyngeal carcinoma from Tunis and Taiwan do in fact contain EBV DNA (J. S. Pagano, G. de Thé, and C. S. Yang, unpublished).

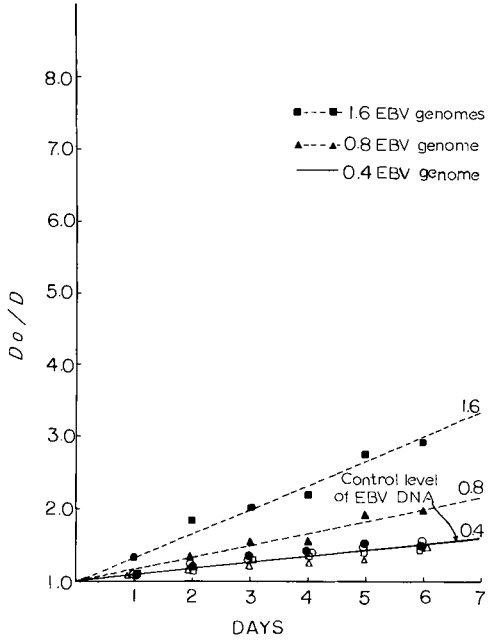

FIG. 3. DNA–DNA renaturation kinetics analysis of Epstein-Barr viral DNA. The kinetics of reannealing of different concentrations of the same radiolabeled Epstein-Barr viral DNA shown in Fig. 2 was determined in the presence of *E. coli* DNA (○) or HEp-2 DNA (□). Doubling and quadrupling of the amount of tritiated viral DNA in the system gave proportional increases in the kinetics of renaturation of the index DNA. The Cot_{50} for 0.4 genome (0.02 μg) was 5.76×10^{-2} mole second/liter; for 0.8 genome (0.04 μg), 2.88×10^{-2} mole second/liter; and for 1.6 genome (0.08 μg), 1.44×10^{-2} mole second/liter. The total DNA concentration in each reaction mixture was equalized by the addition of *E. coli* DNA to 2 mg. The results established that as little as 0.2 of the viral genome in 2 mg of cellular DNA is detectable.

G. Epstein-Barr Virus and Lymphocytes

Direct and indirect evidence (summarized by Klein, 1972) suggests that human lymphocytes cannot be maintained in continuous culture *in vitro* unless the Epstein-Barr viral genome is present. We have tested by cRNA–DNA hybridization a number of lymphoblastoid cell lines established from the peripheral blood. These cell lines were from J. Minowada. All except one of the immunoglobulin-synthesizing lines contained Epstein-Barr viral DNA in amounts ranging between 510 and 5 genome equivalents regardless of whether there was other evidence of the presence of the virus (Table VII). The single cell line without detectable Epstein-Barr viral DNA (RPMI 8226) turns out to be of plasma

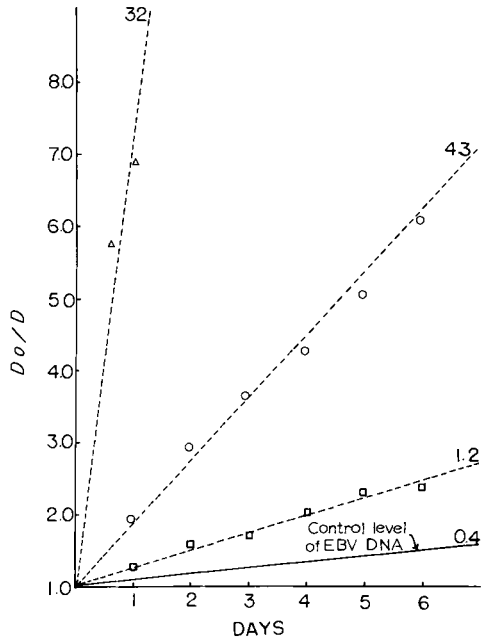

FIG. 4. Analysis of the Raji non-virus-producing cell line for its Epstein-Barr viral DNA content. The conditions were the same as in Figs. 2 and 3. Three concentrations of Raji DNA (40, 150, and 1000 μg) contain 1.2, 4.3, and 32 genomes, respectively. The amount of tritiated EBV DNA used as the index of renaturation was again 0.02 μg, and the total DNA concentration was 2 mg, equalized by the addition of *E. coli* DNA.

cell rather than lymphocyte origin; it is the so-called Simpson line (Minowada *et al.*, 1974).

Therefore all lymphocytic lines with B cell characteristics that we have tested so far harbor Epstein-Barr viral DNA. Leukocytes taken from the umbilical cord of newborn infants cannot be established in culture unless they are first transformed by exposure to Epstein-Barr virus; the transformed lines then contain the viral genome. Lymphoblastoid cell lines originating from tissues of Burkitt's lymphoma are always of B cell origin; the cells harbor Epstein-Barr viral DNA.

H. B versus T Cells

We have discovered that an additional lymphoblastoid cell line, the so-called Molt cells, also does not harbor Epstein-Barr viral DNA. These cells are thymus derived in type, as shown by their capacity to provoke

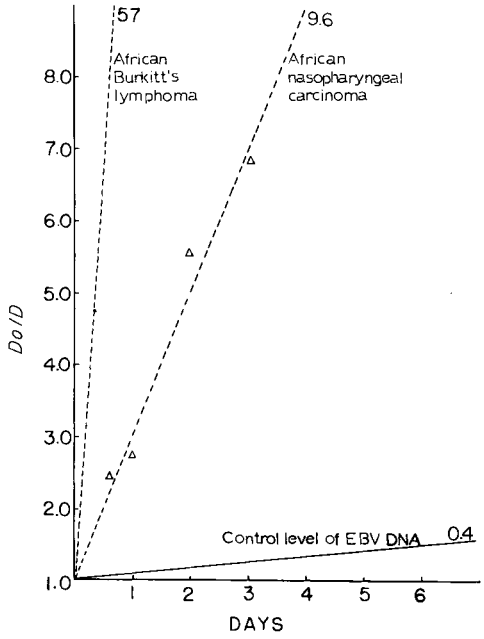

FIG. 5. African Burkitt's lymphoma and nasopharyngeal carcinoma: representative analyses by DNA–DNA renaturation kinetics. The conditions are the same as in the preceding figures. The nasopharyngeal carcinoma DNA (Δ) contained 9.6 EBV genomes in 0.4 mg of cellular DNA (48 genomes/cell), and the Burkitt's lymphoma DNA (---) contained 57 genomes in 2 mg of cellular DNA.

rosette formation with sheep red cells and by surface membrane markers such as complement receptor sites. Continuous lines of lymphocytes of T cell origin are exceedingly rare, but recently we have had the opportunity to study a clone of T cells derived from a line established from a 14-year-old girl with acute lymphocytic leukemia. The cells were established by Dr. George Moore with J. Minowada. Cell lines with B cell characteristics were established from the same patient. Table VIII shows that all three clones of B cell origin contained relatively abundant Epstein-Barr viral DNA as detected by cRNA–DNA hybridization, whereas the lymphocytic line of T cell origin did not (less than 2 genome equivalents). When retested by DNA–DNA renaturation kinetics analyses (Fig. 6), the results were confirmed. There was less than 0.4 and probably less than 0.2 genome of Epstein-Barr viral DNA in the T cell line as well as in the original T cell line (Molt-4). The plasma cell line (Simpson) is also shown in this figure, as is one of the B cell lines in Table VIII.

TABLE VII

Epstein-Barr Virus DNA in Thymus-Independent (B) Lymphocyte Lines cRNA–DNA Hybridization[a,b]

Cell line	Virions (EM)	Antigen (VCA)	No. of EBV genome equivalents/cell	IG synthesis (λ or $\kappa + \delta$, γ, or μ)	Membrane marker receptor site for complement
2 RPMI 7921	+	+	510	+	+
3 RPMI 7711	+	+	235	+	+
5 RPMI 7481	+	+	87	+	+
6 NC-37	?	?	80	+	+
8 RPMI 7551	−	+	60	+	+
9 RPMI 7851	+	+	59	+	+
10 RPMI 6410	−	−	45	+	+
11 RPMI 7281	+	+	40	+	+
13 RPMI 5287	−	+	34	+	+
14 B411-4	+	+	30	+	+
15 RPMI 7881	+	+	29	+	+
17 RPMI 1788	−	−	27	+	+
18 RPMI 7841	−	−	21	+	+
19 RPMI 8235	−	−	5	+	+
20 RPMI 8226[c]	−	−	<2	+(λ only)	−

[a] From J. S. Pagano and J. Minowada (unpublished; see Minowada *et al.*, 1974).
[b] Rosette formation and membrane receptor site for IgG negative in all cells.
[c] Myeloma cell line.

TABLE VIII

Four Lymphocyte Lines, B versus T Cell Origin: cRNA-DNA Hybridization[a,b]

Cell line	cpm hybridized/ 50 μg DNA[c]	No. of EBV genome equivalents/cell
Controls		
Raji	1973,2444,2046	60
HEp-2	210	−
8422 (B)	7036	196
8422 (B)	5746	160
8392 (B)	5830	162
8402 (T)	62	<2

[a] From J. S. Pagano, J. Minowada, and G. Moore (unpublished).
[b] Background of hybridization to HEp-2 DNA has been subtracted.
[c] Input of cRNA 1.2×10^5 cpm/filter.

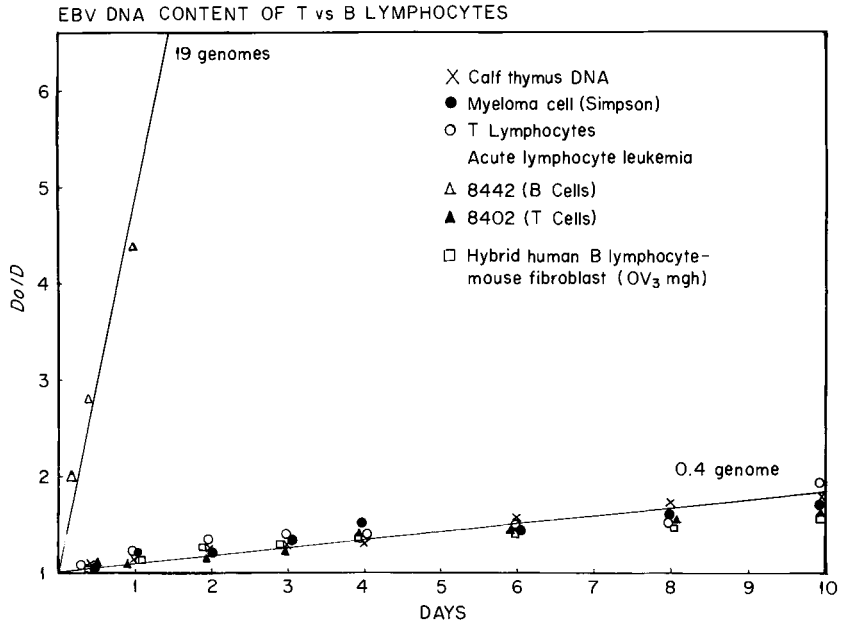

FIG. 6. Analysis of the DNA of T lymphocytes, B lymphocytes, myeloma cells, and a hybrid of human lymphocytic and mouse fibroblastic cells for homology to Epstein-Barr viral DNA. For the B cell line (8442) 0.238 mg of the cellular DNA plus 1.762 mg of calf thymus DNA comprised the renaturation mixture. In all other cases 2 mg of DNA were analyzed in the presence of 0.02 μg of EBV DNA (specific activity, 1.6×10^6 cpm/μg). Single- and double-stranded DNA were distinguished by digestion with S1 enzyme. The Cot_{50} for all the renaturation mixtures was reached in 12 days, except in the case of the B cell line which reached the Cot_{50} value in 6 hours. The B and T cell lines were supplied by J. Minowada and G. Moore and are described in Table VIII. The hybrid cell line was from P. Buchanan. (See Table X and Orkin *et al.*, 1973.)

It therefore appears that lymphocytes of B cell origin require the presence of the viral genome for perpetuation in culture, whereas T cell lines do not (Pattengale *et al.*, 1973). In infection experiments we have been unable to infect the Molt cells with high concentrations of Epstein-Barr virus. These cells do not contain receptors for EBV (Jondal and Klein, 1973).

I. Infectious Mononucleosis

We are engaged in comprehensive studies of patients with acute infectious mononucleosis, drawn mainly from an American student infirmary population administering to patients with mild illness. Some of the results

of the virologic studies are shown in Table IX. In 7 of the patients cell lines were established from the peripheral blood with little difficulty, but never in less than 18 days. The cell lines thus established all harbored Epstein-Barr viral DNA detectable by cRNA–DNA hybridization. The viral DNA in these lines bears at least 90% homology to the HR1K viral DNA (Kawai *et al.*, 1973). Some of these cell lines also exhibited viral capsid antigen by immunofluorescence tests. Filtered throat washings from the patients when applied to leukocytes taken from the umbilical cord of newborn infants, as described by Miller *et al.* (1973), regularly transformed the lymphocytes so that continuous cultures of these cells could be established, usually in 2 to 3 weeks. These new lines of transformed cord cells also contained Epstein-Barr viral DNA detected by cRNA–DNA hybridization, but they did not display viral capsid antigen (Table IX).

Leukocytes were taken from the peripheral blood of nine of these patients. When the DNA of these cells was extracted and tested directly

TABLE IX

Infectious Mononucleosis and Epstein-Barr Virus DNA: cRNA–DNA Hybridization[a,b]

				Cell lines from peripheral blood		Transformed cord leukocytes (throat washings)	
	Antibody titers						
Patient	VCA	EA	Day	Days	No. of genome equivalents	Days	No. of genome equivalents
1	320	160	18?	21–88	60,48,62	Not done	
2	160	80	90	25	27	Not done	
3	160	160	14	21	20	17	15
4	320	80	7	16	37	18	12
5	160	160	7	17	29	17	11
6	320	160	7	17	39	20	11
8	160	160	7	26	34	24	10
9	20	<10	?	36	196	No growth	
10	20	<10	12	No growth		No growth	
7	<10	<10	30	No growth		No growth	

[a] Cytohybridization directly on throat washings and peripheral blood negative for EBV DNA (Pt. Nos. 1 and 3). cRNA-DNA hybridization on membrane filters negative for EBV DNA in all patients (< 2 genome equivalents; not done in Pt. No. 9). Results of studies with material from patients 9, 10, and 7 are shown for comparison. Patients 9 and 10 probably did not have acute disease, the EBV antibody titers being long-standing, and patient 7 did not have EBV infection.

[b] J. S. Pagano and Y.-T. Huang (unpublished).

for the presence of Epstein-Barr viral DNA by membrane hybridization, it did not contain detectable amounts of viral DNA. Some of the same specimens were also examined by the cytohybridization technique with negative results, although the tests are being repeated with more cells screened per slide. Kieff also failed to detect viral DNA by cRNA–DNA hybridization directly in peripheral leukocyte DNA (E. Kieff, personal communication).

J. Infectious Mononucleosis: A Hypothesis

The paradox in these results is that patients with acute infectious mononucleosis invariably exhibit a circulating leukocyte count composed of 40% or more atypical ("transformed") lymphocytes; 80% of the circulating leukocytes in one of our patients were atypical lymphocytes. This fact led to a fairly general assumption that these cells were one of the sites, if not the major site, of replication of the Epstein-Barr virus during the course of the disease. The discovery by Sheldon *et al.* (1973) and Virolainen *et al.* (1973) that the majority of circulating lymphocytes in acute infectious mononucleosis are of T cell origin is peculiarly relevant to the B lymphocyte tropism of the Epstein-Barr virus. These observations stimulate speculations that can be tested experimentally.

We assume that the Epstein-Barr virus replicates in B lymphocytes, perhaps in the bone marrow, in the tonsil, and in the spleen. These lymphocytes bearing the genome are released into the circulation but are largely eliminated by the T lymphocytes, which appear transiently in the circulation. The T cells are immunologically reactive lymphocytes, probably responding to Epstein-Barr virus-specified membrane antigens. The time at which the Epstein-Barr virus-carrying B lymphocytes can be detected in the circulation is unknown, but presumably it is relatively early in the course of the disease, perhaps preceding the symptoms, before the T cell response has been mobilized and the B cells are eliminated. The Epstein-Barr virus-transformed B cells might also be detected in cases of severe infection in which there is an overwhelming discharge of the cells into the circulation. zur Hausen has described such a case, a patient who received a splenectomy, apparently because of an impression of acute malignant lymphoma. In this patient the cellular DNA of the peripheral leukocytes when extracted and tested by cRNA–DNA membrane hybridization disclosed the presence of a large amount of viral DNA; spleen and bone marrow cells also contained Epstein-Barr viral DNA.

However, not all the B lymphocytes bearing the viral genome are eliminated. Continuous lines can be established from the blood of such patients many years after recovery, and the lines that emerge are of the B lymphocyte type bearing the viral genome. It therefore appears that a balance is struck, and a few B lymphocytes persist in the circulation, perhaps by virtue of the presence of a blocking factor and a checking of cytotoxic effects on the residual target (B) cells. Perhaps the persistence of a few of the Epstein-Barr virus-transformed B cells is needed in order to maintain the immune state. The hypothesis suggests that at any time in life following the acute infection, if there is a failure of this immunologic surveillance mechanism, the Epstein-Barr virus-transformed lymphocytes would reemerge *in vivo*, and a B lymphocyte leukemia might result.

K. Relation of the Viral Genome and the Mammalian Cellular Chromosome

The cells selected for these studies have been the Raji line, a non-virus-producing lymphoblastoid cell line originally derived from a specimen of African Burkitt's lymphoma. This cell line was unusual at the time because it did not display evidence of Epstein-Barr virus by electron microscopy or immunofluorescence tests for viral antigens. It did, however, exhibit an "early" (EA) neoantigen when the cells were infected with Epstein-Barr virus *in vitro* (Henle *et al.*, 1970). Although the appearance of the "E" antigen is associated with viral DNA synthesis but not the production of virus in the infected Raji cells (Nonoyama and Pagano, 1972b), viral DNA synthesis is not necessary for its appearance (Gergely *et al.*, 1971). We showed by cRNA–DNA hybridization that the Raji cell line contained 60 genome equivalents per cell of viral DNA, and later confirmed this result by the DNA–DNA renaturation kinetics analysis that indicated that the number of genomes was only slightly lower (52 genomes) than the earlier estimate (Nonoyama and Pagano, 1971, 1973). The striking feature of the association of Epstein-Barr viral DNA and the Raji cells is its constancy. The number of genome equivalents remains the same from one cell generation to the next. zur Hausen has had similar results. Viral DNA synthesis occurs in concert with replication of the cellular chromosome. Moreover viral and cellular DNA replication cannot be dissociated by the use of an inhibitor of protein synthesis, cycloheximide (Pagano *et al.*, 1973a). There must therefore be a mechanism fostering an intimate operational association between virus and cell.

Hampar *et al* (1973) have provided some insight into the association. In virus-producing or nonproducing cell lines the critical period of DNA synthesis that allows either spontaneous or induced activation of the virus begins approximately one hour into the DNA synthetic (S) phase of the cell cycle. Also, in the nonactivated, non-virus-producing cells (Raji) the resident EBV genome is replicated between 1 and 2 hours into the cellular S phase (Hampar *et al* 1974). Thus it appears that the replication of the latent viral DNA is definitely under cellular control.

Having found that all of the viral DNA contained in a culture of Raji cells could be accounted for by the viral DNA content of the cellular chromosomes isolated from cells arrested in metaphase (Nonoyama and Pagano, 1971), we attempted to determine whether the viral DNA was integrated into the cellular DNA by covalent linkage (Nonoyama and Pagano, 1972a). The nuclei were separated from Raji cells and their DNA gently extracted in order to keep shearing of the DNA to a minimum. This high molecular weight DNA was then sedimented through an alkaline glycerol gradient. The distribution of the viral DNA in the gradient was determined by hybridization of each fraction of the gradient with cRNA on membrane filters. Although the cellular DNA sedimented with a characteristic of about 130 S, the viral DNA sedimented in the 70 S to 30 S region, consistent with unit length single strands and nicked single strands under alkaline conditions. All of the viral DNA seemed to be accounted for in this region, but it is possible that a small amount of viral DNA remained in the high molecular weight cellular DNA, undetectable because it was lost in the background of nonspecific hybridization (less than 2 genome equivalents). The possibility that the viral DNA in the 30 S to 70 S region in the gradient originated from a few cells that were replicating Epstein-Barr virus was probably excluded by checking of the culture by cytohybridization; the examination did not reveal a concentration of viral DNA in a few cells, but only the usual widely scattered occasional grains seen in Raji cells with this technique.

The difficulty with this experiment is that it is hard to keep cellular DNA in a region of the gradient away from the expected location of the unit length strands of viral DNA because there is often some fragmentation of the cellular DNA with trailing up through the gradient. Also, there is the real possibility that while the bulk of the viral DNA is indeed freed from the cellular DNA by the alkaline treatment a residuum of the viral DNA with an alkali-stable linkage might still be in the cellular DNA.

The straightforward interpretation of the results of this experiment is that the viral DNA in Raji cells is not covalently integrated into the

cellular chromosome. Experiments that lead to a conclusion somewhat at variance with this one were carried out by Adams *et al.* (1973). Making use of the differences in densities between Epstein-Barr viral DNA and cellular DNA (1.718 as against 1.700 gm/cm^3), these workers fractionated purified Raji DNA by centrifugation in CsCl. The Epstein-Barr viral DNA banded broadly and was also present at the mean density of cellular DNA. When however such fractionated DNA of cellular density was sheared to a size of 10^7 daltons, a class of DNA approaching the density of Epstein-Barr viral DNA could be distinguished in neutral gradients of CsCl. The DNA with a density intermediate between that of cellular DNA and viral DNA hybridized with Epstein-Barr virus-specific cRNA, whereas the lighter DNA did not. It therefore appeared that there might be a linear association between the lighter cellular DNA and the viral DNA.

In alkaline gradients, however, the viral DNA separated completely from the cellular fragments. Therefore it appeared again that the viral and cellular sequences were joined by alkali-labile bonding. Three possibilities have been considered to explain the instability in alkali. The viral and cellular DNA are joined by either RNA linkages or some type of protein or histone bonding, or else there are strategically located nicks in either the viral or cellular DNA such that the viral and cellular sequences are maintained in the linear alignment only by hydrogen bonding of complementary viral and cellular sequences. This interpretation would gain support if Epstein-Barr viral DNA is proved to contain single-stranded nicks in the native state (Nonoyama and Pagano, 1972a,b).

One difficulty with this experiment arises from the possibility that extensive shearing of viral DNA begins to separate adenine+thymidine-rich and guanidine+cytosine-rich regions of the genome so that even pure viral DNA could be found with a density intermediate between that of cellular and viral DNA. The fragments of intermediate density would of course hybridize with the cRNA. This possibility is difficult to exclude rigorously, although Adams and Lindahl did carry out a reconstruction experiment in which a mixture of sheared, purified viral DNA and cellular DNA did not disclose such a density redistribution. It should be noted that the DNA of intermediate density shown in the experiments bands in a rather broad, somewhat skewed, distribution which suggests that the proportion of viral to cellular DNA in these fragments was not constant. This interpretation in turn may suggest that the linkage between viral and cellular sequences was not located at a unique site in these intermediate density molecules.

Experiments to explore these various possibilities are in progress,

including combined studies of velocity sedimentation and isopycnic banding, both under neutral and under alkaline conditions. Experiments to investigate the possibility of RNA and protein linkage are also in process.

L. Homology between Epstein-Barr Viral DNA and Other Herpesviruses

Whether any homology exists between the nucleic acid of the Epstein-Barr virus and some of the other herpes-type viruses, in particular cytomegalovirus and herpes simplex virus type 2, is a question of long standing. All three of these viruses persist in the human host in a latent state for the duration of life after infection – some of the agents infecting tissues or cells of the same type, such as leukocytes, cervix, and seminal cells. It is obviously essential therefore to ascertain whether a given viral cRNA probe might fail to distinguish between related species of viruses. Theoretically the simplest decisive way to examine this possibility is by a determination of homology carried out by DNA–DNA renaturation kinetics analyses. It is now possible to make such comparisons because the radiolabeling of DNA *in vitro* provides viral DNA of sufficiently high radioactivity.

The analysis shown in Fig. 7 indicates no detectable homology between the labeled Epstein-Barr viral DNA, the DNA of a strain of human cytomegalovirus (AD-169), and herpes simplex (types 1 and 2) DNA. In the converse analysis, in which the cytomegalovirus DNA was radiolabeled and the other viral DNA's were unlabeled, the result was the same, that is, there was no detectable homology. The limits of this analysis are such that as little as 5% homology would have been detected, so that the experiments show that there is less than 5% homology between the nucleic acids of these viruses (Huang and Pagano, 1974). Heteroduplex mapping detected by electron microscopy would probably be an even more sensitive, although more cumbersome, method of detecting very small regions of homology, but this method is hardly practicable with EBV DNA.

As indicated earlier the viral sequences found in nasopharyngeal carcinoma show at least 90% relatedness to the standard HR1K viral DNA derived from Burkitt's lymphoma. Also, we have been unable to distinguish any differences in homology between the HR1K viral DNA and the viral DNA in three transformed lymphocyte lines from infectious mononucleosis. These analyses are not considered conclusive until appropriate reciprocal comparisons can be conducted.

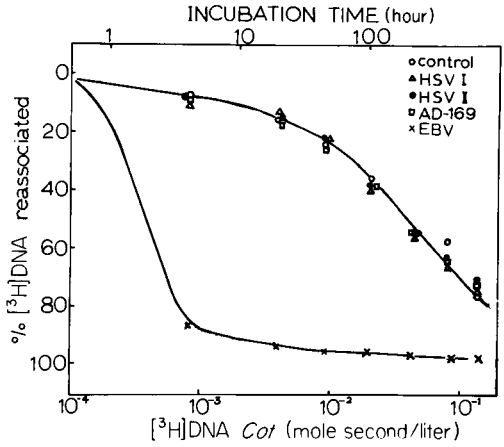

FIG. 7. The lack of homology between Epstein-Barr viral DNA and the DNA of human cytomegalovirus (strain AD-169) and type 1 and type 2 DNA of herpes simplex viruses. Fragmented EBV [^{3}H]DNA 0.02 μg (5.4×10^4 cpm), 20 μg of calf-thymus DNA, and 3 μg of each of the unlabeled viral DNA's were denatured at 100°C in 0.01 *M* Tris-HCl pH 7.4 and 0.0025 *M* EDTA. The salt concentration was then adjusted to 1.2 *N* NaCl. The hybridization was carried out at 66°C. The fraction of reassociated DNA was analyzed by S1 enzyme differential digestion. Calf thymus control DNA (○); HSV 1 DNA (△); HSV II DNA (●); AD-169 DNA (□); EBV DNA (×). (From Huang and Pagano, 1974). Only the addition of the unlabeled CMV DNA accelerated the renaturation of the labeled index CMV DNA.

M. Somatic Cell Hybrids

Hybrids have been produced by Orkin *et al.* (1973) between human lymphocytes and mouse fibroblastic (3T3) cells. The hybrid cells grow as monolayers, but have the unique property of continuing to synthesize human immunoglobulins, namely, λ chains. Clones of the hybrid cells containing between 4 and 24 human chromosomes have been isolated (Table X). These hybrid cell lines are potentially useful in studying the interaction of the Epstein-Barr virus and the mammalian chromosome, since the parental lymphocyte lines (T5-1 and PGLC33H) contain 30 and 60 Epstein-Barr virus genome equivalents, respectively, as measured by cRNA–DNA hybridization. In contrast, eight of the hybrid clones thus far tested contain no detectable Epstein-Barr viral DNA, either by cRNA–DNA hybridization on membrane filters or, in one case, by DNA–DNA renaturation kinetics analysis (Fig. 6). If the viral DNA is associated with a particular human chromosome or several chromosomes then it should be possible to discover the specific chromo-

TABLE X

Human Lymphoblast–Mouse Fibroblast Hybrid Cells[a,b,c]

Cell lines	Mean No. chromosome (range)	Biarmed chromosomes[d] (range)	Genome Equivalents of EBV DNA per cell (cRNA-DNA)
Parental			
T5-1 (human lymphoblasts)	46 (45, 46)	35, 36	30
PGLC33H (human lymphoblasts)	?	?	60
3T3 (mouse fibroblasts)	66 (65–68)	0–2	<2
Hybrids			
T5-1 OV_3	134 (120–156)	17 (10–26)	<2
T5-1 OV_3 BUdR[e]	?	~16	<2
T5-1 OV_3 HMT	?	~17	<2
T5-1 OV_3 MGH	?	~17	<0.4[f]
T5-1 V_1	103 (92–110)	13 (7–15)	<2
T5-1 V_1 MGH	?	~13	<2
PGLC33H L_5	?	18–20	<2
PGLC33H L_5 MGH	?	18–20	<2

[a] From J. S. Pagano, S. Lowry, and P. Buchanan (unpublished).

[b] Both parental lymphoblastic lines and all the T5-1 hybrid clones synthesize human λ chain immunoglobulin. T5-1 is a clone of PGLC33H.

[c] Described in part by Orkin *et al.* (1973).

[d] The biarmed chromosomes with their ranges in parentheses represent an estimate of the minimum number of human chromosomes.

[e] Lacks human chromosome No. 17.

[f] By DNA–DNA renaturation kinetics analysis.

somal association, if such exists, between the Epstein-Barr viral DNA and the mammalian genetic apparatus.

A further possibility is that the bulk of the viral DNA, existing perhaps as a plasmid (Nonoyama and Pagano, 1972a; Pagano *et al.*, 1973a), may have been extruded from the hybrid cells, but a remnant of integrated viral DNA persists in a particular chromosomal linkage if one presumes that the "plasmid" DNA is situated at a different site. So far we have only encountered "all or none" situations, and so there is no evidence to substantiate this hypothesis. It may be difficult to detect a small residuum of integrated viral DNA, since as little as 0.01 of the genome could certainly code for important functions. One clue to persistent viral DNA in the hybrid cells would be the detection of a virus-specified thymidine kinase activity if such exists. These cells lend themselves to such an investigation because a clone of the hybrid cells that

has lost human chromosome number 17, which codes for thymidine kinase, is available (Orkin *et al.*, 1973).

Finally, all the immunoglobulin-synthesizing cell lines that we have studied thus far contain Epstein-Barr viral DNA except the Simpson plasma cell line. However since the hybrid cells do not contain detectable Epstein-Barr viral DNA, the presence of the genome does not appear to be linked to the cellular genes for the synthesis of at least the λ light chain portion of the γ-globulin molecule. Recently Klein *et al.* (1974) have described three lymphoblastoid cell lines with B cell characteristics that apparently do not contain EBV DNA when tested by cRNA–DNA hybridization. The cell lines were established from lymphoreticular malignancies including a lymphosarcoma.

N. Thymidine Kinase

We have been studying the thymidine kinase activity in extracts of Raji cells and also in the somatic cell hybrids. Hampar *et al.* (1972) claimed that in a continuous line of lymphocytes in which virus or viral antigens are inducible by BUdR the level of thymidine kinase activity in the cells is increased after the induction, presumably because the viral genome is then being expressed. This is inferential because thymidine kinases of cellular and viral origin were not distinguished, and there may have been a rebound of the cellular enzymatic activity. Characterization of the enzymatic activity by electrophoresis on polyacrylamide gels is being done (S. Lowry, unpublished). As suggested above we also expect to be able to characterize thymidine kinase coded for by the Epstein-Barr virus genome in hybrid cells that have lost their intrinsic thymidine kinase activity, perhaps after induction of synthesis of viral gene products.

V. Problems and Conclusions

A. Burkitt's Lymphoma

The remarkable association of the viral nucleic acid of the Epstein-Barr virus and two forms of human malignancies, one the African Burkitt's lymphoma and the other nasopharyngeal carcinoma, has been demonstrated and now quantitatively defined in approaching 100% of cases. This demonstration is a stronger link to the virus than the finding of EBV antibodies that are ubiquitous in normal patients as well as those with Burkitt's lymphoma; even those with the disease do not necessarily have high titers of antibodies.

Even if these findings are not a milestone on the road to proof of the viral cause of these tumors, they at least sharply pose an intriguing question, namely, why does the virus single out these two malignancies? Furthermore, if the Epstein-Barr virus is finally shown to cause Burkitt's lymphoma only in a restricted region of East Africa, this is crucial to the study of the cause of cancers elsewhere, for the conclusion seems inescapable that an essential cofactor is operative only in East Africa, since the Epstein-Barr virus is distributed globally. The existence of more than one strain of the Epstein-Barr virus is not substantiated so far either by immunologic markers or by nucleic acid homology studies, but the studies are not conclusive.

With respect to American Burkitt's lymphoma the evidence would now suggest that the disease in the United States, despite its histopathologic and clinical resemblances, is different from the African variety. The fact that the American tumor specimens lack Epstein-Barr viral DNA is confirmed by the latest serologic survey, which fails to disclose a higher mean antibody titer in patients with American Burkitt's lymphoma compared with matched control patients (Hirshaut *et al.*, 1973). Of course the argument could be turned around to suggest that the tumors in Africa and the United States are the same, but that the virus is an accidental passenger in the African situation, perhaps because of a greater prevalence of the agent. This conclusion ignores the mass of epidemiologic observations, especially the age distribution of patients with the tumor and the time–space clustering of cases, that suggests a transmissible cause operating perhaps through a vector.

There is a further possibility. The lymphomas in both areas might be caused by the Epstein-Barr virus, but the amount of viral genome residing in the American tumor tissue is below the limits of detection, which at present approach 0.1 genome. From a comparison with SV40 transforming systems it is clear that as little as 0.01 of the Epstein-Barr viral genome could code for a protein required for transformation.

Finally, since the epidemiology of the American tumors hints at a possible infectious cause, American Burkitt's lymphoma might be due to another virus, as yet undiscovered. Klein has postulated the existence of another agent in those rare tissues of African Burkitt's lymphoma that meet the clinical, histopathologic, and epidemiologic criteria of the African disease but do not contain detectable Epstein-Barr viral DNA.

B. Nasopharyngeal Carcinoma

The association of the Epstein-Barr virus with nasopharyngeal carcinoma is significant on two counts: First, this malignancy is much more

widely distributed throughout the world than the Burkitt's lymphoma, although its frequency is highest in persons of Asiatic origin. Second, the tumor cells are epithelial in origin. The question of global distribution versus peculiar regional association of the virus with malignancy promises to be answered decisively by the nucleic acid hybridization studies underway with this carcinoma. The mean antibody titers to the Epstein-Barr virus in patients with nasopharyngeal carcinoma in parts of the world outside Africa appear to be distinctly higher than patients without the malignancy who live in the same region. The results just now coming in indicate that Epstein-Barr viral DNA is present in specimens of nasopharyngeal carcinoma from outside East Africa (J. S. Pagano, G. de Thé, and C. S. Yang, unpublished data).

The second topic of interest are the findings by Wolf *et al.* (1973) that the Epstein-Barr viral DNA in these carcinomas appears to be localized in the epithelial cells rather than in the lymphocytic elements that characteristically infiltrate this tumor. If these findings are confirmed, they either mean that the Epstein-Barr virus is not exclusively a lymphocytotropic virus or that a cooperative interaction between lymphocytes and carcinomatous cells occurs in this tumor—an interaction that lifts the restriction to the presence of Epstein-Barr viral DNA in cells of epithelial origin. There is a tangential suggestion of such an interaction in the studies of Glaser and Rapp (1972) of somatic cell hybrids.

C. Infectious Mononucleosis and Leukemia

The more we study infectious mononucleosis, the more the adage of Dameshek, that infectious mononucleosis is a self-limited leukemia, seems to be tenable. There is no question that the Epstein-Barr virus has a remarkable tropism for human lymphocytes. Infection of these cells converts them into continuous lines *in vitro,* but this phenomenon appears to take place only with lymphocytes of B cell origin. Such transformed B cells persist in the circulation of persons who have been infected with this virus, either subclinically or during symptomatic infectious mononucleosis, for life. It is therefore certainly true that these transformed cells are held in check in the human organism, probably by a mechanism of immunologic restraints that is lost once the cells are transferred to *in vitro* culture conditions. Such a formulation has been considered for years by G. Klein and by G. Henle and W. Henle. Now a theory as to the specific nature of these restraint mechanisms is beginning to evolve, and I have sketched it out, based on an already substantial body of data, earlier in this chapter. The importance of this whole concept is that virtually all chronic lymphocytic leukemias are of

B cell origin (Aisenberg and Bloch, 1972), and, in fact, certain forms of lymphocytic leukemia are associated with high Epstein-Barr viral antibody titers (Levine *et al.*, 1971).

D. Interaction of the Viral Genome and the Cellular Chromosome

The evidence that the viral DNA in the non-virus-producing Raji cells is not linearly integrated into the cellular chromosome by alkali-stable linkages has been reviewed earlier. Since the viral DNA in the Raji cells appears to be evenly distributed throughout the cells in a given population—although this has not been rigorously proved—it appears unlikely that the constancy in amount of viral DNA in these cells is due to a balance between two populations of cells, one producing viral DNA and the other without replicating Epstein-Barr viral DNA. Moreover examination of the cells by the *in situ* cytohybridization technique failed to reveal concentration of the viral DNA in a few cells. Also, essentially all the cells contain the EBNA. In functional terms, therefore, one might consider that the state of the viral genome in the Raji cells is tantamount to that of a plasmid (Pagano *et al.*, 1973a). The so-called plasmid hypothesis is attractive as a mechanism to explain the relative ease of reactivation of the viral genome in the Raji cells. It is consistent with the fact that unit length viral DNA can be distinguished in the Raji cells (Nonoyama and Pagano, 1972a). The hypothesis might also predict that this cell line could be "cured" of its viral DNA, but efforts in this direction thus far have been unsuccessful. Moreover since Raji cells have the characteristics of B lymphoblastoid cells, their survival in culture after curing might well be terminated.

Another possibility is that the viral DNA in the Raji cells and perhaps in other non-virus-producing lymphoblastoid cell lines exists both in an integrated state and as a plasmid. The plasmid DNA would be the biologic basis for reactivation phenomena, so characteristic of herpes-type infections, whereas linearly integrated DNA, present in small quantities, might be necessary for the transforming relation between virus and cell. In the Raji cells both relations might coexist because Raji cells are transformed and also exhibit reactivation of the genome when presented with certain stimuli. It would thus be unnecessary to invoke the integration of 50 copies of a genome with a molecular weight of 10^8 daltons into the chromosomes of every Raji cell. The integration of a fragment of the genome would suffice to maintain the malignant state. The bulk of the viral DNA, although its replication is somehow controlled by the replication cycle of the cellular DNA, might then not be

essential to the survival of lymphoblastoid cell lines *in vitro*. The somatic cell hybrids developed by Orkin *et al.* (1973) may provide the avenue for the investigations needed to test this concept.

The argument for causality is likely to take fascinating turns in an uncharted quest, but now clearly it is important to ascertain not only that the viral DNA is in the tissue but also the nature of the relation between the viral genome and the cellular chromosome. It is not difficult to imagine that a casual or "passenger" relation between virus and cell might have different hallmarks from an etiological association. These ideas frame one of the key current approaches to assessing the significance of the relation between the DNA of the Epstein-Barr virus and the malignancies with which it carries such a striking association.

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CHAPTER 5

Comparison of Genome Replication Mechanisms of Oncogenic DNA Viruses

PIERRE BOURGAUX

I. Introduction

Oncogenic DNA viruses kill the cell in which they multiply; thus virus-transformed cultures are generally free of virus. Under certain circumstances, however, cells transformed by these viruses will yield the transforming agent and, of course, die. Therefore, oncogenic DNA viruses are not unlike temperate phages. Since structurally they appear to be an extremely heterogeneous collection of viruses, their genomes

have been examined for the existence of common properties. Both in molecular weight and structure (secondary and tertiary), the nucleic acids of these viruses were found to cover a wide spectrum. The possibility still remained, however, that some of the intracellular forms of their genomes would be similar in some respect. With this in mind, people began to search the infected cell for forms of the viral DNA—namely, replicative forms—yet undetected in the virion. Such a search was necessarily restricted to those viruses that could be grown satisfactorily in tissue cultures, namely, certain papova-, adeno-, and herpesviruses. Among these viruses, the two papovaviruses, SV40 and polyoma, offered numerous advantages under the circumstances. Not unexpectedly, they were the first ones to be subjected to extensive study. Herpesviruses, on the other hand, contain a DNA with a molecular weight in excess of 10^8 daltons. This DNA's great sensitivity to shearing represents a serious handicap for those attempting to characterize its intracellular forms. It is thus not surprising that due to lack of sufficient information, herpesvirus DNA replication will not be dealt with here.

II. Papovaviruses

A. Facts and Predictions

Purified preparations of polyoma virus and SV40 contain three molecular forms of double-stranded DNA (Weil and Vinograd, 1963; Crawford and Black, 1964), namely, components or forms I, II, and III (Fig. 1). Component I may be regarded as the intact viral genome since, at least in the case of polyoma virus, it has been conclusively shown to be the component endowed with maximal biological activity, i.e., infectivity and transforming activity (Dulbecco and Vogt, 1963; Crawford *et al.*, 1964; Bourgaux *et al.*, 1965). It is a circular molecule (Dulbecco and Vogt, 1963; Weil and Vinograd, 1963) with separately continuous complementary chains (Weil and Vinograd, 1963; Vinograd *et al.*, 1965) of molecular weight 3×10^6. When unconstrained, such a molecule containing about 5000 nucleotide pairs should have 500 (*duplex winding number* or β) Watson–Crick or duplex turns in the B configuration (Vinograd and Lebowitz, 1966). When the synthesis of component I is completed in the cell, the molecule comprises only 480 to 485 (*topological winding number* or α) turns, depending on the origin of the DNA. The α of the molecule cannot be changed, as long as the continuity of the polynucleotide chains is preserved. In solution, the molecule

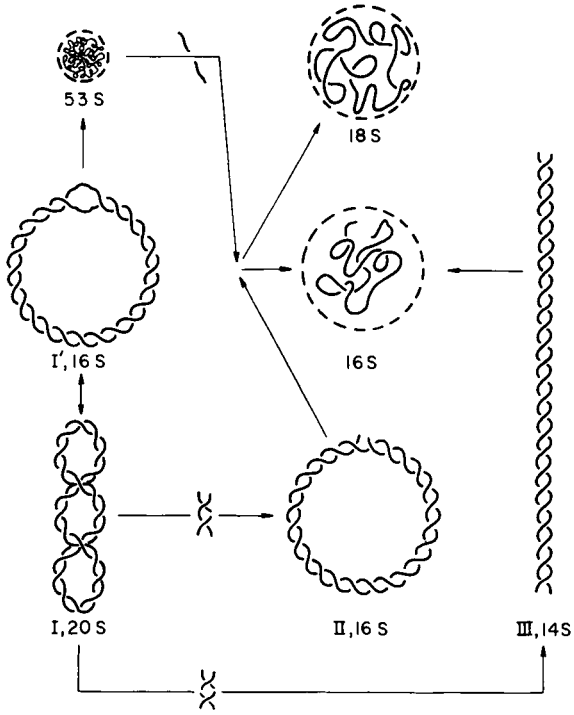

FIG. 1. Diagrammatic representation of the several forms of polyoma DNA (courtesy of Dr. Jerome Vinograd). After Vinograd *et al.* (1965), with permission of *Proc. Nat. Acad. Sci. U.S.*

tends to acquire a structure with 500 secondary turns, and, as a result, *negative* twists or supercoils, i.e., tertiary turns, appear. Components I from polyoma virus and SV40 DNA contain, respectively, 16 and 19 (*superhelix winding number* or τ) such twists (Gray *et al.*, 1971). The structure of component I is thus defined by the equation (Vinograd *et al.*, 1968)

$$\tau = \alpha - \beta \tag{1}$$

One single-stranded break or nick converts component I into component II (Vinograd *et al.*, 1965). One chain being able to rotate about the axis of the duplex, component II thus contains no twists ($\alpha = \beta \rightarrow \tau = 0$). These changes in configuration account for the differences in sedimentation coefficients observed between component I (20 S) and component II (16 S) in neutral solution. Under the same conditions, the viral DNA would sediment at 14.5 S (Weil and Vinograd,

1963) when in the linear configuration. Such linear duplexes, as well as linear duplexes of shorter length, are found in SV40 and polyoma virus preparations (component III). Most of them, however, appear to be of cellular rather than viral origin (Winocour, 1967; Kaye and Winocour, 1967; Michel *et al.*, 1967; Trilling and Axelrod, 1970).

When sedimented in alkaline solution, the three components again show distinctive properties. Both components II and III are abruptly denatured at a critical pH. The former then gives rise to one circular single strand and one linear single strand, sedimenting, respectively, at 18 S and 16 S in alkaline solution (Vinograd *et al.*, 1965), while the latter yields linear single strands exclusively. When centrifuged for short periods of time in alkaline solution, the two discrete components arising from component II appear as a single band sedimenting at 17 S. In striking contrast, the sedimentation coefficient of component I varies widely between the pH values of 11.5 and 12.5 (Vinograd *et al.*, 1965). As pH is raised the helix—as well as the superhelix initially—unwinds. When the number of remaining duplex turns is equal to α, no *negative* twists are left, so that the molecule is endowed with a decreased sedimentation coefficient similar to that of component II (Fig. 1). Further unwinding of the duplex at still higher pH values causes the appearance of *positive* twists, with a corresponding increase in S value. Upon complete titration of the duplex, the complementary chains remain together, held by the topological bond that results from their continuity. Since α is not changed after denaturation, fully titrated component I assumes a very compact configuration and sediments at 53 S (Vinograd *et al.*, 1965). Intercalating drugs also unwind duplex DNA (Crawford and Waring, 1967; Radloff *et al.*, 1967). Up to a certain point, increasing doses of these drugs act on the configuration and S value of component I as increasing pH values (Crawford and Waring, 1967; Bauer and Vinograd, 1968). In addition, they decrease the buoyant density of DNA in caesium chloride (CsCl) solution. Since the continuity of the chains in component I represents a restriction to unwinding or intercalation—at high drug concentration at least (Bauer and Vinograd, 1968)—component I binds less drug and is thus denser than component II or III in caesium chloride solution containing ethidium bromide (EthBr–CsCl) (Radloff *et al.*, 1967) or propidium iodide (PDI–CsCl) (Hudson *et al.*, 1969; Eason and Vinograd, 1971). It had been known for a long time that large amounts of component I were synthesized in productively infected cells (Weil, 1963; Weil *et al.*, 1965; Sheinin, 1966; Hirt, 1967). The identification of replicating DNA of these viruses could then be envisaged in the following way. Productively infected cells would be subjected to short pulses of radioactive thymidine during the phase of mature DNA synthesis. Since cellular DNA synthesis is stimulated by the

viral infection (Dulbecco *et al.*, 1965; Weil *et al.*, 1965; Winocour *et al.*, 1965), viral DNA should then be isolated from the cells using a method that would allow the separation from cellular and also preferably from encapsidated viral DNA. This should be possible, since it was expected that the two latter DNA's would be either strongly bound to protein and/or part of large protein DNA complexes. Since the mature forms of the DNA have well-defined physical properties, the presence of replicating molecules with other distinct characteristics could then be envisaged in the fraction of the extract containing the relatively low molecular weight viral DNA. Replicating molecules were expected to have a higher molecular weight than mature ones, and thus to sediment more rapidly. Short pulses would preferentially label growing chains, which would necessarily sediment in alkaline solution as linear single strands, the size of which could be easily compared to that of those present in component II. Long pulses would also label template chains; if cyclic, those would be detected as 18 S sedimenting material in alkaline solution. The presumably progressive unwinding of the template duplex implied by the semiconservative nature of the replication process (Hirt, 1966) required that at least one of the template chains be nicked, permanently or intermittently. A low density in EthBr–CsCl solution therefore would not have been an unexpected feature for a precursor of mature DNA. These were predictions one could make at the time the experimental work started.

B. Nature and Architecture of the Replicative Intermediates

1. Polyoma Virus

In order to study viral DNA replication, monolayer cultures of whole mouse embryo in resting state (Fried and Pitts, 1968) were infected with either large-plaque (Bourgaux *et al.*, 1969) or small-plaque (Bourgaux *et al.*, 1971) polyoma virus. The cultures were pulse labeled for various lengths of time with radioactive thymidine 30 hours after infection, i.e., at the time mature viral DNA (form I) accumulated intracellularly at a maximal rate (Dulbecco *et al.*, 1965). The monolayers were then covered with a 0.25% (w/v) sodium deoxycholate solution and maintained for 10 minutes at room temperature. The resulting cell lysate was collected from the plates and clarified after centrifugation at 30,000 *g* for 20 minutes. The clear supernatant obtained under these conditions contained what was referred to as the DOC-extracted DNA, consisting almost exclusively of free, nonencapsidated viral DNA (Bourgaux *et al.*, 1969).

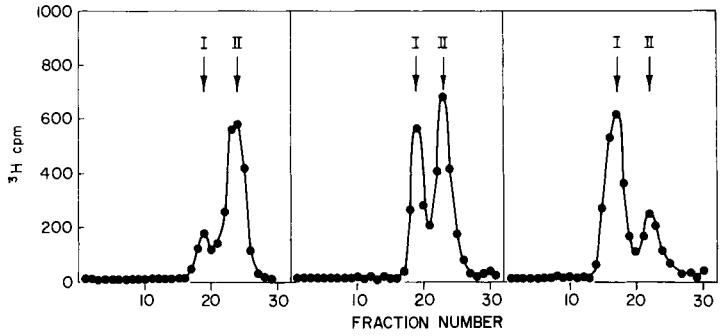

FIG. 2. Radioactivity distribution after dye buoyant density gradient centrifugation of DOC-extracted DNA. DNA was extracted from infected cells either after a 5-minute pulse (left), after a 5-minute pulse followed by a 5-minute (center), or 15-minute chase (right), before being centrifuged to equilibrium in EthBr–CsCl solution. The arrows indicate the positions in such gradients of mature viral DNA form I (dense) and form II (light).

As shown by Fig. 2, most of the DOC-extracted DNA labeled after a short pulse of [^{3}H]thymidine exhibits a light density in EthBr–CsCl solution, while this radioactivity can be chased quantitatively into material with the high density characteristic of form I (Bourgaux *et al.*, 1969). When subjected to velocity sedimentation in neutral solution, the radioactive material with the light density was shown to consist of form II and of a heretofore unidentified component, sedimenting more rapidly and more heterogeneously. The latter component, which, after short labeling periods, represented most of the radioactivity recovered in the light band, was shown to hybridize with purified cold polyoma virus DNA. It was thus likely to represent a replicative intermediate of the viral DNA and was designated as component, or form, II* (Bourgaux *et al.*, 1969).

When form II* was labeled after 3 to 15 minute pulses of [^{3}H] thymidine and sedimented through alkaline sucrose or CsCl gradients, the radioactive profiles registered were those expected for a collection of uniformly labeled linear chains of various lengths, but were never greater than the chains present in mature viral DNA (Fig. 3). These highly reproducible and clear-cut findings (Bourgaux *et al.*, 1969, 1971) suggested the following conclusions: (1) Form II* actually consisted of replicating molecules, since a significant fraction of the radioactivity present in it after a short labeling period was recovered in alkaline solution as slowly sedimenting single strands, presumed to represent growing chains from "young" replicating molecules. (2) The

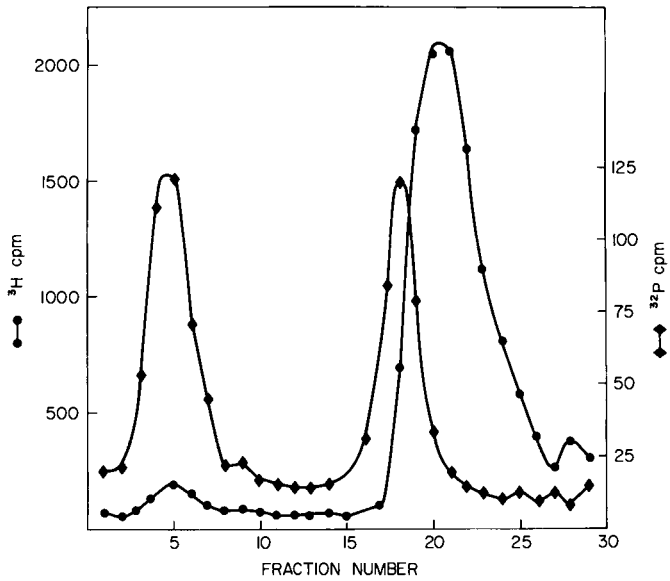

FIG. 3. Velocity sedimentation of DOC-extracted DNA in alkaline CsCl solution. DNA was extracted from infected cells at the end of a 3.5-minute pulse and mixed with ^{32}P-labeled mature viral DNA (form I + form II) before being analyzed. Sedimentation is from right to left. The approximate position of linear single chains of one genome length in this gradient is given by denatured component II, i.e., the slower moving ^{32}P-labeled band. From Bourgaux *et al.* (1969).

duplication time of form II* was somewhat shorter than 5 minutes, for after a pulse of such length, the activity within the largest single chains was already maximal. (3) Replication did not involve the elongation of the template chains, for no labeled linear single chains larger than those present in mature viral DNA were detected.

Later, form II* was purified on benzoylated-naphthoylated DEAE (BND) cellulose (Gillam *et al.*, 1967), as already done by Levine *et al.* (1970) for replicating SV40 DNA. The validity of the aforementioned conclusions was then verified (Bourgaux *et al.*, 1971). When examined under the electron microscope, purified preparations of form II* were found to consist of cyclic molecules with three branches, two branch points, and no visible end (Fig. 4), similar in all respects to those already isolated by Hirt (1969) from polyoma virus-infected cells. Two of the branches were approximately equal in length ($a = b$), while their mean length $(a + b)/2$, added to that of the third branch (c), gave a value $[(a + b)/2 + c]$ equal to that of the contour length of mature polyoma

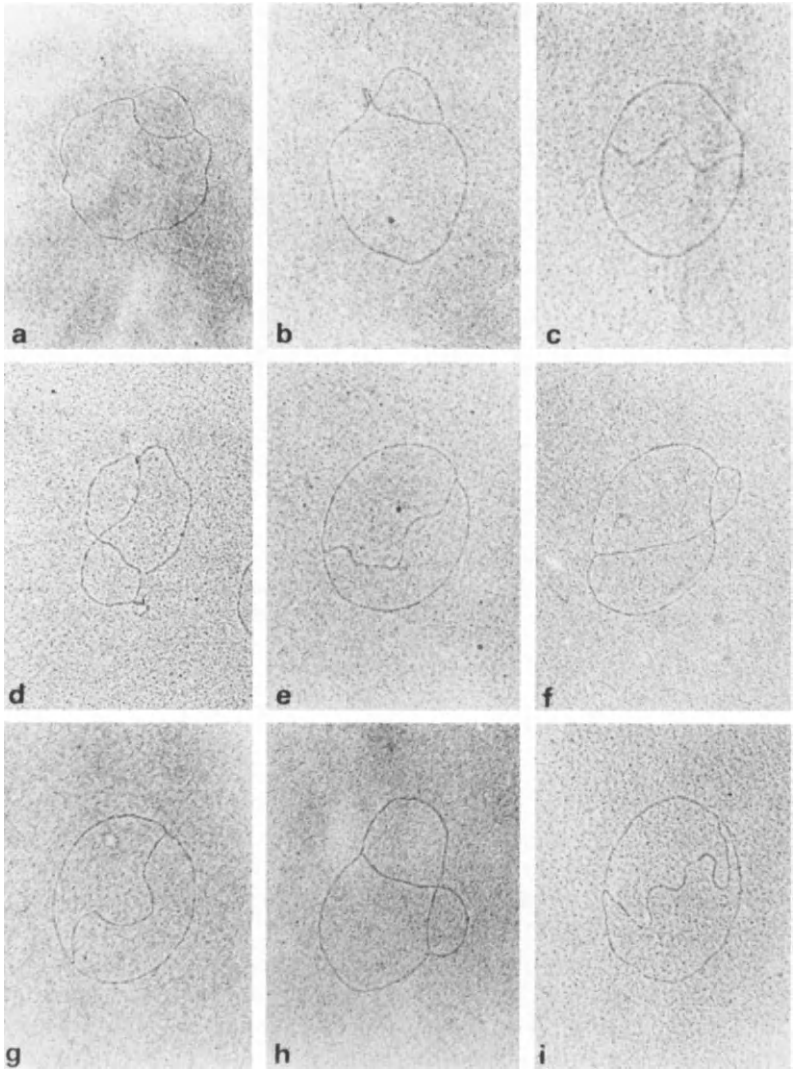

FIG. 4. Electron micrographs of form II*. × 35,600. From Bourgaux *et al.* (1971).

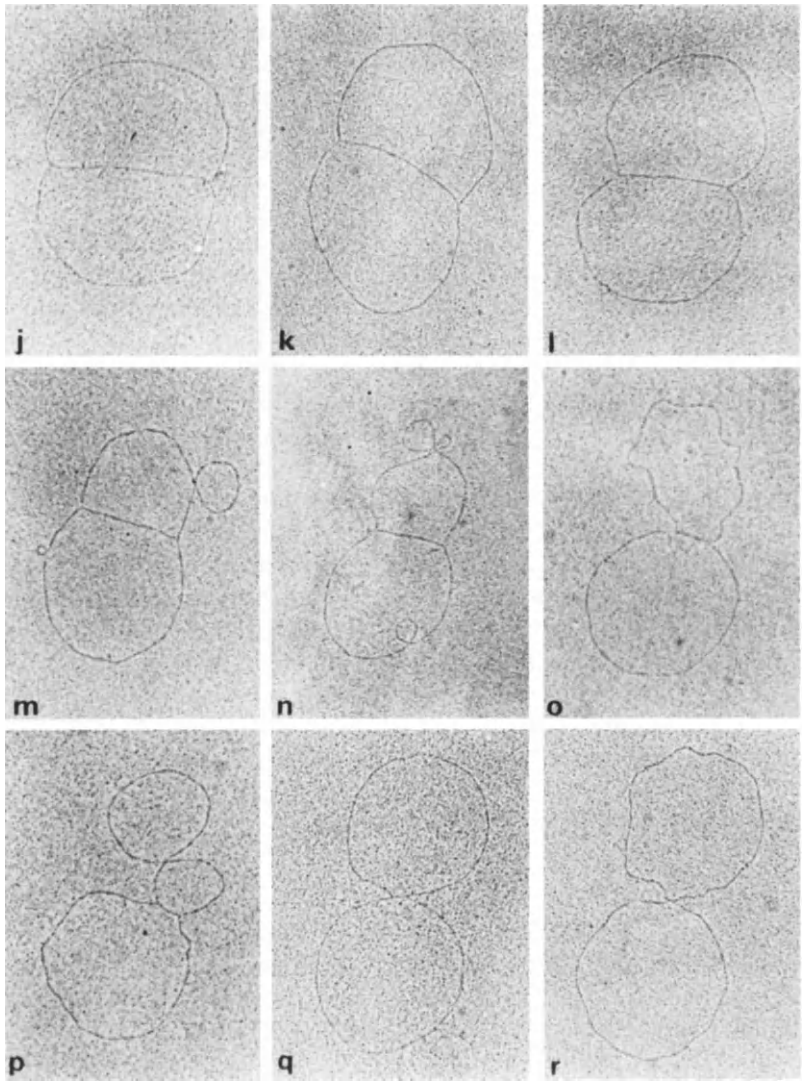

FIG. 4. *Continued*

virus DNA. It was possible to estimate for each such molecule the fraction of genome that was replicated by calculating the ratio: $(a + b)/2$ over $(a + b)/2 + c$. Such an analysis seemed to indicate that molecules near completion of replication were slightly more numerous than those in which replication had just been initiated. Such a finding was difficult to interpret, since the latter molecules obviously were bound to appear less conspicuously under the microscope.

2. SV40

Similar findings on SV40 DNA replication were reported by Levine *et al.* (1970). Chromatography on BND cellulose yielded preparations of replicating viral DNA which, when examined under the electron microscope, were found to contain molecules similar to those observed by Hirt (1969) in cells infected with polyoma virus. The preponderance of largely replicated molecules in those preparations was even more marked than in the case of form II* from polyoma virus DNA. Although this finding was further substantiated later (Sebring *et al.*, 1971), its significance is still unclear.

When labeled after short pulses of [^{3}H]thymidine and sedimented in alkaline sucrose solution, replicating SV40 DNA produced radioactive patterns (Levine *et al.*, 1970) that were clearly reminiscent of those observed for similarly labeled polyoma form II*. Unfortunately, the sedimentation properties of the daughter chains were not accurately determined in those early studies, and, as a result, the general mechanism of replication could not be assessed at that stage.

C. Origin and Direction of Replication

1. Polyoma Virus

Replication DNA's from both SV40 (Levine *et al.*, 1970) and polyoma virus (Bourgaux *et al.*, 1971) were thus successfully purified on BND cellulose (Gillam *et al.*, 1967). Elution of these molecules from the columns required solutions containing caffeine, while that of the corresponding mature double-stranded DNA could be achieved using buffered saline solutions. These chromatographic properties suggested that the replicating molecules included single-stranded regions. Such regions had already been visualized in other replicating genomes and found at the forks presumed to represent growing points (cf. Watson, 1972). It thus appeared logical to think that information regarding the magnitude, location, and number of these regions might be useful for the understanding of the replication mechanism. Form II* was therefore sub-

jected to the action of a nuclease preparation extracted from *Neurospora crassa* conidia, which had been shown to include exonucleolytic and endonucleolytic activities and to be highly specific for single-stranded polynucleotides (Fraser *et al.*, 1970; Rabin and Fraser, 1970). It was found that (1) single-stranded material in form II* represented very little of its total mass, for only 2% of the radioactivity in uniformly labeled preparations were rendered acid-soluble after prolonged exposure to the nuclease (Bourgaux-Ramoisy, 1971). (2) Form II*, sedimenting at about 25 S in neutral sucrose solution, was converted by the nuclease first into a 19 S intermediate product, and then into a 16 S final product (Fig. 5). As judged from their affinity for BND cellulose, the intermediate product was still partly single stranded, while the final product was not. Since the nuclease did not generate any DNA fragment shorter than polyoma DNA form III (14.5 S), these changes in sedimentation coefficients could only reflect changes in configuration, such as those that would result from the digestion of *two* single-stranded regions,

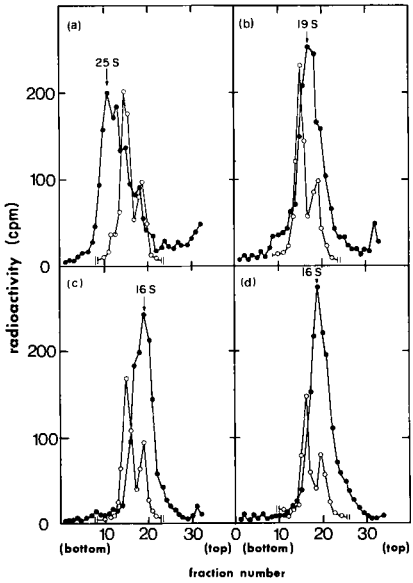

FIG. 5. Sedimentation properties of nuclease-digested replicating polyoma virus DNA (form II*) in neutral sucrose solution. Form II* was purified from viral DNA that had been labeled for 15 minutes with [^{3}H]thymidine and treated with nuclease for (a) 0, (b) 10, (c) 30, or (d) 120 minutes before being centrifuged through neutral sucrose solutions. The sedimentation marker is a mixture of ^{14}C-labeled form I and form II. ●—●, ^{3}H radioactivity; ○—○, ^{14}C radioactivity. From Bourgaux and Bourgaux-Ramoisy (1971).

each located on a different loop of the molecule (Bourgaux, 1971). (3) Under the electron microscope, the intermediate product appeared as circular structures having one tail (Fig. 6). The respective lengths of the tail and circular portions of these structures were those that one would have expected, assuming that the digested single-stranded regions had

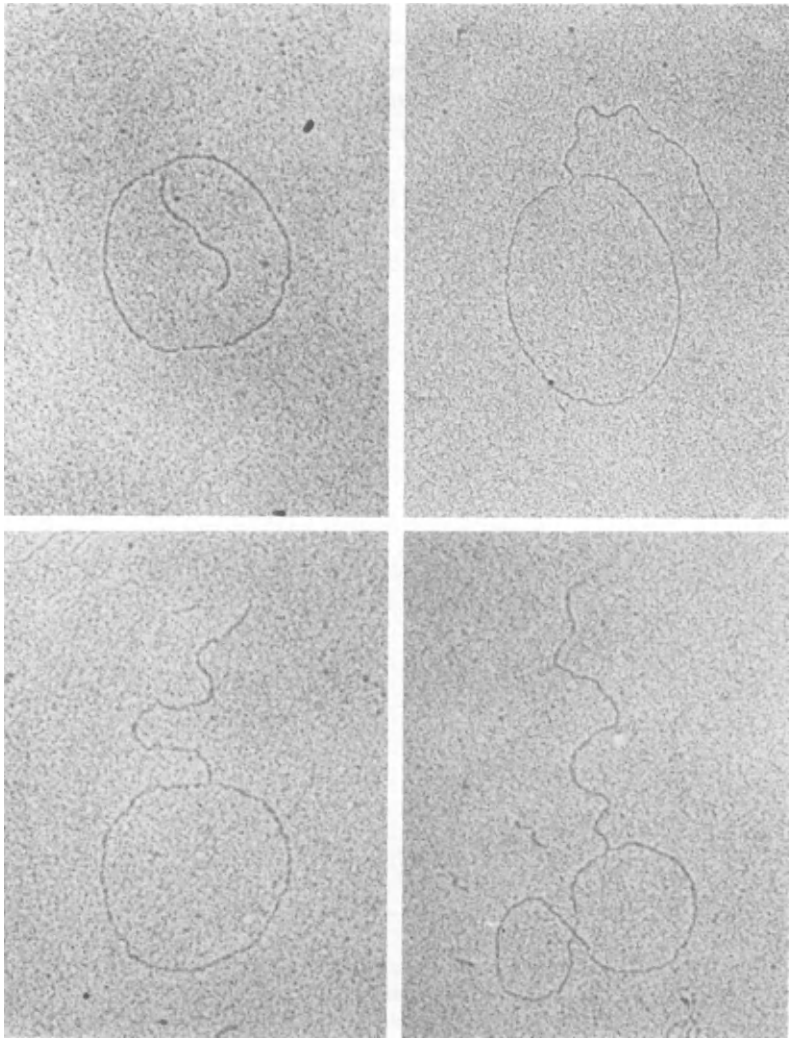

FIG. 6. Cyclic duplexes with one tail (intermediate product) generated by the *Neurospora crassa* nuclease. ×47,100. From Bourgaux and Bourgaux-Ramoisy (1971).

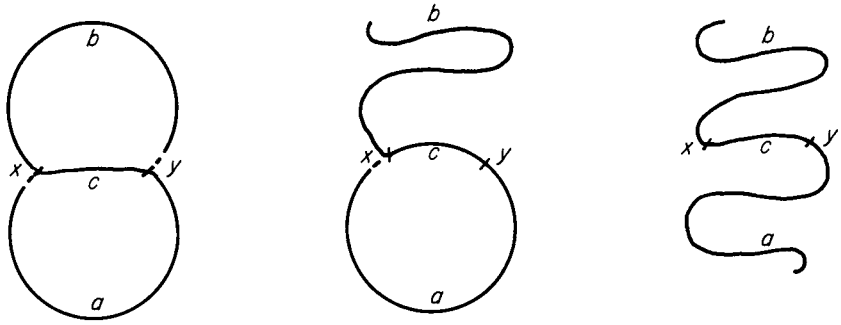

FIG. 7. Nuclease-induced configurational changes in form II*. x, y, branch points; *a*, *b*, replicated branches; *c*, unreplicated branch. Since *a* equals *b*, $a + c$ equals $b + c$ equals a constant. In the cyclic structures with tails which were actually observed (see Fig. 6), it was verified that the length of the circular portion ($a + c$) was constant, while that of the tail (*b*) varied as already observed for $(a + b)/2$ in the intact molecules. From Bourgaux and Bourgaux-Ramoisy, (1971).

been located on one of the two replicated branches, close to one branch point (Fig. 7). The electron microscopic examination also suggested that the final product was a nonbranched linear duplex, as expected if the second nuclease-susceptible site had been located on the other replicated branch, close to the other branch point (Fig. 7). As a conclusion to these observations (Bourgaux and Bourgaux-Ramoisy, 1971), the following replication model was proposed (Fig. 8). Replication of polyoma DNA is initiated at one, presumably specific, point on the molecule and proceeds from there in both directions. As the DNA polymerases synthesize polynucleotidic chains in the 5′ → 3′ direction only (Mitra *et al.*, 1967; Kornberg and Gefter, 1971), the synthesis of the two daughter chains at the growing point cannot be strictly synchronous (Guild, 1968; Richardson, 1969). Since complementary chains have opposite polarities, the fact that elongation of chain W would lag behind that of chain C at one growing point would imply that the opposite situation would prevail at the other growing point. This would explain why the two regions where template material is exposed in a single-stranded form are located trans on the molecule. Such a location of single-stranded regions has since been observed in various DNA genomes replicating bidirectionally (Watson, 1972).

2. *SV40*

SV40 DNA was shown to be cleaved into eleven specific fragments, separable by polyacrylamide gel electrophoresis, when exposed to a

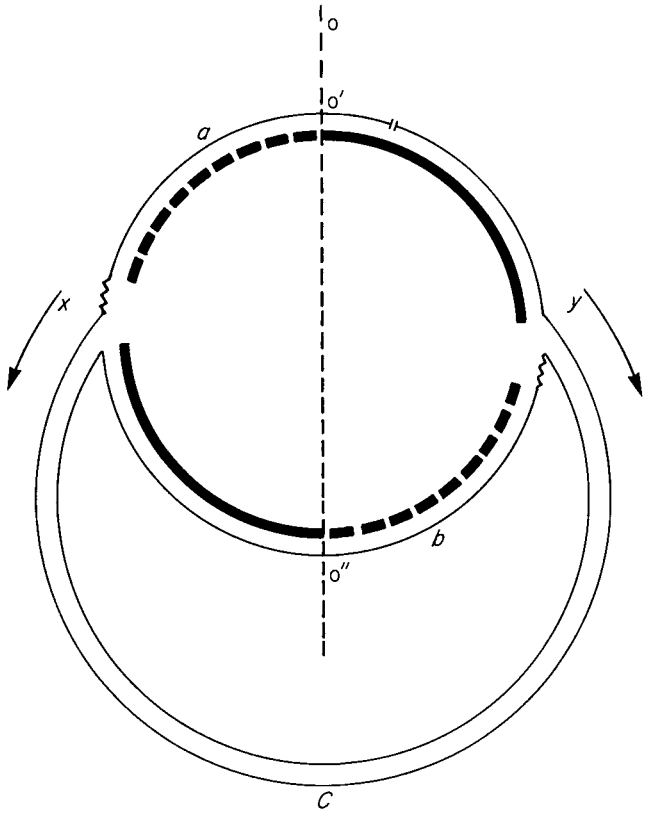

FIG. 8. Model for the structure of form II*. The template chains are drawn as thin lines, the jagged portions representing single-stranded material. The parts of the growing chains, having elongated in the overall 5′ → 3′ direction are shown as thick continuous lines, while the thick discontinuous lines indicate the parts having elongated in the 3′ → 5′ direction. From Bourgaux and Bourgaux-Ramoisy (1971).

restriction endonuclease from *Haemophilus influenzae* (Danna and Nathans, 1971). If one assumes that replication starts from a specific origin, a population of replicating molecules should be enriched in those sequences that are close to the starting point. Nathans and Danna (1972) concluded that replication of SV40 DNA was indeed initiated at a unique site after having observed (1) a temporal gradient in the labeling of the various fragments obtained from replicating molecules and (2) an opposite temporal gradient in the labeling of the fragments from completed molecules. This was also expected, since after pulses shorter than one replication cycle, radioactivity in mature molecules should be con-

fined to those sequences that are close to the point where replication stops. Using a somewhat similar reasoning, Thoren *et al.* (1972) approached the problem in a slightly different way. These authors separated the growing chains of replicating SV40 DNA according to size after velocity sedimentation through alkaline sucrose solution, and followed the renaturation of these chains within each size class. After making the corrections required by the differences in molecular weight of the chains, it was found that the growing chains originating from the "young" replicating molecules renatured more rapidly than those from the "old" replicating molecules, indicating that the former chains represented a less diverse collection of sequences. Such a difference in genetic complexity would not have been expected if replication had been initiated at random sites, since then even the shortest growing chains would have represented all the sequences present in the viral genome.

The experiments briefly discussed allow the conclusion that replication does not start at random sites throughout the SV40 genome. However, they could neither yield precise information on the location of the starting point, nor formally exclude the existence of a second alternative starting point, nor distinguish unequivocally between unidirectional and bidirectional replication. Fareed *et al.* (1972) exposed replicating SV40 DNA to yet another restriction endonuclease, R_1 from *Escherichia coli,* which introduces one double-strand break in SV40 DNA at a specific site. The nuclease-treated molecules were then characterized by sedimentation and electron microscopy. When the two branch points were positioned with respect to the free ends produced by the enzyme, it was found that the origin of replication is 33% of the genome length from the R_1 cleavage site and that both branch points are growing points. In addition to locating the origin of replication with satisfying accuracy, these data showed that SV40 DNA replication was indeed bidirectional.

D. The Mechanism of Chain Elongation

1. SV40

When replicating SV40 DNA was labeled after pulses of 45 seconds or less and centrifuged in alkaline sucrose solution, a clearly bimodal distribution of the radioactivity was observed in the gradient (Fareed and Salzman, 1972). Besides the already described broad peak representing daughter chains at various stages of elongation, a second, more homogeneous, 4 S sedimenting peak was noticeable. This second peak became less conspicuous as the length of the radioactive pulse was increased or when the pulse was followed by a chase with cold thymi-

dine. As we shall see in the next section, "young" and "old" replicating molecules can be separated after dye buoyant density gradient centrifugation in EthBr–CsCl or PDI–CsCl solutions. When replicating molecules labeled after a 45-second pulse were fractionated in this way and then analyzed in alkaline sucrose gradients, both early and late replicating molecules were found to include 4 S sedimenting material. Fareed and Salzman (1972) concluded from these observations that SV40 DNA chain elongation occurs by a discontinuous mechanism in which short chains, 150 deoxyribonucleotides in length, are first formed and then joined to the growing daughter chains. The fact that these short chains did extensively self-anneal further suggested that *both* daughter chains are synthesized discontinuously (Fareed *et al.*, 1973b). After a 1-hour 5-fluorodeoxyuridine block followed by a 1-minute [^{3}H]thymidine pulse, most of the radioactivity incorporated into replicating SV40 DNA sedimented at 4 S in alkaline gradients, as if joining of the 4 S chains to form the daughter chains was inhibited by the analogue (Salzman and Thoren, 1973). From this observation it was suggested that replication of SV40 DNA might require two distinct DNA polymerases: a replicase and a second polymerase, which would bridge the gaps between the 4 S chains and the growing daughter chains.

It should be pointed out that these conclusions were essentially based upon the analysis of DNA in alkaline solution. Since RNA is alkali-labile, it is of course unclear whether the gaps which are referred to are true gaps or stretches of RNA within the DNA chains.

2. *Polyoma Virus*

Reichard and co-workers have developed a system that allows the study of the replication of polyoma DNA in nuclei isolated from virus-infected cells (Winnacker *et al.*, 1972; Magnusson *et al.*, 1972). They have shown recently (Magnusson *et al.*, 1973) that the elongation of daughter chains (initiation of new rounds of replication does not take place in this system) involves the transient formation of short chains (4 S–5 S). Ribonucleoside triphosphates stimulate the formation of these short chains, which include an RNA moiety attached to the 5′ end of the DNA sequence. The latter conclusion was drawn from two observed features of the 4 S–5 S chains: (1) They show a greater buoyant density than chains from mature DNA in cesium sulfate solutions. (2) When α-^{32}P-labeled deoxyribonucleoside triphosphates are added to this *in vitro* system, the label is recovered in 2′(3′)-ribonucleotides after alkaline hydrolysis of the replicating viral DNA. Here again starvation from deoxyribonucleotides, induced by treatment of the cells with hydroxyurea instead of 5-fluorodeoxyuridine, caused an accumulation of short

chains. These short chains, however, were deficient in RNA. This again suggests that deoxyribonucleotide starvation interferes with chain elongation by inhibiting a process concerned with filling gaps between DNA sequences, or rather with substituting deoxyribonucleotides to ribonucleotides.

Preliminary evidence for the presence of DNA-linked RNA in *in vivo* replicating polyoma virus DNA has also been reported (Sadoff and Cheevers, 1973).

E. The Physical State of Template Chains in Replicating Molecules

It was first demonstrated in the case of SV40 DNA that replicating molecules often included *two* cyclic template chains (Sebring *et al.*, 1971; Jaenisch *et al.*, 1971). Although initially undetected (Hirt, 1969; Bourgaux *et al.*, 1969, 1971), this feature was later described for replicating polyoma virus DNA also (Bourgaux and Bourgaux-Ramoisy, 1972b). Since the findings were similar in both systems, there is little point in considering them separately.

Viral DNA was double-labeled with [^{14}C]- and [^{3}H]thymidine, under conditions in which most tritium counts incorporated were present in the growing chains of the replicating molecules (Fig. 9). When the viral DNA preparation was subjected to dye buoyant density gradient centrifugation essentially all of the tritium-labeled DNA was found to form a broad band, with a peak fraction at the position where nicked mature DNA was expected (see also Fig. 10) but markedly asymmetric towards the higher densities. When successive fractions containing the tritium-labeled DNA were pooled and analyzed by velocity sedimentation through alkaline sucrose solutions, it was found that the denser replicating molecules contained shorter growing chains (Fig. 9b), while the lighter molecules contained longer growing chains (Fig. 9d). This indicated that the early or "young" replicating molecules bound less dye than the late or "old" replicating molecules. One possible explanation for this was that the unreplicated and replicated portions of the replicating molecules bound different amounts of dye per unit length of DNA, as expected if the former portion behaved as a covalently closed duplex, while the latter behaved as a nicked duplex. In agreement with this explanation was the observation that treatments known to introduce nicks in DNA were found to convert replicating DNA into material with the density characteristic of nicked mature DNA in PDI–CsCl or EthBr–CsCl gradients (Jaenisch *et al.*, 1971; Bourgaux and Bourgaux-Ramoisy, 1972b).

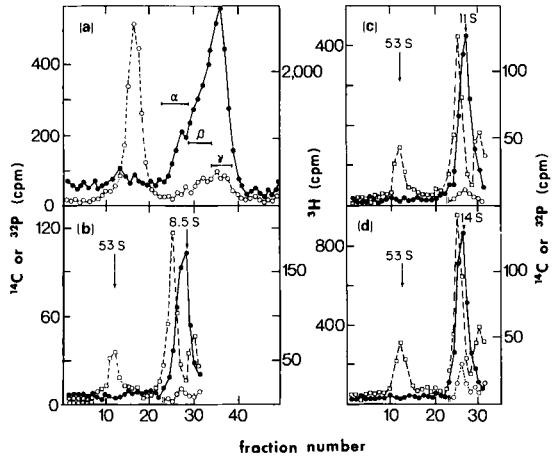

FIG. 9. Characterization of replicating polyoma virus DNA (form II*) labeled in growing chains. After incubation of the infected cells with [^{14}C]thymidine for 3 hours and then with [^{3}H]thymidine for 90 seconds, viral DNA was selectively extracted and centrifuged to equilibrium in PDI-CsCl solution (a). Note that most of the ^{14}C-labeled DNA forms a sharp dense band, as expected for mature DNA (form I). Successive fractions containing ^{3}H-labeled DNA were mixed, added to ^{32}P-labeled marker DNA (I + II), and subjected to velocity sedimentation in alkaline sucrose solutions. (b) pool α; (c) pool β; (d) pool γ. Note the sedimentation coefficients of the ^{3}H-labeled material from these various pools. ●–●, ^{3}H radioactivity; ○–○, ^{14}C radioactivity; □–□, ^{32}P radioactivity. From Bourgaux and Bourgaux-Ramoisy (1972b).

Clarification of this situation resulted from the characterization of replicating molecules labeled in their *template* chains. For instance, viral DNA was labeled after a 4 hour pulse with [^{3}H]thymidine, followed by a 30 minute chase with cold thymidine, and fractionated on BND cellulose columns. The replicating DNA from the caffeine fraction was mixed with ^{14}C-labeled mature DNA and centrifuged to equilibrium in EthBr–CsCl solution (Fig. 10). Early and late replicating molecules recovered from this gradient were then centrifuged through alkaline sucrose gradients. Most of the radioactive material found in the latter gradients sedimented with an S value in excess of 17 S, confirming that little label, if any, was present in the growing chains. In contrast, it formed a rather broad band sedimenting with a peak fraction at 45 S and 33 S in the case of the early and late replicating molecules, respectively (Fig. 10c and d). While the minor 17 S component suggested the presence of molecules with two template chains including at least one alkali-labile bond, the major 45 S, or 33 S, component could only represent submolecules comprising two template chains held together by a topo-

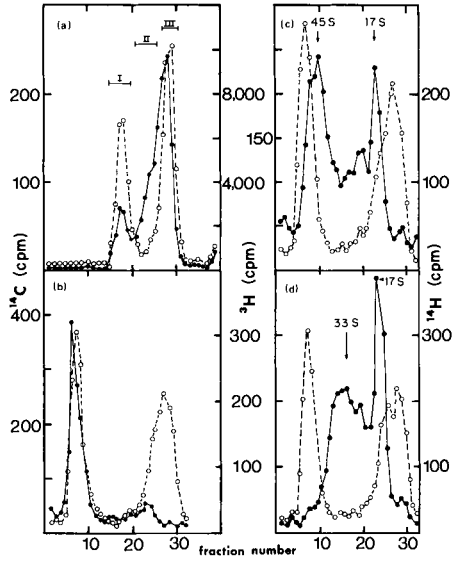

FIG. 10. Characterization of replicating polyoma virus DNA (form II*) labeled in template chains. Purified form II*, labeled as indicated in the text, was mixed with ^{14}C-labeled mature viral DNA used as a marker and centrifuged at equilibrium in EthBr–CsCl solution (a). Successive fractions were pooled, mixed with an additional amount of marker, and subjected to velocity sedimentation in alkaline sucrose solution. Under the conditions used here, cyclic (18 S) and linear (16 S) chains of mature DNA size appear as a single 17 S sedimenting band in alkaline solution. (b) pool I; (c) pool II; (d) pool III. ●–●, ^{3}H radioactivity; ○–○, ^{14}C radioactivity. From Bourgaux and Bourgaux-Ramoisy (1972b).

logical (and no hydrogen) bond. Indeed, introducing at least one nick into every molecule caused the conversion of this major component into 17 S sedimenting material (now shown). Why the sedimentation coefficient of the topologically bonded template chains is inferior to that of mature DNA form I and decreases with the extent of replication can be explained if we go back to Section I,A. The S value registered in alkaline solution for fully titrated, covalently closed duplexes of fixed molecular weight varies with α, i.e., the *topological winding number*. As replication proceeds, the two template chains must come apart, and α decreases. But α cannot decrease unless the template duplex is nicked, unwound, and sealed again. Champoux and Dulbecco (1972) have recently isolated from uninfected cells an activity that could perform this whole operation.

A more direct demonstration of the circularity of both template chains in replicating DNA was provided by the electron microscope (Fig. 11).

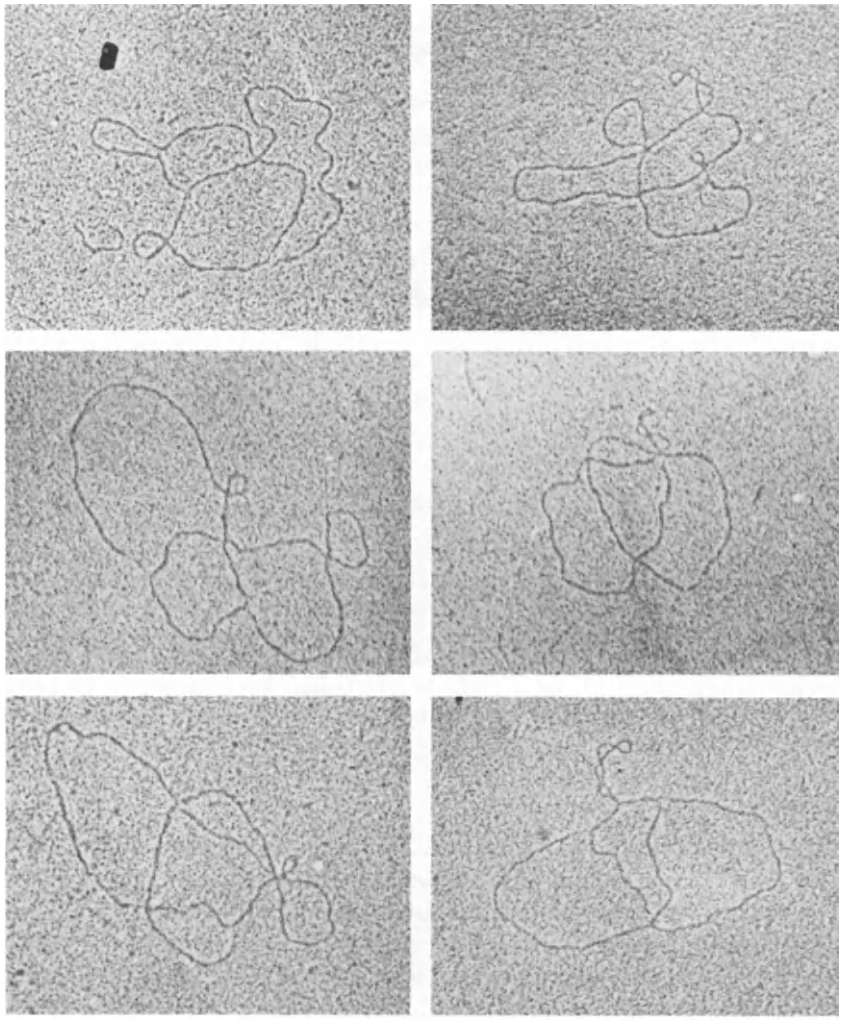

FIG. 11. Molecules with twists or cross overs in form II*. ×55,000. From Bourgaux and Bourgaux-Ramoisy (1972b).

In addition to the more conventional ones already described, molecules with two equal relaxed branches and a third twisted branch were observed. The length of the former branches was shown to be directly correlated with the extent of replication (Sebring *et al.*, 1971).

Replicating molecules with two cyclic template chains offered a

unique opportunity to try and produce covalently closed cyclic duplexes having an unusually low α, since the growing chains could easily be separated from the template chains under denaturation conditions that would allow the latter chains to remain partly hydrogen bonded. In spite of a partially preserved base pairing, cyclic, complementary template chains were found to be incapable of regaining a conventional double-stranded structure under various renaturation conditions (Bourgaux and Bourgaux-Ramoisy, 1972b), suggesting that the deficit in α was probably in excess of what could be compensated for by a mere increase in τ [Eq. (1)].

The observed continuity of template chains provided further confirmation that the replication of the genomes of SV40 and polyoma virus could not proceed as predicted by the rolling circle model, since this continuity precluded the existence of a covalent linkage between template chains and growing chains. In addition, this structural feature allowed the fractionation of replicating molecules with respect to their extent of replication. Finally, it should be pointed out that the assumed periodic unwinding of the template duplex should be kept in mind when assessing the significance of the discontinuous synthesis of the growing chains.

F. The Replication Cycle

In the first paper that appeared on the labeling and characterization of a replicative intermediate of papovavirus DNA, Bourgaux *et al.* (1969) proposed a scheme (Fig. 12) accounting for the formation of the three major molecular forms of viral DNA present in productively infected cells. It was supported by the kinetics of labeling of these three components (Table I). Although still largely valid, this scheme should be commented upon in the light of the information available at present. The process obviously starts with component I, which originates from the infecting particle. Whether or not this molecule is converted into component II prior to the onset of replication is still unclear, although the fact that replicating molecules generally contain intact template chains suggests that this might be unnecessary. Yet both polyoma virus (Cuzin *et al.*, 1971) and SV40 (Kaplan *et al.*, 1972) appear to contain a specific endonucleolytic activity, which, at least in the case of the former, seems to be absent from some temperature-sensitive mutants (cf. Chapter 6 by Cuzin) that are defective for a function concerned with the initiation of both viral DNA synthesis and transformation. A specific endonucleolytic cleavage of one or both chains of the viral DNA might thus still be required to allow, for instance, the initiation of the rounds

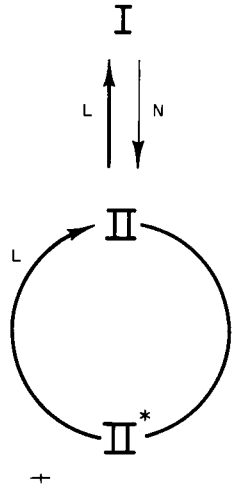

FIG. 12. Replication cycle of polyoma virus DNA. Besides polymerase(s), a ligase (L), and a nuclease (N) may intervene. From Bourgaux *et al.* (1969).

of replication to occur at a specific site on the molecule. Therefore, replication of the viral genome gives rise to the structures we have just described. As suggested by the results in Table I and by similar subsequent results (Bourgaux *et al.*, 1971), the initial product of polyoma virus DNA replication might be a molecule with the sedimentation properties of component II, comprising presumably one cyclic template chain and one still incomplete daughter chain. Fareed *et al.* (1973a) have recently reported that this is indeed the case in SV40. This component II would then be sealed by a ligase, thereby giving rise to new component I. The latter will either be involved in new rounds of replication or become encapsidated and appear in progeny virus. As again suggested by the kinetics of labeling of the various components (P. Bourgaux, unpublished), the former route is followed more frequently early in the productive cycle, while the latter becomes preponderant late in infection.

These are roughly the mechanisms through which the pools of replicating and mature viral molecules (especially the latter) increase in size during a productive infection. The picture would not be complete, however, if two additional, poorly understood pathways were not mentioned.

1. Integration–Excision

This is very much beyond the scope of this chapter. However, there is good multifarious evidence showing that viral DNA is both integrated

TABLE I

Kinetics of Labeling of Forms I, II, and II*[a]

	Length of labeling					
	5 minutes		20 minutes		60 minutes	
	Total cpm	Proportion	Total cpm	Proportion	Total cpm	Proportion
Radioactivity in I + II + II*	26,820		74,600		413,269	
Radioactivity in II	1,340	0.05	8,952	0.12	49,592	0.12
Radioactivity in II*	23,335	0.87	35,062	0.47	74,388	0.18
Radioactivity in I	2,145	0.08	30,586	0.41	289,289	0.70
Ratio II/I	0.62		0.29		0.17	
Ratio II/II*	0.05		0.25		0.66	

[a] From Bourgaut *et al.* (1969), with permission of *Proc. Nat. Acad. Sci. U.S.*

into and excised from host DNA in productively infected cells. Integration and excision are, of course, used here in a broad sense, since at present it is virtually impossible to distinguish, under these circumstances, between the transfer of viral sequences and that of functional viral genomes. However, this process clearly raises a number of questions regarding both the origin and fate of the replicating molecules described. For instance, how much of the viral DNA progeny fails to become encapsidated, being lost from the viral DNA pool through integration into cell DNA? Does integration followed by excision represent a prerequisite for viral DNA replication? If this were the case, is replication of the viral genome initiated before or after its excision? With respect to the last two questions, it is worth recalling that the temperature-sensitive mutants mentioned above seem to lose the ability of initiating either new rounds of viral DNA replication or transformation—which is presumed to require integration of the viral genome—when the infected cells are shifted to the temperature at which the viral defect is expressed (Fried, 1965; Tegtmeyer, 1972; Francke and Eckhart, 1973).

2. *Oligomers*

Circular and catenated oligomers of polyoma and SV40 DNA are produced either when the synthesis of virus is induced in transformed permissive cells (Cuzin *et al.*, 1970) or during acute productive infection (Meinke and Goldstein, 1971; Jaenisch and Levine, 1971). Conceivably, such molecules could arise either through recombination between monomers and/or oligomers of lower order, or by an aberrant replication mechanism, or through excision and replication of viral molecules that were integrated in tandem into the host genome.

It has been shown that inhibition of protein synthesis causes a rapid reduction in the rate of synthesis of viral DNA in cells productively infected by both polyoma virus (Branton *et al.*, 1970) and SV40 (Kit and Nakajima, 1971). Simultaneously, it reduces the superhelix density of the viral molecules still being synthesized (Bourgaux and Bourgaux-Ramoisy, 1972a; White and Eason, 1973; Jaenisch and Levine, 1973) and increases the occurrence of the oligomers of viral DNA (Jaenisch and Levine, 1972; Bourgaux, 1973).

Under conditions where only some of the molecules synthesized exhibit abnormalities in tertiary structures, these abnormalities are clearly more frequent among the oligomers (Bourgaux, 1973). Having cyclic template chains, replicating viral molecules are subjected to the kind of topological constraints that are imposed on intact mature molecules. It is thus likely that alteration in these constraints, such as those

that would result from changes in the amount or nature of the proteins bound to the DNA, would result in the formation of a mature product with an altered tertiary structure, which sometimes would fail to segregate into two daughter molecules (Bourgaux, 1973). Most of the oligomers of viral DNA produced during acute productive infection are thus likely to be formed through replication errors.

III. Adenoviruses

A. Facts and Predictions

As compared to papovaviruses, adenoviruses provide an interesting alternative model for the study of DNA replication. Although only certain serotypes were found to be oncogenic *in vivo*, all types seem able to transform hamster and rat cells *in vitro* (cf. Green, 1970). Adenoviruses contain linear, double-stranded DNA molecules with molecular weights ranging from 20 to 24 $\times$ 10^6 daltons, depending on the serotype (Green *et al.*, 1967). At least part of the viral genome is known to be present in adenovirus-transformed cells (cf. Green, 1970). Since the circularity of the viral DNA was often regarded as a prerequisite to its integration into the genetic material of the host, adenovirus DNA was thus examined for the existence of a terminal redundancy, with initially negative results (Green *et al.*, 1967; van der Eb *et al.*, 1969; Doerfler and Kleinschmidt, 1970). Recently, however, the existence of an unusual terminal repetition was detected in the DNA's extracted from adenoviruses 1, 2, 3, 7, 18, and 31 (Garon *et al.*, 1972; Wolfson and Dressler, 1972). When DNA from these viruses is denatured and then subjected to renaturation conditions *at low DNA concentration,* cyclic structures of one genome length are formed which appear single stranded over most of their circumferences. In these structures, the ends of the chains are held together by a short duplex segment, indicating the existence of two complementary sequences, 200 to 500 bases in length, within each adenovirus DNA chain (Wolfson and Dressler, 1972). To many people, this finding came as a relief, not only because integration of adenovirus DNA could then be explained in classical terms, but also for another reason. Considering what is known of the enzymes that appear to be involved, it is difficult to envisage how the replication of a linear duplex DNA, which would be devoid of terminal repetition, could actually take place. Since DNA polymerases do require a primer to which to add deoxyribonucleotides, there is no way in which a growing chain elongating in the 3′ $\rightarrow$ 5′ direction could reach the 3′ end of a linear template (Watson, 1972). Terminal

repetition thus appears as an essential feature of any linear DNA behaving as a replicon, for it makes possible the formation without any nonnucleotide linker, of cyclic and/or concatemeric forms, which both allow the duplication of the molecular ends of the genome (Watson, 1972). Replicative intermediates of adenovirus DNA were thus expected to include cyclic and/or concatemeric forms.

B. Unit Length Linear Replicating Molecules

Although numerous groups are engaged in an effort to shed light on adenovirus DNA replication, a coherent picture has not emerged yet. The experimental models selected most often include adenovirus type 5 (Ad 5) and adenovirus type 2 (Ad 2). The conditions selected for multiplying the viruses and labeling and extracting the DNA's differ so widely that it is difficult at present to account for many of the inconsistencies that have been recorded. The results reported by three different groups (Sussenbach and van der Vliet, 1972; van der Vliet and Sussenbach, 1972; Sussenbach *et al.*, 1972; van der Eb, 1973; Robin *et al.*, 1973; Bourgaux-Ramoisy *et al.*, 1974) however are in agreement on several important points. When cells productively infected with Ad 2 or Ad 5, or nuclei isolated from these cells, are incubated for short periods of time in the presence of a radioactive precursor of DNA, radioactivity is recovered both in mature viral DNA and in another molecular form of the viral DNA, which we shall refer to here as DNA-R. The kinetics of labeling of these two forms suggest that DNA-R is a precursor of mature viral DNA. This DNA-R sediments in neutral sucrose solution as a collection of linear duplexes with molecular weights ranging from 1 to 2 times that of mature viral DNA. When DNA-R is labeled after short radioactive pulses, fractionated according to molecular weight after velocity sedimentation through neutral sucrose gradients, and then analyzed in alkaline sucrose solution, it is found that the heavier molecules yield the longer radioactive chains after denaturation. When examined under the electron microscope, preparations of DNA-R are found to contain unit length branched molecules, showing only one branch point (Y-shaped molecules). No "eye forms" (Dressler *et al.*, 1972) have been described so far. The replication of Ad 5 and Ad 2 thus appears to proceed unidirectionally.

C. Single-Stranded Regions in Replicating Adenovirus DNA

As with the other replicating DNA's, DNA-R contains single-stranded regions and can thus be purified using BND cellulose. A point of conten-

tion concerns the magnitude of these single-stranded regions. According to Sussenbach and van der Vliet (1972) and to van der Eb (1973), the density of DNA-R in neutral CsCl is increased up to 0.008 gm/cm^3 over that of mature viral DNA, indicating the presence of extended single-stranded regions. Such regions can be detected in preparations of DNA-R examined under the electron microscope, both in the linear branched structures described above and in nonbranched structures of one genome length. Sussenbach *et al.* (1972) have presented a model for the replication of adenovirus DNA which takes into account the occurrence of these long single-stranded regions. These authors suggest that the replication of adenovirus DNA involves first the synthesis of only one daughter chain that displaces one of the two template chains from the parental duplex. The synthesis of the second daughter chain takes place subsequently, either before or after completion of that of the first one. The latter alternative would account for the occurrence of nonbranched partly or fully single-stranded structures.

Robin *et al.* (1973) and Bourgaux-Ramoisy *et al.* (1974) find little single-stranded material in DNA-R. Although the reason for this discrepancy is not clear, there is another serious difference that seems worth bringing in the discussion at this stage. The results from the other laboratories indicate the presence, in DNA preparations extracted from nuclei after short labeling periods, of a radioactive material sedimenting slowly in neutral sucrose solution (cf. van der Eb, 1973, Fig. 3; Sussenbach and van der Vliet, 1972, Fig. 2a); some of this material at least is viral in origin and single-stranded in nature (Sussenbach and van der Vliet, 1972). This material, which Bourgaux-Ramoisy *et al.* (1974) do not find in Ad 2 infected cells, might represent nascent DNA chains, which, at the time of extraction, were not yet covalently bound to growing chains and became released from their template, leaving it in a single-stranded form. This interpretation is in agreement with the following observations.

1. As already mentioned earlier, inhibition of the joining of the 4 S pieces in replicating viral DNA occurs after treatment of either SV40-infected cells with 5-fluorodeoxyuridine (Salzman and Thoren, 1973) or polyoma virus-infected cells with hydroxyurea (Magnusson, 1973a,b). Under these conditions, small molecular weight fragments of viral DNA accumulated in the cell. During extraction of the DNA by a procedure similar to that used by van der Eb, and by Sussenbach and co-workers, some of these fragments were released from the replicative intermediates (Magnusson, 1973a). The fragments became associated with preformed replicating strands upon removal of the inhibitor (Magnusson, 1973b).

2. After inhibition of Ad 5 DNA synthesis by hydroxyurea, replicative intermediates containing even more extended single-stranded regions were isolated by Sussenbach and van der Vliet (1973). Under these conditions, DNA sedimenting slowly in neutral solution represented the most prominent radioactive material detected in the cell extracts after short pulses of [^{3}H]thymidine (cf. Sussenbach and van der Vliet, 1973, Fig. 2).

Whatever its exact biological significance, the presence of extended single-stranded regions in replicating adenovirus DNA turned out to be a remarkably useful feature. Sussenbach *et al.* (1973) showed that these regions were of parental origin and hybridized exclusively with the light chain (Doerfler and Kleinschmidt, 1970) of the mature viral DNA. This suggests that adenovirus DNA replication starts from one unique molecular end of the genome and proceeds in the direction of the other end. In this process, the two daughter chains would be synthesized by two distinct mechanisms, presumably in accordance with their polarities. Whether or not these two mechanisms operate in a completely asynchronous fashion is still presently open to question.

D. Other Intracellular Forms of Adenovirus DNA?

The fact that only unit length replicating molecules of adenovirus DNA have been described so far might appear disturbing, although such forms have already been observed for T7 DNA (Dressler *et al.*, 1972). This is hardly surprising, however, for the methods of extraction of the DNA which have been used in the studies summarized would select against any rapidly sedimenting form of the viral DNA, such as circular or concatemeric forms. It is important to find out whether such forms of the viral DNA exist in the cell. In doing this, it will be worth keeping in mind the possibility, at first glance unlikely, that the molecular ends of the viral genome could be held together by a non-nucleotide linker allowing the formation of either monomeric circles or multiple length structures. Whatever the nature of the bond holding together the ends of the adenovirus genome, the search for these yet uncovered forms of the viral DNA will not be an easy task.

IV. Concluding Remarks

While the replication mechanisms appear remarkably similar for SV40 DNA and polyoma virus DNA, adenovirus DNA replication, although

incompletely understood, seems to follow a very different pattern. It remains to be seen whether it will be possible to reconciliate the structural features of some intracellular forms of these DNA's with their known common oncogenic properties.

There is one aspect of adenovirus DNA replication which seems better understood than that of papovirus DNA. Indeed, the two complementary strands of adenovirus DNA are clearly synthesized by different mechanisms. It would be ironic if that had been found because of the use of extraction conditions that allow a partial breakdown of the structure of replicating adenovirus DNA. The 4 S nascent chains of SV40 DNA do self-anneal (Fareed *et al.*, 1973b), suggesting that both daughter strands are synthesized in a discontinuous fashion. Yet, there seems to exist no way in which they could be synthesized simultaneously at the growing point. The existence of single-stranded regions with an apparently specific location at these points (Bourgaux and Bourgaux-Ramoisy, 1971) seems to indicate that elongation of the complementary chains is actually asynchronous.

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CHAPTER 6

Early Proteins of Oncogenic Papovaviruses

FRANÇOIS CUZIN

I. Introduction

The small oncogenic DNA viruses of the papova group (polyoma, SV40) are able to effect in mammalian cells a permanent modification in the control of cell division and DNA synthesis. The *transformed* cell will no longer respond either to certain stimuli originating at the cell surface ("topoinhibition") or to nutritional conditions (serum factors), which control the growth of normal cells. Although a number of agents, such as chemical carcinogens, are able to modify the cell in the same general way, oncogenic viruses do offer a unique possibility for an approach at the molecular level. Biochemical analysis of such highly complex systems might, in fact, be considered as a hopeless task if not for the availability in this particular system of two powerful tools: immunological and genetic analysis.

Genetic analysis seems especially worthwhile in the case of polyoma and SV40, both viruses carrying a very limited genetic information (coding capacity in the range of 150–200,000 daltons of proteins). Our understanding of the genetic structure of eukaryotic cells and their

viruses is still primitive; some originally unsuspected limitation may well reduce the power of a genetic approach by methods derived from phage studies: for example, due to the apparent lack, or poor efficiency, in *in vivo* recombination in the polyoma–SV40 system, new technologies have to be devised for genetic and structural mapping of the DNA (Danna and Nathans, 1971; Morrow and Berg, 1972). However, our present knowledge of polyoma and SV40 genetics is already sufficient to provide a framework and operational criteria for investigating the nature and functions of the viral gene products. The propositions that product(s) of one or more early viral genes are necessary for the establishment of the transformed phenotype, as well as for its maintenance in a transformed line, have been experimentally substantiated (Dulbecco and Eckhart, 1970).

Genetic studies in this system have been recently reviewed (Eckhart, 1972), and we will mainly discuss results of immunological and biochemical work on the nature of early gene products.

II. Early and Pre-early Functions

The term "early genes" will be used hereafter with the general meaning derived from phage analysis, namely, viral genes whose products are transcribed, translated, and act before the onset of viral DNA synthesis. Conditional lethal mutants for these genes will not synthesize viral DNA under nonpermissive conditions. Only these gene products will be synthesized if cells are treated with specific DNA synthesis inhibitors (FUdR, ara-C) immediately after infection.

Recent findings in several animal virus systems have pointed out that a class of viral gene products may be functional at a still earlier stage of infection ("pre-early"). These proteins are enclosed in viral particles, as for instance vaccinia virus RNA polymerase (Kates and McAuslan, 1967) or oncornavirus reverse transcriptase (Baltimore, 1970; Temin and Mizutani, 1970). Such enzymes, although they may in theory be synthesized at a late stage of the cycle, will only act during the next growth cycle immediately after removal of the external coat and before any translation of viral genes. The possibility of a situation of this type in the papova system is suggested by two lines of evidence (although pure viral DNA is infectious, its specific infectivity is at least 1000-fold lower than that of complete particles): (a) We have observed (Cuzin *et al.*, 1971, 1973) an endonuclease associated with pure polyoma virions, which specifically cleaves the viral DNA at a unique site. (b) It has been reported that for both the ts-3 mutant of polyoma and the ts-101 mutant

of SV40, infection with virus particles is inefficient at high temperature, while infection with pure DNA is not temperature-dependent (Robb and Martin, 1972; W. Eckhart, personal communication). This may be explained if a protein present in the particle is necessary for initiating a normal cycle.

III. Intranuclear and Plasma Membrane Events

All criteria of the transformed state fall into either one of two classes: (1) membrane structure modification, (2) DNA synthesis regulation. It is likely that in higher cells, as well as in bacteria, there is a close connection between these two. These considerations raise the problem of the primary target of the viral gene product(s) triggering the transformation process. It seems equally possible *a priori* that a viral protein acts at the membrane level, e.g., by a specific modification of a receptor for external stimuli, and that a different pattern of DNA synthesis is a secondary result of this change or, alternatively, that the primary target of viral enzyme(s) is a specific site on cellular DNA (initiation of DNA synthesis, integration of viral genomes, etc.). The latter type of model may be easier to test first, for there are well-established techniques for dealing with proteins endowed with specific recognition of nucleotide sequences, while analytical biochemistry of membranes still is at a very early stage of development.

The reader is referred to a number of recent reviews for more complete discussions and detailed specific information (Green, 1970; Butel *et al.*, 1972; Eckhart, 1972; Sambrook, 1972).

IV. Intranuclear Immunological Changes after Virus Infection

Among the new antigens that are detected during the early phase of the virus cycle, some are located on the periplasmic membrane, like the tumor-specific transplantation antigen (TSTA) or the surface (S) antigen, while others are only, or mainly, of intranuclear localization. All of them are continuously expressed in a permanently virus-transformed line.

1. T Antigen

T antigen was first demonstrated by Huebner *et al.* (1963) by complement fixation, using sera from SV40-induced tumor bearing hamsters.

Antigen is present both in productively infected cells (where its expression is not dependent upon viral DNA replication) and in transformed cells. Using antibodies labeled with either fluorescein (Pope and Rowe, 1964) or various enzymes (Wicker and Avrameas, 1969), one finds the label associated in most cases with the intranuclear compartment of the cell (nucleoli excepted); however, electron microscopic examination after treatment with peroxidase-coupled antibodies reveals some antigen bound to the perinuclear endoplasmic membranes (Meyer, 1971), which may correspond to the pool of nascent polypeptides.

The T immunospecificity is correlated with the virus; polyoma-infected or -transformed mouse or hamster cells react to the same serum, while SV40-infected mouse, hamster, monkey, human, or even lizard cells (Clark and Jensen, 1970) show a different and common specificity.

Although T antigen is the only polyoma early antigen known so far, other specificities have been recently identified in the case of SV40.

2. U Antigen

Human and monkey cells infected by a nondefective adenovirus 2–SV40 hybrid ($Ad2^+ND_1$) contain an SV40-specific intranuclear antigen, different from T antigen on the basis of serum specificity, heat stability of the antigen, and sensitivity of its synthesis to cytosine arabinoside. Induction of U antigen by $Ad2^+ND_1$ hybrid is due to the presence of a short segment of SV40 DNA, and anti-U antibodies are also present in most sera from hamsters carrying SV40-induced tumors. In the case of SV40, U antigen behaves as an early gene product (its synthesis is not inhibited by FUdR and ara-C), while in cells infected by $Ad2^+ND$, U antigen synthesis requires viral DNA synthesis. This discrepancy is explained by the fact that the SV40 part of the hybrid is located in the late region of the adenovirus genome (Lewis *et al.*, 1969; Lewis and Rowe, 1971).

V. Are Early Intranuclear Antigens Polypeptides of Viral Information?

The constant correlation between virus type and immunological specificity suggests, but does not prove, that the antigenic site is carried by a polypeptide of viral information. Infection with UV-irradiated virus leads to a decreased fraction of T-positive cells, demonstrating a specific requirement for a viral gene to be expressed. The relative target size is of the order of 0.5 genome, as compared with 0.2 for cell transformation or induction of membrane (S) antigen (see Sambrook, 1972).

More recently, Lewis *et al.* (1973), Henry *et al.* (1973), and Morrow and Berg (1972) studied nondefective adenovirus–SV40 hybrids, which carry various segments of the SV40 genome, their length being correlated with the number of SV40-specific antigens they can induce. Respective amounts of 0.18, 0.32 and 0.43 SV40 genome correspond to hybrids that induce either U antigen alone, or U and TSTA, or U, TSTA, and T.

Taken together with an estimated molecular weight of 70,000 for the T antigen polypeptide (Del Villano and Defendi, 1973; cf. Section VI) and with the above-mentioned radioinactivation data, it seems that most, if not all, of the early SV40 region is necessary for coding the T polypeptide. Other antigens (U, TSTA) could then correspond either to induced cellular proteins, to various modifications of a common peptidic backbone, to different peptides encoded in the same DNA sequence (multiple initiation sites and/or read-through), or to specific cleavage products of a large precursor with T specificity.

Early mutants of the ts-A complementation group of polyoma fail to induce T antigen synthesis under nonpermissive (high-temperature) conditions (Oxman *et al.*, 1972). Although this result strongly substantiates the hypothesis of a viral gene product being specifically required for T antigen appearance, it does not prove by itself that T antigen is the actual product of this viral gene. It is to some extent unexpected that all the minor defects in the protein structure due to several independent ts mutations would completely abolish its immunological reactivity, rather than produce cross-reacting material. One can then either assume that the immunological and the functional sites are very close to each other on the molecule, or alternatively that a functional product of this viral gene is necessary for inducing T antigen synthesis by another gene, either viral or cellular.

VI. Identification and Attempts to Purify Early Viral Proteins

Polyacrylamide gel electrophoresis in SDS of whole protein extracts of polyoma- and SV40-infected permissive cells reveals a modified electrophoretic pattern (Anderson and Gesteland, 1972; Walter *et al.*, 1972). New protein species have been tentatively identified in this way. An important background of cellular proteins, however, and discrimination only on the basis of peptide molecular weight, did not allow a definite distinction between new peptides of viral information and virus-induced cellular proteins. Results of this type of approach are summarized in Table I.

TABLE I

Nomenclatures and Molecular Weights ($\times 10^{-3}$) of Proteins Observed in Viral Particles and in Infected Cell Extracts

Viral particles			Infected cells	
Girard *et al.*, 1970; Estes *et al.*, 1971	Walter *et al.*, 1972	Hirt and Gesteland, 1971	Anderson and Gesteland, 1971	Walter *et al.*, 1972
43 (VP1)	44 (VP1)	42	40 (IVP1)	70 (NVP1)
32 (VP2)	31 (VP3)	35		60 (NVP2)
23 (VP3)		25		8 (NVP3)
14 (VP4)	14 (VP4)	16		
12.5 (VP5)	12 (VP5)	11,5		
11 (VP6)	11 (VP6)			

Several attempts have been made to purify SV40 T antigen using complement fixation as an assay and conventional protein fractionation procedures (see Meyer, 1971, for review). They have not yet been successful, as judged only by the wide discrepancies in estimated molecular weights of the antigen (from 60,000 to 600,000). Technical difficulties arise from the small amounts of material usually available in these systems, which prevent good recoveries in most fractionation procedures, and from the fact that T antigen is likely to be associated *in vivo* with high molecular weight material (RNA?).

Del Villano and Defendi (1973) used an immunochemical method for SV40 T antigen purification, eluting the radioactive protein fraction bound to insolubilized anti-T IgG immunoglobulins. This material migrated as a single band of 60,000 to 70,000 daltons in SDS polyacrylamide gel electrophoresis. Criticism of these techniques is usually based on the poor recovery of the biological (antigenic in this case) activity, due to drastic conditions of elution of the antibody bound molecules (very low pH or high ionic strength).

Experiments in progress in our laboratory (Paulin *et al.*, 1974) try to circumvent this objection: The principle is to use the same type of immunochemical techniques but only for removal of contaminant host proteins; partially purified viral proteins are left in solution. A broad spectrum antiserum against soluble proteins from noninfected mouse cells was prepared. Only the protein fraction extracted at low ionic strength from the nuclei was used. After repeated injections of this material into sheeps, a serum was obtained which recognizes a number of cellular antigens. A solid phase immunoadsorbent prepared from the serum specifically removed 70 to 80% of cellular components from this protein fraction (Fig. 1).

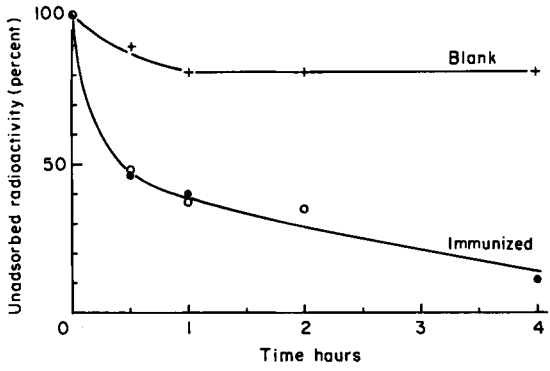

FIG. 1. Adsorption of nuclear proteins extracted from polyoma infected (○) or from normal mouse cells (●) on a solid phase immunoadsorbent directed against host nuclear proteins. A solid phase immunoadsorbent was prepared by cross-linking with glutaraldehyde (Avrameas and Ternynck, 1969) from sheep serum after immunization against mouse nuclear proteins (see text). A similarly prepared extract from normal or polyoma-infected (20 hours after infection) 3T6 mouse fibroblasts, labeled with [^{14}C]amino acids, was incubated in the presence of an excess immunoadsorbent with gentle stirring for the indicated time at room temperature. After pelleting the adsorbent particles at high speed, the radioactivity left in the supernatant was counted. As a control an identical immunoadsorbent was prepared, using serum from a nonimmunized animal. The level of nonspecific adsorption observed never exceeded 10–15%.

This procedure has been applied to extracts from polyoma-infected mouse cells (3T6 Swiss mouse fibroblasts, infected at a multiplicity of 50 PFU/cell and harvested 20 to 24 hours after infection) (Fig. 2). Gel electrophoretic analysis of the radioactive proteins left in the supernatant (unadsorbed material corresponding either to viral or to virus-induced cellular peptides) shows four main bands (A to D) with respective molecular weights in the range of 40,000, 46,000, 70,000 and 90,000 daltons (Fig. 3). An additional small size component (10,000–12,000) could also be seen, although it was often more difficult to resolve from other small peptides (histones).

Minor bands (a,b, and d, Fig. 3) of the same electrophoretic mobility as bands, A, B, and D could be detected in similarly treated extracts from uninfected cells; band C probably corresponds to the main capsid protein.

Experiments are in progress to distinguish viral gene products from induced cellular components (using extracts from cells infected with viral mutants) and also early from late viral polypeptides (extracts from cells infected in the presence of inhibitors of DNA synthesis).

This method allows a partial purification with good recoveries and has two main advantages over conventional fractionation methods: (1) It is

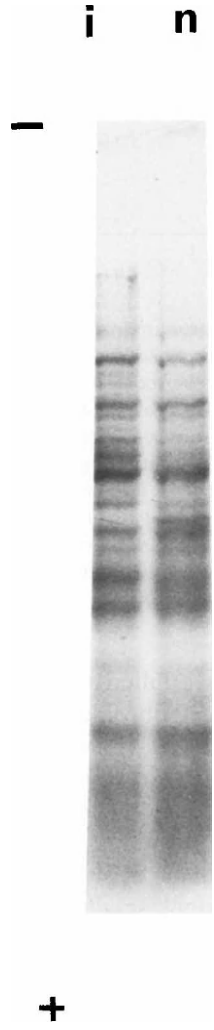

FIG. 2. Gel electrophoresis in SDS of extracts from polyoma infected (i) and normal (n) 3T6 cell nuclei. [^{14}C]Amino acid-labeled proteins extracted from purified nuclei were analyzed by SDS electrophoresis according to Laemmli (1970) on slab gels (15% polyacrylamide; 0.1% SDS). Autoradiography on Kodirex X-ray film for 2 weeks.

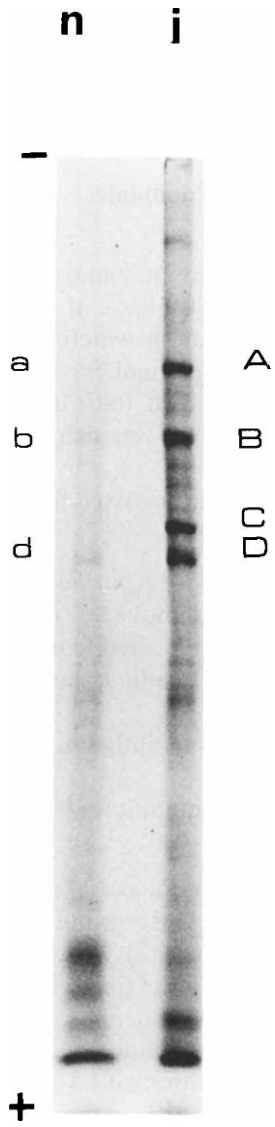

FIG. 3. Gel electrophoresis in SDS of the same extracts as in Fig. 2, but after immunoadsorption of cell proteins. Analysis of the radioactive material left in the supernatant after 2 hours of adsorption under the same conditions as in Fig. 1.

relatively indifferent to protein concentration and still efficient at a microscale level; (2) it leaves the viral proteins untouched, under mild conditions, and suitable for recovery of enzymological and immunological activities.

VII. Viral Enzymes: Possible Candidates

The search for virus-specified enzymatic activities in extracts from infected cells is complicated by the lack of a rational purification method (see above) from crude extracts in which many activities may interfere with the assay. In addition, a number of cellular enzymes are derepressed during the early phase of the viral cycle (see review, Green, 1970) and may be difficult to distinguish from "new" virus-coded enzymes.

Some operational criteria may be used for identification of a given enzyme with a viral gene product.

1. The enzyme must have been purified, at least to a step at which most interfering activities are removed.
2. The activity can be distinguished from analogous cellular enzymes by biochemical differences (kinetics, heat stability, cofactors, ionic requirements, etc.).
3. Antibodies recognizing viral antigens (T, U, etc.) have an inhibitory effect on the reaction.
4. Cells infected by a ts mutant exhibit a temperature-dependent activity.

It is obvious that in the case of polyoma and SV40, no enzyme can yet be definitely identified on these grounds as a viral gene product. A possible candidate in the recent past was thymidine kinase (TK), which has been shown to be different from the cellular activity both in polyoma-infected mouse cells (Sheinin, 1966) and in SV40-infected monkey cells (Kit *et al.*, 1966; Hatanaka and Dulbecco, 1967), by heat inactivation kinetics, and sensitivity to 5-FUR. No data, however, are available on properties of purified enzymes or on extracts of cells infected with ts mutants, which could substantiate the hypothesis of a virus coded thymidine kinase. On the contrary, the work of Basilico *et al.* (1969) showed that thymidine kinase-less (TK^-) mouse cell variants are susceptible to polyoma infection and that this infection does not lead to appearance of TK activity. Although the possibility is still open to some extent (especially in the case of SV40, where no TK^- variant of monkey cells is available) that at least part of the enzyme structure

might be derived from viral genetic information, thymidine kinase is no longer one of the leading candidates.

Another enzyme of possible viral genetic origin is the virion-associated endonuclease, which has been discovered in our laboratory in the case of polyoma (Cuzin *et al.*, 1971, 1973; Rouget *et al.*, 1974) and has also been studied in SV40 by Kaplan *et al.* (1972) and Kidwell *et al.* (1972). This activity was found in CsCl purified wild-type polyoma virions of either the large plaque, or the small plaque type (Fig. 4). In competition experiments it showed (Fig. 5) a higher (40- to 100-fold) affinity for polyoma DNA than for *Escherichia coli* DNA or poly(dA-dT). No activity could be detected in several preparations of two mutants of the same early viral complementation group (ts-A and ts-25). As previously discussed (Cuzin *et al.*, 1973), the absence of any detectable activity *in vitro*, even under permissive conditions (low temperature), is consistent with, but does not prove that, the enzyme is a viral gene product. The possibility is still open of a cellular enzyme induced only by wild-type polyoma infection.

The enzyme produces only one break on each strand of circular polyoma DNA, either at the same or at very close sites, generating linear double-stranded duplexes. Uniqueness of the cleavage site on the molecule has been demonstrated (1) by the fact that a dimeric polyoma DNA molecule (Cuzin *et al.*, 1970) is cleaved twice at symmetric sites, generating monomeric linears (Fig. 5), and (2) by analysis of the product

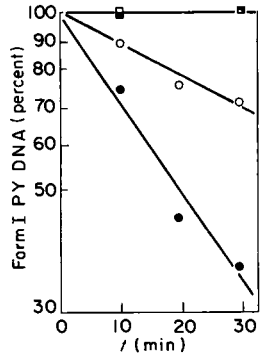

FIG. 4. Endonuclease activity of purified polyoma virus particle (Cuzin *et al.*, 1973). Pure polyoma virus particles were used as an enzyme source in a reaction mixture containing Tris-HCl buffer pH 7.4 (10 m*M*), $MgCl_2$ (5 m*M*), and ^{3}H-labeled form I polyoma DNA as substrate. Conversion of form I (superhelical) DNA into linear or relaxed circular molecules measured the endonuclease activity. □ and ■, control without addition of polyoma; ○, control with addition of polyoma; ●, reaction in the presence of *Escherichia coli* tRNA (200 μg/ml).

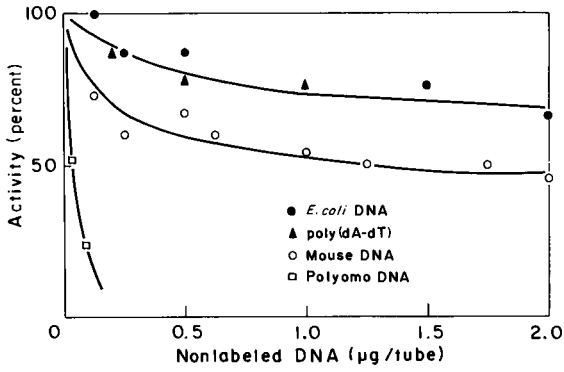

FIG. 5. Competition by heterologous DNA's. To a standard reaction mixture (see Fig. 4), activity taken as 100, are added increasing amounts of cold DNA of various sources. All DNA's have been extensively purified and reduced by ultrasonic treatment to the same average molecular size. ^{3}H-labeled polyoma DNA (substrate) concentration, 0.03 µg/tube.

after successive hydrolysis with the virion nuclease and RI restriction endonuclease, which cleaves polyoma DNA at a unique site (Fig. 6) (Mertz and Davis, 1972; Yaniv *et al.*, 1974).

Presence of the enzyme in the viral particle may indicate a "pre-early" function (e.g., integration of the viral DNA into host DNA could be required as a very first step in the cycle, for instance, for subsequent transcription of part of the viral message from a cellular promotor). On the other hand, the enzyme may be gratuitously present in virus particles, simply as a result of its high affinity for polyoma DNA, to which it could remain associated throughout the maturation process.

Although the enzyme activity on host cell (mouse) DNA has not yet been thoroughly studied, its affinity appears to be relatively high (Fig. 5), suggesting that the recognition site present on polyoma DNA might also exist on cellular DNA. Such a situation would be expected for an enzyme involved in the integration process, which, according to the model of Campbell (1962), requires cleavage on both strands of the viral and the cellular DNA's. This hypothesis would be strengthened if a relationship could be more clearly established between the endonuclease and the ts-A gene of polyoma, since a defect in the ability of the ts-A mutant to integrate has been suggested by several independent results (Stoker and Dulbecco, 1969; Cuzin *et al.*, 1970).

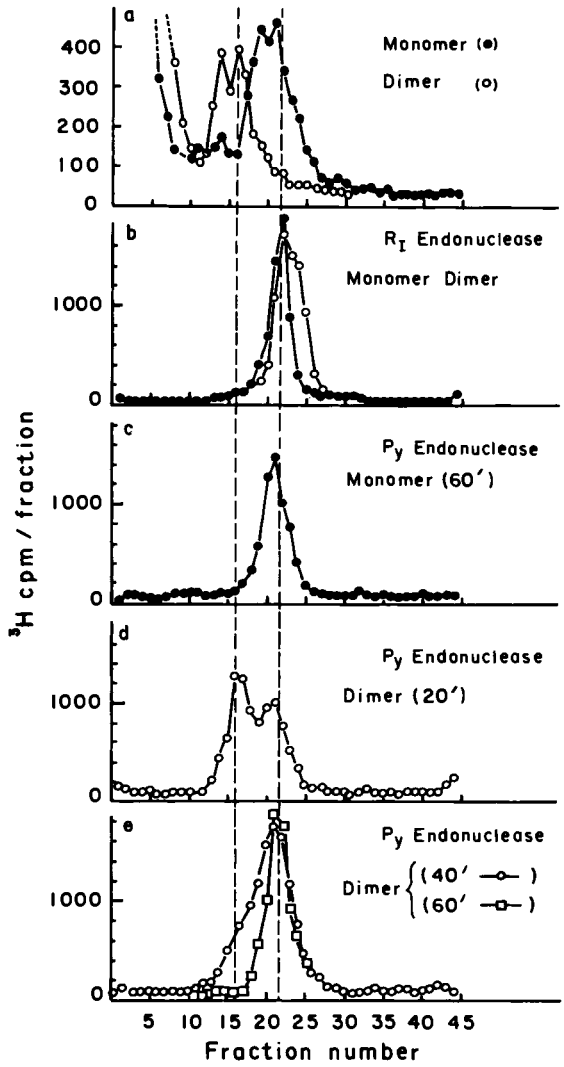

FIG. 6. Alkaline sucrose sedimentation of the products of endonucleolytic cleavage of monomeric and dimeric polyoma DNA, by RI restriction enzyme and polyoma endonuclease. (a) Sedimentation of monomeric and dimeric polyoma DNA without enzyme treatment. Vertical dotted lines indicate the calculated position in the tube of, respectively, monomeric and dimeric linear single strands. Most of the DNA (90%) is under the supercoiled configuration and sediments at the bottom of the tube. The remaining 10% sediment as two close bands for each DNA, corresponding, respectively, to circular and linear single strands. (b) R_I endonuclease product from monomer and dimer form I DNA's. (c) Polyoma endonuclease product from monomeric DNA. (d) and (e) Polyoma endonuclease action on dimer at three successive incubation times. Sedimentation in 5–20% sucrose gradient, 0.2 *N* NaOH, 0.8 *M* NaCl, at 5°C, for 240 minutes at 48,000 rpm in a Spinco SW 50-1 rotor.

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CHAPTER 7

Adenovirus Genes and Cancer

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I. Introduction

The stated title of this chapter is undoubtedly too pretentious. Its only justification is the assumption that cell transformation is the *in vitro* counterpart of malignancy. A more modest and accurate reflection of the subject matter to be discussed would be the title "Adenovirus Gene Functions: A Study of Regulation of Productive Infections and Cell Transformation." The experimental approaches that permit a study of control of viral replication as well as the cellular consequences of virus–cell interactions are possible because of two qualities of adenoviruses:

(1) They produce a unique variety of effects, ranging from productive infections and cell death to abortive viral replication and cell transformation, and in animals the viral encounter may result in acute disease or under some circumstances in malignancies. (2) The viral gene products in the virion, the viral proteins, have been rather well defined according to their biological functions and their structures.

The Virion and Its Replication

The viral genome, a double-stranded, linear DNA with a molecular weight of 20 to 25×10^6 daltons (depending upon the viral species) (Green *et al.*, 1967; van der Eb *et al.*, 1969), can theoretically code for 25 to 50 proteins. In fact, many fewer virus-specific proteins have been definitely identified in purified virions and virus-infected cell extracts. The icosahedral viral capsid is composed of 240 hexons and 12 pentons, each of which consists of a base and a fiber. It has also been proposed that an additional small protein is closely associated with the hexons (Everitt *et al.*, 1973). Structurally the hexons form the sides and faces of the 20 equilaterial triangles (Wilcox *et al.*, 1963; Valentine and Pereira, 1965), and immunologically this major protein contains the antigenic reactive groups responsible for type-specific neutralizing antibodies (Wilcox and Ginsberg, 1963b; Kjellin and Pereira, 1968; Pereira and Laver, 1970) and the cross-reactive family antibodies (Wilcox and Ginsberg, 1961, 1963a). Each hexon is composed of three identical polypeptide chains of 93,000–105,000 daltons each (Cornick *et al.*, 1973; Ginsberg *et al.*, 1974). The intact pentons form the vertices of the icosahedral capsid, and the fiber component of each penton serves as the virion's organ of attachment to susceptible cells (Levine and Ginsberg, 1967). The fiber is type-specific (Wilcox and Ginsberg, 1961); it consists of three polypeptide chains of similar sizes, i.e., 61,000 daltons (Sundquist *et al.*, 1973; Dorsett and Ginsberg, 1974), but one of the polypeptides is chemically different from the other two (Dorsett and Ginsberg, 1974). The penton base has not been chemically characterized, but physically it is composed of protomers of about 70,000 daltons each, and immunologically it shows extensive family cross-reactivity (Wilcox and Ginsberg, 1961). Associated with the viral DNA are at least two small internal proteins, one of which has an arginine content of 21 to 23 moles% (Laver, 1970; Prage and Pettersson, 1971).

Replication of the virion's constituents proceeds in a well-ordered

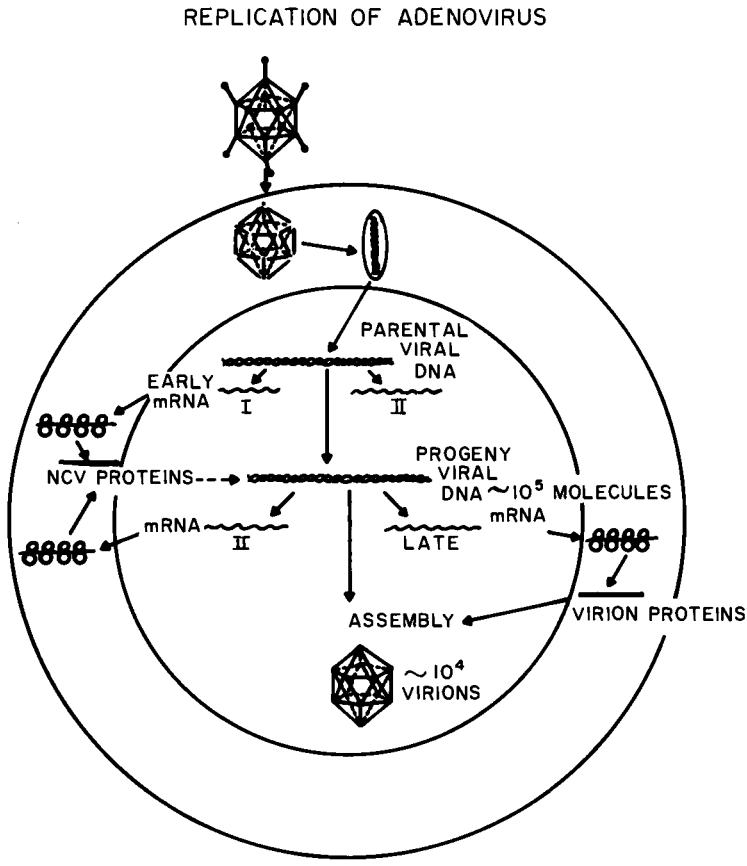

FIG. 1. A diagrammatic representation of the biochemical events initiated with "uncoating" of the viral genome and terminated by assembly of the virions from viral subunits.

series of biosynthetic reactions, which culminate in assembly of the subunits into a perfect icosahedral virion. It is the regulation of these biochemical events which was the stimulus for the investigations to be summarized in this communication. The replication events, diagrammatically described in Fig. 1, are initiated with the uncoating of the viral genome and the subsequent transcription of early species of virus-specific RNA's. At least some of these early transcripts, which are synthesized in the absence of protein synthesis (Parsons and Green, 1971) and prior to replication of the viral DNA (Thomas and Green, 1969;

Lucas and Ginsberg, 1971), serve as messenger RNA's (mRNA's) for virus-coded nonvirion proteins. However, the function and structure of these essential viral gene products have still not been defined. The asymmetric, semiconservative, linear replication of the viral DNA (Sussenbach *et al.*, 1972), which occurs within infected nuclei (van der Vliet and Sussenbach, 1972), is dependent upon the prior synthesis of early proteins (Wilcox and Ginsberg, 1963c). It is possible that at least one of the early class I mRNA's, which are only transcribed early (Lucas and Ginsberg, 1971), is translated into a protein essential for initiation of viral DNA replication, whereas one or more of the class II mRNA's, which are transcribed continuously during viral synthesis (Lucas and Ginsberg, 1971), are translated into nonvirion proteins necessary for late as well as early biosynthetic reactions. Late mRNA's can only be transcribed after replication of viral DNA has begun (Bello and Ginsberg, 1969), although continued replication of the viral genome is unnecessary for prolonged synthesis of late transcripts and late viral proteins (J. J. Lucas and H. S. Ginsberg, unpublished data). Viral mRNA's, which are transcribed and processed in the nucleus (Parsons *et al.*, 1971; Wall *et al.*, 1972), must be transported into the cytoplasm (Parsons *et al.*, 1971; Parsons and Green, 1971; Lucas and Ginsberg, 1971) where they are translated (Thomas and Green, 1966; Velicer and Ginsberg, 1968; 1970). The nascent polypeptide chains are subsequently transported into the nucleus where they form multimeric proteins that are assembled into infectious viral particles (Velicer and Ginsberg, 1970). Although the sequential biosynthetic events leading to morphogenesis of the virion are orderly and the final yield of infectious virions may be large, the assembly process is inefficient. For example, infection of KB cells with type 5 adenovirus yields as many as 10^4 infectious particles per cell, but only about 10% of the viral DNA and capsid proteins synthesized are actually packaged into viral particles (Ginsberg and Dixon, 1959).

To reveal which proteins essential for viral synthesis are viral gene products and to understand the mechanisms regulating the biosynthetic events described above, techniques other than conventional biochemical measurements appeared to be required. Conditionally lethal mutants proved to be invaluable for understanding the details of biosynthesis of bacteriophages (Epstein *et al.*, 1963), and it seemed likely that similar studies using temperature-sensitive (ts) mutants of adenoviruses would be equally revealing. The experiments to be summarized in this chapter employed selected ts mutants of types 5 and 12 adenoviruses, the former virus because it has been extensively utilized for biochemical and structural investigations, the latter because it readily transforms primary rodent cells *in vitro* and is highly oncogenic *in vivo*.

II. Conditionally Lethal Temperature-Sensitive (ts) Mutants of Type 5 Adenovirus

A. Mutagenesis and Selection of Mutants

The acquisition of wild-type (Wt) virus and its multiplication characteristics at 32°C (permissive temperature) and 39.5°C (restrictive temperature), the techniques of mutagenesis, and the selection of ts mutants have been described in detail elsewhere (Ensinger and Ginsberg, 1972). Briefly, mutagenesis was carried out with 0.1 *M* $NaNO_2$ at pH 4.0 for 45 minutes, or 1 *M* hydroxylamine at pH 7.0 for 26 hours, or by propagating the virus in the presence of 25 μg/ml of nitrosoguanidine for 60 hours at 32°C. A two-step procedure was employed for selection of mutants: (1) the plaque-enlargement technique (Edgar and Lielausis, 1964) and (2) determination of the potential of the mutants to produce complete cytopathic effects in monolayers of KB cells at 39.5°C. Fifteen mutants with reversion frequencies of less than 10^{-5} were selected for characterization.

Nomenclature of ts Mutants

In accordance with an agreement reached by investigators working with adenovirus mutants, a defined system of nomenclature will be employed (Ginsberg *et al.*, 1973). The terminology is similar to that also accepted for use with SV40 mutants (Robb *et al.*, 1972). Thus, type 5 adenovirus temperature-sensitive mutants will be termed H5ts (number of mutant), and type 12 ts mutants will be indicated as H12ts–.* The complementation group designation, which should be a term of the official name of a mutant (A, B, C, etc.) will not be presented for the mutants to be discussed in this chapter because most of the mutants have not yet been crossed with those isolated in other laboratories (Williams *et al.*, 1971; Lundholm and Doerfler, 1971; Shiroki *et al.*, 1972; Suzuki *et al.*, 1972) and therefore a final complementation group designation cannot be made. For convenience the complementation groups detected will be temporarily noted as I, II, III, etc.

B. Complementation Analysis

Characterization of the ts mutants was initiated by classifying the mutants into complementation groups according to their affected cistrons. Cultures were doubly infected with 15 PFU per cell of each mutant and

* – = mutant number.

TABLE I

Complementation between Representative ts Mutants of Type 5 Adenovirus

ts 125 (I)	[0.061]					
ts 116 (II)	1.6[a]	[0.016][b]				
ts 135 (III)	73	30	[0.037]			
ts 142 (IV)	146	36	55	[0.017]		
ts 147 (V)	60	15	0.45	338	[0.045]	
ts 149 (VI)	12	1.0	11	223	195	[0.010]
	ts 125	ts 116	ts 135	ts 142	ts 147	ts 149

[a] Suspension cultures of KB cells were infected at 39.5°C with 15 PFU of each of two mutants per cell and the final yield was assayed at 32°C. The final yields are expressed at fluorescent focus units (FFU) per cell.

[b] Yield from cultures infected with 15 PFU of only one mutant per cell. The yield from Wt virus at 39.5°C was 2 to 4.4 × 10^3 FFU/cell.

incubated at 39.5°C for 40 hours (Ensinger and Ginsberg, 1972). Table I illustrates the yields obtained with double and single infections using representative mutants and the consequent classification of the 15 ts mutants analyzed into 6 complementation groups. It is important to note that the mutants selected did not separate randomly into the groups identified: 11 of the 15 mutants segregated into complementation groups II and III (Table II).

C. Recombination

Adenoviruses were considered to be ideal for study of genetic recombination between viruses because (1) they have a linear, double-stranded, unsegmented DNA genome and therefore avoid the problems of reassortment encountered with viruses having segmented genomes (i.e., reoviruses and influenza viruses) and (2) they assemble in the nuclei of cells without budding and thus avoid the difficulties of heterozygote formation (e.g., herpes simplex and Newcastle disease viruses). It was found, however, that adenoviruses possess their own imperfections for ideal recombination studies. In addition to clear Wt plaques, small, faint "miniplaques" resulted from crosses between members of different complementation groups or between viruses of complementation group III. The miniplaques appear to result from complementation on the plate (Ensinger, 1973). This phenomenon was avoided when recombination was studied with viruses of group II; these

TABLE II

Properties of Temperature-Sensitive Mutants of Type 5 Adenovirus

Complementation group	Number of mutants	Phenotype
I	1	DNA minus
II	6[a]	Hexon minus
III	5	Assembly minus
IV	1	Fiber minus
V	1	Hexon transport minus
VI	1	DNA minus

[a] Two may be double mutants.

intracistronic recombination crosses demonstrated the linearity of the genetic map. Despite the difficulties encountered with the appearance of miniplaques, counting only normal-sized plaques, recombination crosses were done between all of the first eight ts mutants (members of the first three complementation groups) which have been extensively characterized. The data obtained suggest that the entire map is linear and that the three complementation groups studied are arranged in the following sequence: group II–group I–group III. The recombination frequencies ranged from about 0.19% to 15.9% and were consistently higher for crosses between ts mutants of different complementation groups (Ensinger, 1973). The four ts mutants of group II can be more precisely arranged. ts 115–ts 116–ts 132–ts 114.

D. Characterization of ts Mutants

Biochemical and immunological techniques were utilized to describe the phenotype and thus reveal the functional defect of each mutant. Thirteen of the 15 mutants had only a single detectable defective gene function that was common to all members of the specific complementation group (summarized according to the complementation groups in Table II). Two of the viruses appeared to be double ts mutants.

Only 2 mutants were isolated that were unable to synthesize their viral DNA at the nonpermissive temperature. They complemented each other effectively, indicating that there are at least two distinct gene products necessary for replication of viral DNA. One (ts 149) did not complement the DNA minus mutant (ts 36) isolated by Williams and his colleagues (Williams *et al.*, 1971; Wilkie *et al.*, 1973). It is striking that if the group I mutant (ts 125)-infected cells were incubated at 32°C, to

permit viral DNA replication to begin, the DNA synthesis rapidly ceased when the infected cells were shifted to 39.5°C. When infected cells were shifted down from 39.5°C to the permissive temperature, synthesis of viral DNA was resumed within 1 hour. It should be further noted that viral capsid proteins were not produced in cells infected at 39.5°C with the DNA minus mutants, adding additional confirmation to the observations that transcription of late mRNA's and synthesis of late proteins are dependent upon prior replication of viral DNA (Flanagan and Ginsberg, 1962; Bello and Ginsberg, 1969).

Group II mutants, which were commonly found, synthesized normal amounts of viral DNA, produced viral pentons and the internal arginine-rich protein, but did not make immunologically reactive hexon protein at the nonpermissive temperature. Polyacrylamide gel electrophoresis of the infected cell extracts, however, showed that the appropriately sized polypeptide chains were made, although they were detected in reduced amounts. Indeed, all of the viral proteins were present in smaller quantities than in wild-type infections, owing to degradation, inefficient extraction, or decreased rate of synthesis. Group III mutants, which were also commonly isolated, appeared to make all known components of the virion; the capsid proteins and the arginine-rich internal protein were immunologically reactive, but virions did not form—they appear to be assembly mutants. The single group IV mutant was defective only in the fiber protein. The group V mutant has an intriguing defect: all capsid proteins, including the hexon protein, were synthesized and were immunologically active; the fiber and penton base proteins moved normally into the nucleus, but the hexon protein could not be transported into the nucleus at the nonpermissive temperature (R. S. Kauffman, unpublished data).

III. Temperature-Sensitive Mutants of Type 12 Adenovirus

A. Mutagenesis and Selection

The mutants to be described were obtained after propagation of type 12 Wt adenovirus in KB cell monolayers in the presence of 15 μg 5-bromo-2-deoxyuridine (BUdR) for 62 hours at 32°C in the dark. The mutagenized virus was plated on KB cells at 32°C; plaques were picked and tested at low multiplicity (0.1–0.01 PFU/cell) for cytopathic effects at 32° and 39.5°C. Initially three mutants were obtained from 165 plaques tested (a mutation rate of 1.89%). The three ts mutants were plaque-purified three times. The mutants had 32°/39.5° plating efficiency

ratios greater than 10^7 to 10^8 and similarly low reversion frequencies (Rubenstein *et al.*, 1973).

B. Complementation Analysis

The three ts mutants studied proved to complement each other excellently so that the yield of each double infection was several hundred times greater than the sum of the single infections. Thus, each ts mutant belonged to a separate, nonoverlapping complementation group. The three unique mutants were then characterized according to their biochemical defects in the lytic cycle, their ability to transform rat embryo cells at 32° and 39.5°C, and their replication in the rodent cells.

C. Characterization of H12ts Mutants in KB Cells

Using procedures similar to those described for the study of type 5 ts mutants (Ensinger and Ginsberg, 1972), the three type 12 ts mutants were found to have the following defects under nonpermissive conditions of viral replication.

1. The mutant ts 307 did not replicate its viral DNA and, as would be predicted, did not synthesize late proteins at 39.5°C. T antigen, however, was produced in the infected cells. It was also striking that synthesis of host DNA was only reduced about 25%, in sharp contrast to the DNA minus type 5 mutants studied which decreased replication of host DNA 75%. Biosynthesis of host proteins was not altered in the infected cells.
2. The mutant ts 333 showed reduced replication of its DNA (about 45% as compared to Wt virus), shut-off synthesis of host cell DNA, made T antigen normally, but had markedly reduced production of late viral proteins and did not effectively shut-off synthesis of host proteins.
3. The mutant ts 303 made defective hexon protein that was immunologically unreactive. Indeed, its phenotype was similar to the complementation group II type 5 ts mutants (Table II).

D. Transformation Analysis

The Wt type 12 adenovirus was found to transform primary rat embryo fibroblasts at both 39.5° and 32°C, which afforded an opportunity to explore the mechanism of viral transformation using the ts mutants described. It was extremely good fortune that the three distinct mutants

had significantly different transformation frequencies at the nonpermissive temperature. Thus, ts 303 transformed at the frequency of wild-type virus (about 2 to 4×10^{-4} transformations per infected cell); ts 333 essentially did not transform cells at 39.5°C; and ts 307 transformed at a frequency of 2.5 to 4 times greater than Wt virus. It should also be noted that ts 333 at 32°C characteristically produced clear plaques that were at least twice the size of Wt plaques or the plaques induced by the other ts mutants. This large plaque characteristic of ts 333 is similar to Takemori's type 12 cyt mutants, which also have reduced transforming capability (Takemori *et al.*, 1968, 1969).

E. Replication of H12 Wt and ts Mutants in Rat Embryo Cells

Type 12 adenovirus cannot multiply in hamster cells, which it effectively transforms (Strohl *et al.*, 1966). Investigation of the capacity of the Wt virus and the three ts mutants to multiply in rat embryo cells showed that none of these viruses produced infectious virus at either 32°C or 39.5°C. Furthermore, when the DNA of infected cells was examined by isopycnic centrifugation in cesium chloride it was shown that none of the four viral DNA's were synthesized at either temperature.

IV. Intertypic Complementation with Type 5 and Type 12 ts Mutants

Although each type of adenovirus has specific immunologically reactive proteins, the structures of the proteins of the different types appear to be similar in size and configuration. It seemed possible therefore that double infection of KB cells at 39.5°C with ts mutants of type 5 and type 12 might result in complementation if their defects were in different cistrons. This hypothesis proved to be valid, as exemplified by the crosses between each of the type 12 ts mutants and the two type 5 DNA minus mutants (Table III). Clearly H5ts125 and H12ts307 have defects in the same cistron so that synthesis of viral DNA was not permitted in the doubly infected cells at 39.5°C. In sharp contrast, these data demonstrate that the defective gene product of H5ts149 is coded in a different cistron from that affected in H5ts125 and H12ts307. It should also be pointed out, that this type of analysis detects similarities and differences in protein defects not observed with intratypic complementation analy-

TABLE III

Complementation between DNA Minus ts Mutants of Type 5 and ts Mutants of Type 12 Adenoviruses

	H12ts303	H12ts307	H12ts333
H5ts125	3.2(+)[a]	0.013(−)	1.3(+)
H5ts149	14(+)	17(+)	29(+)

[a] Maximum yield (FFU/cell) from spinner cultures of KB cells infected at 39.5°C with 15 PFU/cell of each mutant and assayed at 32°C.

sis. Thus H12ts303 did not complement H5ts116 (type 5 complementation group II mutants), hexon minus mutants, and H5ts147, the hexon transport minus mutant (type 5 complementation group V). But H5ts 116 and H5ts147 did complement each other (Table I). Moreover, H5ts116 did not complement either H12ts303 or H12ts333, but the two type 12 mutants complemented each other effectively.

V. Transformation of Rat Embryo Cells by Type 5 Adenoviruses

Type 5 adenovirus is said to be nononcogenic in newborn hamsters, and it can only transform rat embryo cells at a very low frequency. The finding that H5ts125 was defective in the same cistron as H12ts307, a "supertransformer," suggested that the DNA minus type 5 mutant might also be a good transformer if the defective cistron was involved in the virus-induced cellular changes. Transformation analysis of rat embryo

TABLE IV

Transformation of Rat Embryo Cells by DNA Minus ts Mutants of Types 5 and 12 Adenoviruses

Virus	Foci per culture[a]	Transformation frequency
None	0, 0, 0, 0	10^{-4}
H12ts307	10, 9, 11, 7	0.9×10^{-3}
H5Wt	1, 1, 0, 1	0.75×10^{-4}
H5ts125	16, 16, 12, 15	0.15×10^{-2}

[a] Each culture was seeded with 10^4 cells 24 hours after infection with 50 PFU/cell and incubated at 36°C for 40 days.

cells was carried out with H5ts125, H5Wt, and H12ts307. The results of these experiments, summarized in Table IV, show that H5ts125 transformed cells at an even higher frequency than the type 12 "super-transformer" ts 307. As was expected, H5Wt transformed at a very low frequency.

VI. Perspectives

This chapter summarizes the beginnings of studies of conditionally lethal temperature-sensitive mutants of types 5 and 12 adenoviruses. The data presented furnish evidence that the prophecies can be fulfilled that such investigations will permit a better understanding of the regulation of adenovirus replication and cell transformation. Similar mutants of types 5 (Williams *et al.*, 1971; Takahasi, 1972), 12 (Lundholm and Doerfler, 1971; Shiroki *et al.*, 1972), 31 (Suzuki *et al.*, 1972), and CELO adenoviruses (Ishibashi, 1971) have also been described, and the numerous reports indicate that it will be possible to obtain useful mutants involving the major functional cistrons. The demonstration that intertypic complementation is possible greatly extends the range of mutants available for prospective biological, biochemical, and structural investigations.

The few ts mutants of type 5 adenovirus available already permit detailed studies of several critical regulated reactions, which can be identified in the biochemical events of viral replication diagrammatically summarized in Fig. 1. The two distinct DNA minus mutants demonstrate that at least two viral gene products are required for DNA replication. At least one of these (H5ts125) represents a cistron whose product is essential for continued DNA biosynthesis, probably for initiation of each round of replication. The DNA mutants further substantiate prior evidence, obtained with 5-fluoro-2-deoxyuridine (FUdR), indicating that progeny viral DNA molecules must be made in order to transcribe late mRNA's (Bello and Ginsberg, 1969) that are translated into capsid proteins (Flanagan and Ginsberg, 1962).

Earlier studies had demonstrated that if cells were infected with type 5 adenovirus in the presence of FUdR and the analogue-inhibition reversed with thymidine 10 to 12 hours later, viral DNA replication commenced but synthesis of host DNA was not resumed (Ginsberg *et al.*, 1967). These data implied that neither viral nor host DNA synthesis was essential for host cell DNA replication to be blocked in adenovirus-infected cells. The DNA minus ts mutants studied (H5ts125, H12ts307)

strengthened the conclusion that the inhibition of host cell DNA biosynthesis is independent of viral DNA replication.

It has been demonstrated that adenovirus DNA is synthesized in the nucleus, capsid proteins accumulate in the nucleus, and the virions are assembled in the nucleus. The viral proteins, however, are synthesized on polyribosomes in the cytoplasm and rapidly transported after completion into the nucleus (Velicer and Ginsberg, 1968, 1970). It was shown that energy was essential for transport, but detailed studies of the mechanism involved have been unsatisfactory (Velicer and Ginsberg, 1970). Clarification of the transport process should now be possible using the mutants whose hexon protein cannot be transported into the nucleus at the nonpermissive temperature, although the fiber and penton base are transported without restriction [such as H5ts147 and those described by Ishibashi (1970) and Russell *et al.* (1972)]. The transport minus mutants described suggest the following hypotheses: (1) A specific gene product controls the transport of each protein; (2) each protein has a specific region or site which must be intact for successful transport; or (3) each protein must assume a particular conformation through secondary or tertiary folding to permit its transport. The latter possibility seems the most likely, but all of these notions can now be tested.

Despite the numerous investigations of viral transformation of eukaryotic cells, the reactions responsible for the cellular changes have not yet been exposed. Temperature-sensitive mutants of both DNA and RNA viruses are now being widely employed for these studies. The lucky selection of three type 12 adenovirus ts mutants which cover the spectrum from nontransformation to increased efficiency of transformation offers considerable promise that these viruses will be useful in discovering some of the gene products responsible for transformation. The use of intertypic complementation may be particularly valuable in detecting the viral cistron or cistrons implicated in effecting the cellular changes. For example, the finding that H5ts125 has the same gene lesion as H12ts307, which transforms with increased efficiency, led to the evidence that H5ts125 is also a "supertransformer" and that the affected gene, which is essential for viral DNA replication, provides a product or initiates reactions that reduce the possibility of virus-induced cell transformation. These studies may be viewed with optimism since they suggest that the tools are at hand to study the molecular regulation of transformation. But the viruses evolved from such experiments also must be used with caution, since they indicate that the alteration of a single gene can all too readily convert a virus with low-transforming potential into a highly efficient transforming virus.

These data further suggest that all adenoviruses have the propensity

to transform cells, perhaps to be oncogenic. To fulfill the oncogenic potential it may be necessary to alter only one control reaction. This reaction may only change the virus so that it cannot replicate or so that it is less cytocidal (e.g., H5ts125); the decreased lethality may only permit the whole or part of the viral genome to persist in the cell, either integrated or as a free episome. If this model has validity, the present drive to obtain attenuated viruses, which can persist in the host inducing immunity against wild-type pathogenic viruses, could have undesirable, as well as apparent beneficial, consequences.

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CHAPTER 8

Herpesvirus Etiology of Abnormal Growth

FRED RAPP AND ROGER W. KOMENT

I. Introduction

The potential of herpesviruses to engage in multiple interactions with susceptible host cells and organisms has recently refocused attention on these ubiquitous viruses. In addition to productive infections, herpesviruses can establish latent and persistent infections in natural and experimental hosts (Rapp and Jerkofsky, 1973). The concept of an oncogenic potential for these viruses is relatively new, although not unexpected, and has been generated and supported by data derived from a

TABLE I

Transforming Herpesviruses with Known or Suspected Oncogenic Potential

Virus	Cell transformation	Host cell DNA stimulation	Oncogenicity
Marek's Disease Virus (chicken)	Leukocytes	Yes	Lymphoma (chicken)
Herpesvirus saimiri and *H. ateles* (monkey)	Leukocytes	Unknown	Lymphoma (monkey)
Lucké frog virus	Unknown (has not been replicated *in vitro*)	Unknown	Adenocarcinoma (frog)
Herpesvirus sylvilagus (rabbit)	Leukocytes	Unknown	Lymphoma (rabbit)
Guinea pig herpesvirus	Leukocytes	Unknown	Suspected leukemia (guinea pig)
Epstein-Barr virus (man)	Leukocytes	Yes	Suspected lymphoma (man)
Herpes simplex virus (man)	Hamster, mouse, and human fibroblasts	Unknown	Adenocarcinoma and fibrosarcoma (hamster)
Cytomegalovirus (man)	Hamster embryo fibroblasts	Yes	Fibrosarcoma (hamster)

variety of independent investigations. The possibility that these viruses play an etiologic role in human neoplasia is of major importance. However, because of obvious ethical limitations, research efforts have concentrated on animal model and cell culture systems. Fortunately, for the cancer researcher, there is a complete phylogenetic range of reservoir animal hosts carrying herpesviruses, many associated with neoplasia in their natural host, from which analogy and insight may be derived (Table I). Each system has unique and vexing problems which, when solved and assimilated, should expand the known properties of these viruses (Fig. 1). More important, each system offers a handle to molecular events that control virus–cell and virus–host interactions.

II. Oncogenicity of Animal Herpesviruses

A. Frog Adenocarcinoma

At the base of the phylogenetic tree of herpesvirus-associated neoplasias is the frog renal adenocarcinoma (Lucké tumor). This neoplasia was described by Lucké in 1934, who implied a virus etiology after ob-

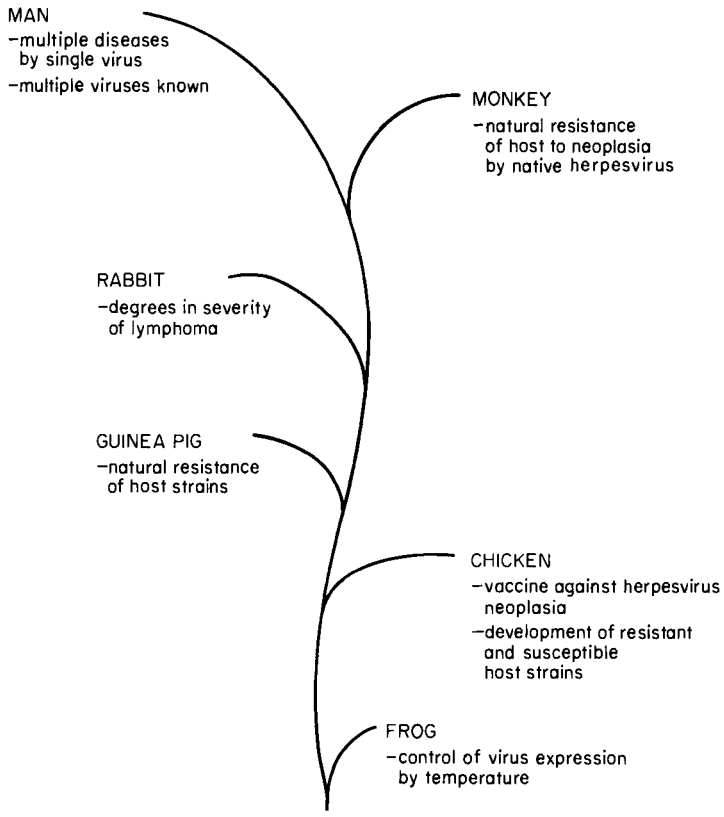

FIG. 1. Unique aspects of various animal herpesvirus neoplasias.

serving intranuclear inclusions in a large number of tumor cells. Lunger (1964) isolated a herpesvirus from the Lucké tumor years after particles with herpesvirus morphology were observed in these tumor cells (Fawcett, 1956).

The Lucké system has unique features that may yield information relevant to the control of virus expression. The host, *Rana pipiens*, hibernates with alternate periods at 20°–25°C (summer) and at 4°–9°C (winter). Consequently, tumors from frogs caught in the summer or maintained in the laboratory at 20°–25°C are designated "summer" tumors, and those from frogs caught in the winter or held at 4°–9°C are called "winter" tumors. While "summer" tumors contain neither virus inclusion bodies nor virus particles, "winter" tumor cells exhibit inclusions, tumor cell degeneration, and release of infectious virus. Accordingly, Mizell (1972) has observed that host cell DNA synthesis is

suppressed concomitant with virus production when tumors are maintained at 7.5°C, but not at 20°–25°C.

Induction of Lucké virus replication by prolonged incubation of "virus-free" tumor-bearing frogs or tumor tissue at low temperature is well documented. Rafferty (1965) demonstrated that nuclear inclusions appeared in biopsies of "summer" tumors maintained at low temperatures. Subsequently, Mizell *et al.* (1968) and Stackpole (1969) reported virus production in "summer" tumor tissue transplanted into the anterior eye chamber of frogs held at low temperature. To determine whether virus induction required unknown host factors, Breidenbach *et al.* (1971) maintained explants from "summer" tumors on agar slants at 7.5°C and, after 3 months, observed virus production. The fact that virus expression occurred independent of the intact host suggested that the complete virus genome was present and latent in these cells. Recently, Collard *et al.* (1973b) detected mRNA transcripts homologous to a portion of the Lucké virus genome in "summer" tumor cells. Thus, the Lucké virus genome appears to be latent in "summer" tumor cells and can be induced to replicate virus following prolonged incubation at low temperature.

B. Lymphoma of Chickens

Marek's disease (MD), a lymphoproliferative disorder of chickens, has been recognized since the beginning of this century (Marek, 1907). Until recently, the egg producer has had to accept a 10 to 20% flock mortality. In fact, Marek's disease has been responsible for major economic loss to the poultry industry, which perhaps explains why such rapid progress has been made in controlling this disease.

Groups working independently in England (Churchill and Biggs, 1967) and in the United States (Solomon *et al.*, 1968; Nazerian *et al.*, 1968) isolated a herpes-like virus [Marek's disease virus (MDV)], which was subsequently shown to be the etiologic agent of the disease. Similar to other herpesvirus-associated neoplasias, virus particles were not observed in blood, tumor, or kidney cells taken from infected birds, but appeared after duck embryo fibroblast or chicken kidney cell cultures were cocultivated with these cells. Subsequently, Calnek *et al.* (1970) and Nazerian and Witter (1970) demonstrated that MDV acquired its envelope, and hence matured, only in cells of the feather follicle epithelium. This explained how the highly cell-associated virus replicated and was transmitted to susceptible chicks.

The etiology of Marek's disease was definitively proved when vacci-

nation of 1-day-old chicks with attenuated MDV prevented neoplastic proliferation (Churchill *et al.*, 1969b). Four strains of virus are currently used to produce vaccines against MDV (Nazerian, 1973b), the most effective to date being an antigenically related herpesvirus of turkeys (HVT) (Kawamura *et al.*, 1969; Witter *et al.*, 1970). Vaccination with HVT does not protect against infection by the virulent virus but does prevent the virus-induced neoplasia. A persistent infection is established within the host, and both HVT and virulent MDV are continuously shed in the feather follicle epithelium (Eidson *et al.*, 1971). Therefore, although a low-grade infection is established, the vaccinated host is protected by an unknown mechanism from developing the lymphoma characteristic of acute Marek's disease.

C. Lymphoma of Guinea Pigs

A herpesvirus of guinea pigs (GPHV) has been isolated by Hsiung *et al.* (1971) and Nayak (1971) from leukemic lymphoblasts of strain-2 guinea pigs and from their nonleukemic counterparts. Guinea pigs of the leukemia-resistant Hartley strain, however, rarely harbor this agent (Hsiung *et al.*, 1971). Like other herpesviruses, GPHV persists in infected animals, despite the presence of neutralizing antibody (Tenser and Hsiung, 1973) and is readily detected in cultured lymphoblasts, but not in fresh leukemic tissue (Nayak, 1972).

The guinea pig herpesvirus appears to have the potential to transform primary hamster cells *in vitro*. In one report, cultured cells that survived cytopathic effects induced by the virus were morphologically transformed to epithelioid cells and contained virus-specific cytoplasmic antigens (Fong and Hsiung, 1973). Extension of these observations to an oncogenic potential for GPHV is complicated by the simultaneous presence of C type virus particles in the leukemic cells. Hsiung *et al.* (1973) recently observed that neoplastic disease could be induced in guinea pigs inoculated with both viruses, but not with either alone, and suggested a synergistic relationship between these agents.

D. Lymphoma of Rabbits

Herpesvirus sylvilagus has been isolated from the kidneys of wild, cottontail rabbits (Hinze, 1971; Hinze and Wegner, 1973). Unlike other herpesviruses, *H. sylvilagus* appears to be host-specific and has not shown infectivity for other laboratory animals. Infection produces a persistent, low grade viremia with concomitant stimulation of the lymphoid

tissue, which varies in severity from lymphoid hyperplasia to malignant lymphoma. Virus can be induced by cocultivation of infected leukocytes with susceptible rabbit kidney cells. A small percentage (0.1 to 1.0%) of cultured leukocytes contained virus antigens detectable by immunofluorescence techniques. To date, these leukocytes have not shown the capacity to proliferate indefinitely in culture that is characteristic of lymphoblastoid cells from patients with Burkitt lymphoma or infectious mononucleosis.

E. Lymphoma of Monkeys

Herpesvirus saimiri (HVS) and *Herpesvirus ateles* (HVA) are the first herpesviruses with oncogenic potential in primate hosts. HVS and HVA were originally isolated by Meléndez *et al.* (1968, 1972b) from apparently healthy squirrel and spider monkeys, respectively. No clinical disease in the natural host has been attributed to either of these simian herpesviruses, although HVS is capable of inducing neoplasia in several primate species and in New Zealand white rabbits. HVA appears to be truly oncogenic only in the cotton-top marmoset (Meléndez *et al.*, 1972a). Similar to simian virus 40 (SV40), these viruses appear to be carried in a latent state within the kidneys of their natural hosts and emerge from degenerating cell cultures.

III. Association of Human Herpesviruses with Neoplasia

A. Epstein-Barr Virus

By seroepidemiological studies, the Epstein-Barr virus (EBV) was first associated with Burkitt lymphoma (BL) and infectious mononucleosis (IM). Several excellent reviews detail the evidence supporting the association of EBV with BL and the fortuitous observations linking EBV etiologically to IM (Epstein, 1970; Klein, 1971, 1972). More recently, this virus has also been associated with nasopharyngeal carcinoma (de-Thé, 1972), Hodgkin's disease (Levine *et al.*, 1971), and the Guillain-Barré syndrome (Grose and Feorino, 1972). If the relationship between EBV and these diseases is causal, EBV can be designated as a prototype, multipotential herpesvirus. Accordingly, similarities between MDV, *H. sylvilagus*, and EBV—clinical manifestations and host response—can be probed for clues to human pathology.

B. Herpes Simplex Virus

Two subtypes of herpes simplex virus (Table II) have been differentiated by biological, biochemical, and biophysical parameters (Nahmias and Dowdle, 1968). Herpes simplex virus type 1 (HSV-1) is generally isolated from the respiratory tract. Herpes simplex virus type 2 (HSV-2) is transmitted by sexual contact, has been isolated from the male genitourinary tract (Centifanto *et al.*, 1972), and produces genital herpetic lesions. John Astuc, a French physician, first published an account of herpes simplex as a venereal disease in 1736 (Goodheart, 1970), and, noting a high frequency in prostitutes, designated the condition a "vocational disease of women."

Herpes simplex viruses are ubiquitous, commonly produce subclinical childhood infections, and persist in the host in a latent state despite the presence of humoral antibody. Several viral pathology studies have indicated that genital herpetic infection (HSV-2) often precedes cervical dysplasia, currently considered a precursor of cervical carcinoma (Naib *et al.*, 1966; Josey *et al.*, 1968; Rawls *et al.*, 1969; Sprecher-Goldberger *et al.*, 1970; Catalano and Johnson, 1971). Extensive seroepidemio-

TABLE II

Comparative Properties of Herpes Simplex Viruses

Property	Type 1	Type 2
Natural site in man	Nongenital areas	Anogenital areas
Latency in man	Trigeminal ganglia; brain cells	Suspected cervical cells
Virus inactivation by		
Heat	Less sensitive	More sensitive
Ultraviolet light	Less sensitive	More sensitive
Neutral red and light	Equally sensitive	Equally sensitive
Thymidine kinase inactivation by heat	Less sensitive	More sensitive
DNA (linear, 100×10^6 molecular weight)		
Buoyant density (gm/ml)	1.726	1.728
Guanine + cytosine (%)	67	69
Unique sequence (%)	95	84
Homology, % of type 1 vs. type 2	47–50	47–50
In vitro transformation of mammalian cells	Hamster and mouse fibroblasts	Hamster and mouse fibroblasts; human embryonic lung cells

logical studies (Rawls *et al.*, 1972, 1973) have likewise linked HSV-2 with cervical carcinoma.

Royston and Aurelian (1970) reported the presence of herpesvirus antigens in exfoliated anaplastic cells from patients with cervical carcinoma, but not in biopsy material taken from such patients (Aurelian *et al.*, 1972). By manipulation of growth requirements (specifically pH) a virus identified as HSV-2 was rescued from a degenerating culture of cervical carcinoma cells (Aurelian *et al.*, 1971). Although this report has not been confirmed, the detection by Frenkel *et al.* (1972) of a fragment of HSV-2 DNA in cervical tumor cells from one patient supports the etiologic role of HSV-2 in cervical carcinoma.

C. Cytomegalovirus

Although human cytomegalovirus (CMV) has not been etiologically associated with any known human neoplasia, it shares many properties common to other suspected neoplastic herpesviruses. CMV has been implicated in an atypical form of infectious mononucleosis and compared with the Epstein-Barr virus (Hanshaw, 1969). Common antigens between CMV and EBV (Hanshaw, 1970) and between CMV and HSV-2 (Wentworth and French, 1970) have been reported.

Infection may be either congenital (Hildebrandt and Monif, 1969; Birnbaum *et al.*, 1969) or acquired as indicated by the presence of serum antibody in the majority of the population by early adulthood. Similar to current ideas with HSV-2, CMV may be transmitted by sexual intercourse. Evidence for this is derived from the work of Lang and Kummer (1972) who recovered CMV in high titers from human semen. A recent clinical study of women with venereal disease demonstrated a high frequency of CMV isolation (Jordan *et al.*, 1973).

After primary infection, CMV can establish a latent infection within leukocytes (Stulberg *et al.*, 1966; Diosi *et al.*, 1969; Perham *et al.*, 1971). The establishment of an *in vitro* system using arabinofuranosylcytosine to study CMV latency has been described by Gönczöl and Váczi (1973).

CMV has been isolated from patients dying of malignant or chronic degenerative diseases, such as childhood leukemia (Cangir and Sullivan, 1966; Sullivan *et al.*, 1968), but to date is considered only an opportunistic parasite. CMV has also been commonly isolated from the immunologically compromised host undergoing renal and cardiac transplants and from patients with malignant neoplasias (Merigan and Stevens, 1971).

IV. Transformation by Human Herpesviruses

Of the three human herpesviruses discussed in this chapter (EBV, HSV, and CMV), only EBV can transform cells *in vitro* without prior inactivation of the virus to overcome the deleterious effects of cytopathology (Table III). Indeed, EBV exhibits no classical cytopathology other than chromatin margination that can be compared to other members of the herpesvirus group. Failure of the virus to grow to high titer in cell cultures has also hampered biochemical studies.

Henle *et al.* (1967) first demonstrated that X-irradiated BL cells could transform normal human leukocytes into cells that would proliferate indefinitely. Similar results were obtained when purified preparations of EBV were used to inoculate normal adult leukocyte cultures (Gerber *et al.*, 1969), fetal lymphoid cells (Pope *et al.*, 1968), and squirrel monkey and marmoset leukocytes (Miller *et al.*, 1972). Cells transformed *in vitro* by EBV as well as those derived from Burkitt tumors demonstrate a constriction of the number 10 chromosome (Miles and O'Neill, 1966, 1967; Kohn *et al.*, 1967; Henle *et al.*, 1967). Although the number of transformants synthesizing virus-specific antigens is low (< 10% of total cell population), cloning studies have demonstrated that each cell contains

TABLE III

In Vitro Transformation of Cells by Human Herpesviruses

Virus[a]	Mode of inactivation	Cell transformed	Reference
EBV	None	Leukocyte	Gerber and Monroe (1968)
HSV-1	Ultraviolet	Mouse LTK^-	Munyon *et al.* (1971)
HSV-2	Ultraviolet	Hamster embryo fibroblast	Duff and Rapp (1971b)
HSV-1	Ultraviolet	Hamster embryo fibroblast	Rapp and Duff (1973)
HSV-2	None	Human embryonic lung	Darai and Munk (1973)
HSV-2	Ultraviolet	Hamster embryo fibroblast	Kutmová *et al.* (1973)
HSV-2	Photodynamic	Hamster embryo fibroblast	Rapp *et al.* (1973)
CMV	Ultraviolet	Hamster embryo fibroblast	Albrecht and Rapp (1973)

[a] EBV, Epstein-Barr virus; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; CMV, cytomegalovirus.

the EBV genome necessary to produce a cell population that again contains a low percentage of cells with demonstrable virus antigens (Maurer *et al.*, 1970).

The association of HSV-2 with human cervical carcinoma has given impetus to research with this virus. Early efforts to induce neoplastic changes by inoculation of newborn rodents with HSV were unsuccessful due to neurovirulence and high animal mortality. One exception was reported by Nahmias *et al.* (1970) who observed a few sarcomas in hamsters following inoculation with HSV. However, no virus markers were detected in the tumors or cells cultured from them, and spontaneous malignancy or activation of a latent hamster virus could not be ruled out.

Likewise, the rapid onset of cytopathology masked *in vitro* transformation of cultured cells by wild-type virus. Demonstration that some DNA tumor viruses lost their cytocidal capabilities more rapidly than their transforming ability when subjected to ultraviolet (UV) irradiation (Latarjet *et al.*, 1967; Defendi and Jensen, 1967; Duff *et al.*, 1972) suggested that this procedure might be applied to HSV. Munyon *et al.* (1971) demonstrated transformation of mouse cells genetically deficient in thymidine kinase (LTK^-) to LTK^+ using ultraviolet irradiated HSV-1. Similar results have since been obtained with HSV-2 (W. Munyon, personal communication). Independently, hamster embryo fibroblasts (HEF) were morphologically transformed by lethally UV-irradiated HSV-2 (Duff and Rapp, 1971a) and subsequently produced malignant tumors when reimplanted into syngeneic hamsters (Duff and Rapp, 1971b). In a similar manner, HEF have been transformed to malignancy by UV-irradiated HSV-1 (Rapp and Duff, 1973). No virus has been isolated from these transformed cells, a characteristic of many cells transformed by DNA viruses (Macpherson, 1970), although virus-specific mRNA (Collard *et al.*, 1973a) (Section V,B) and antigens (Rapp and Duff, 1973) have been detected (Fig. 2).

Kutinová *et al.* (1973) exposed an established hamster cell line to inactivated HSV-2 and obtained cells that were highly oncogenic when inoculated into hamsters. HSV-associated antigens were detected by conventional immunofluorescence techniques, and HSV-2 was neutralized by tumor-bearing hamster sera, indicating the presence of virus-induced proteins in the transformed cells.

Darai and Munk (1973) exposed human embryonic lung cells to nonirradiated HSV-2, shifted the cultures to a temperature nonpermissive for virus replication (42°C), and recovered transformants. This is the first report of transformation of human cells with nonirradiated herpes simplex virus.

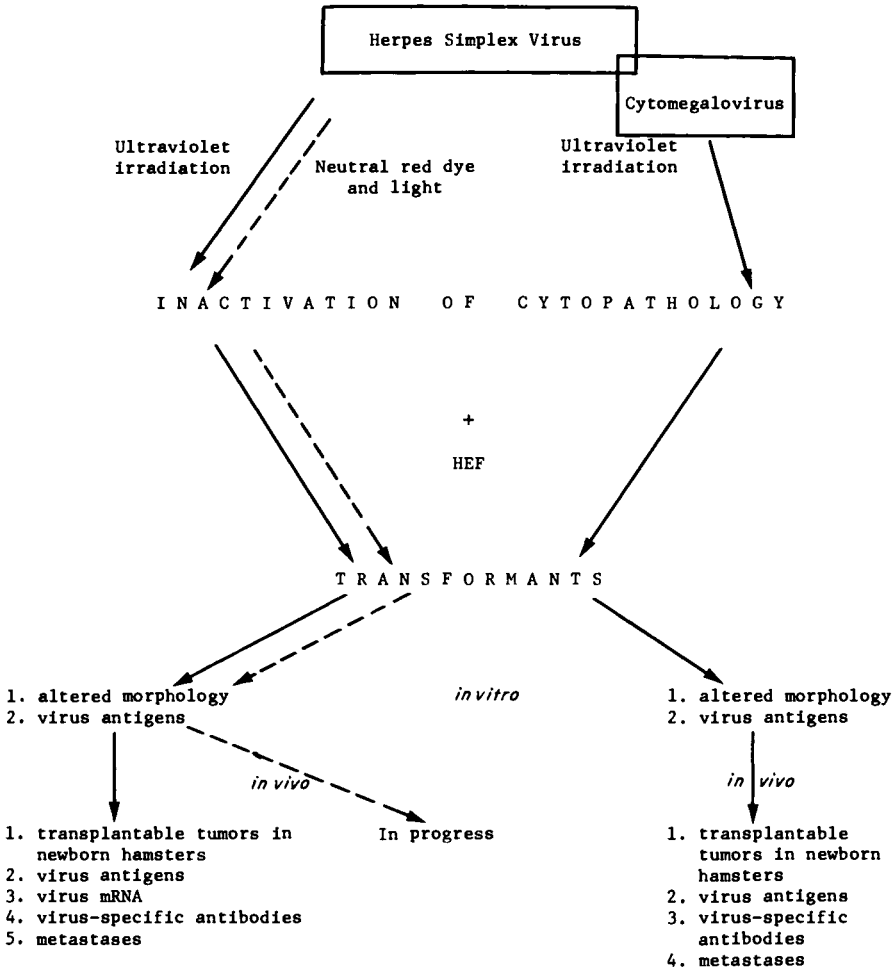

FIG. 2. Transformation of hamster embryo fibroblasts to malignancy by inactivated human herpesviruses.

Another technique that inactivates herpes simplex virus infectivity, permitting cell transformation *in vitro*, is photodynamic inactivation (Rapp *et al.*, 1973). When hamster embryo fibroblasts were exposed to HSV-1 or HSV-2 that had replicated in the presence of neutral red dye prior to inactivation by visible light, foci of transformed cells appeared within 4 weeks and became established cell lines when cloned. Virus-specific antigens were present in the cytoplasm of 8 to 10% of a given cell population (Table IV). The suspected oncogenicity of these cells is

TABLE IV

Presence of Herpes Simplex Virus (HSV)-Specific Antigens in HSV-Transformed and -Infected Hamster Embryo Fibroblasts (HEF) and in Tumor Cells

Cell type	Sera		
	Anti-HSV-1 or anti-HSV-2[a]	HSV-2 tumor[b]	Normal hamster
HEF transformants			
NR-HSV-1[c]	+	+	−
NR-HSV-2	+	+	−
UV-HSV-1[d]	+	+	−
UV-HSV-2	+	+	−
HEF infected with			
HSV-1	+	+	−
HSV-2	+	+	−
Tumor cells			
UV-HSV-1	+	+	−
UV-HSV-2	+	+	−
HEF	−	−	−

[a] Pooled sera from hamsters immunized with HSV-1 or HSV-2.
[b] Pooled sera from hamsters bearing tumor induced by UV-inactivated HSV-2.
[c] NR-HSV, cells transformed by neutral red-light inactivated HSV-1 or HSV-2.
[d] UV-HSV, cells transformed by ultraviolet light inactivated HSV-1 or HSV-2.

being tested. These observations suggested that application of photodynamic inactivation to the treatment of HSV-1 infections (Friedrich, 1973; Felber *et al.*, 1973) should be cautiously evaluated.

The ability of UV-inactivated human cytomegalovirus to transform HEF to malignancy has recently been demonstrated in our laboratory (Albrecht and Rapp, 1973). Foci of transformed cells were observed within 20 days after inoculation of cultures with inactivated CMV and, when cloned, established a proliferating cell line designated Cx-90-3B. These cells were poorly oncogenic in newborn and adult hamsters, but the tumor cells became highly oncogenic upon passage, metastasizing to lungs, kidneys, and other organs. All tumor-bearing hamsters had demonstrable antibody to CMV antigens.

The preceding discussion clearly demonstrates that three human herpesviruses can transform cells *in vitro*, some of which express virus functions (mRNA, antigens), and may be oncogenic in syngeneic hosts. The possibility that some, if not all, human herpesviruses also have this potential for human cells must be considered. Figure 3 depicts a model herpesvirus-transformed cell showing limited virus expression. Virus-specific proteins present in the cytoplasm and on the cell surface are

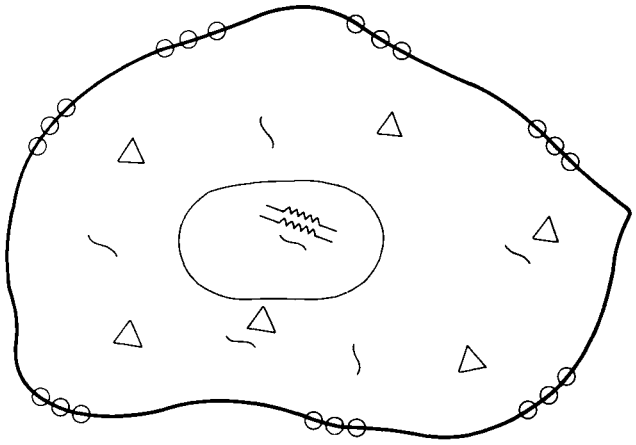

FIG. 3. Expression of the virus genome in transformed cells. ⋀⋀, virus and cell DNA; ~, virus mRNA; ○, virus-specific proteins at the cell surface; △, virus-specific proteins in the cytoplasm.

translated from virus-specific messenger RNA, but no complete virus particles are present.

V. Role of the Herpesvirus Genome in Oncogenicity

During productive infection cycles, herpesviruses rapidly suppress host macromolecular synthesis (Kaplan, 1973) and replicate with annihilation of infected cells. Consequently, the extensive cytopathology in a given cell culture would preclude detection of a chance transformational event. Thus, a prerequisite for oncogenicity is the establishment of a stable relationship between the virus and its host (Table V). To effect this, genetic information coding for transformation, notably stimulation of cell DNA synthesis, must be independent of that coding for host shutdown. Accordingly, reports that EBV (Gerber and Hoyer, 1971), MDV (Lee, 1972), and CMV (St. Jeor *et al.*, 1973) can stimulate the induction of host cell DNA synthesis add significant support to the contention that these may be oncogenic viruses.

Although mature virus particles can be detected in some herpesvirus-transformed neoplastic cells (Table VI), virus expression is generally restricted by unknown mechanisms to early functions (pre-DNA synthesis). A variety of techniques have been used to localize and potentially identify virus functions expressed in transformed cells.

TABLE V

Postulated Herpesvirus–Cell Interactions

Event	Replication	Latency	Transformation
Early virus RNA and protein synthesis	Yes	Limited	Limited
Host DNA synthesis	Inhibited	Not affected	Stimulated
Virus genome replication	Yes	May be associated with chromosomes (intact)	May be associated with chromosomes (fragment)
Virus release	Yes	No	No

A. Synthesis of Antigens

The herpesvirus genome is endowed with the potential of about 250 gene products (Fenner, 1970) derived from a molecular weight of 10^8 daltons. Of this number it seems safe to conclude that only a few will be required to initiate and/or maintain the transformed state and, thereby, be detectable in or on the transformed cell. Such initiation and maintenance factors are currently being studied in the polyoma (Fried, 1965; Dulbecco and Eckhart, 1970; Eckhart *et al.*, 1971) and Rous sarcoma (Toyoshima and Vogt, 1969; Martin, 1970; Kawai and Hanafusa, 1971; Biquard and Vigier, 1972) virus systems by the use of temperature-sensitive mutants. In many herpesvirus systems, appearance of virus-specific antigens has been well documented. With certain exceptions, these consist of capsid antigens that need not necessarily mature into complete, visible particles. Such antigens, and the subsequent heightened host response, were the first indication of the association of herpesviruses with cervical carcinoma, Burkitt lymphoma, nasopharyngeal car-

TABLE VI

Expression of Herpesviruses in Selected Neoplasias

System	Cell type	Virus genome	Virus antigens	Infectious
Marek's disease	Lymphoid tumor	Yes	No	No
Lucké adenocarcinoma	Adenocarcinoma	Yes	No	Yes[a]
Burkitt lymphoma	Lymphoid tumor	Yes	No[b]	No[b]
Cervical cancer	Epithelial carcinoma	Yes	Yes	Yes[c]

[a] Temperature dependent.
[b] Detected in cultured cells from the tumor. Virus DNA present in the cells of the tumor.
[c] Detected in degenerating cells from one tumor.

cinoma, and infectious mononucleosis. Although these are important, characterization of early herpesvirus antigens may reveal which virus functions are critical to initiation and maintenance of cell transformation.

Perhaps the most advanced work along these lines has been carried out with EBV. The first true early antigen of EBV was reported by Henle *et al.* (1970) and termed "early antigen" (EA). This antigen is expressed prior to virus DNA synthesis and is, therefore, differentiated from virus capsid antigen (VCA), which is regarded as a late (post-virus DNA synthesis) virus product (Gergely *et al.*, 1971a,b). Elevated antibody titer to EA has been demonstrated in BL patients with tumors when compared to cases with complete tumor regression (Henle *et al.*, 1971). Further, this antigen is prognostic in BL; a high antibody titer to EA is indicative of poor prognosis, while low or undetectable antibody titers indicate a favorable prognosis.

Recently, Reedman and Klein (1973) demonstrated an EBV-associated nuclear antigen (EBNA) by anticomplement immunofluorescence (ACIF) in more than 90% of the Burkitt tumor cells and lymphoblastoid lines examined, including those derived from patients with nasopharyngeal carcinoma. Analogy to the T antigen of oncogenic papovaviruses and adenoviruses was suggested.

When Burkitt tumor cells that had been cultured *in vitro* were studied by immunofluorescence techniques (Klein *et al.*, 1966, 1967), a second early antigen of EBV was described. This antigen, which resides at the cell surface, was termed membrane antigen (MA). MA can also be demonstrated in "EBV negative" lines (Klein *et al.*, 1967). Unlike EA, MA has no inhibitory effects on host cell macromolecular synthesis (Gergely *et al.*, 1971a).

Likewise, in the HSV system, membrane antigens have been described on the surface of infected cells. These antigens were similar to those found on the surface of herpes simplex virions (Roane and Roizman, 1964; Roizman and Spring, 1967).

Reports (Hollinshead and Tarro, 1973; Sabin and Tarro, 1973) that sera from patients with eight different cancers contain complement-fixing antibodies to herpesvirus nonvirion antigens have generated hope that these reagents will serve as diagnostic and analytic tools to study herpesvirus-mediated malignant transformation. Aurelian *et al.* (1973) have described an antigen of HSV, termed Ag-4, which appears 4 hours after infection of HEp-2 cells and reacts with sera from patients with cervical carcinoma. Since the presence of reacting antibodies correlates with the presence and extent of the tumor, its use as a diagnostic and prognostic tool was suggested.

Using an immunoprecipitin test to screen sera from MDV-infected

chickens, Churchill *et al.* (1969a) described the presence of three distinct antigens. The strongest precipitin band detected in the supernatant fluid of MDV-infected cultures was termed the "A" antigen and regarded as "soluble." "B" and "C" antigens were designated "insoluble" as they were cell-associated and released into the medium only upon cell destruction. At high virus passage (20 to 30), the "A" antigen was gradually lost and, subsequently (passage 33), pathogenicity and oncogenicity for chicks were also lost. Whether the loss of oncogenicity is due to loss of the "A" antigen or is merely a coincidental occurrence remains unresolved.

B. Synthesis of Nucleic Acids

The presence of virus protein in transformed cells forces the hypothesis that virus information is retained and conserved in the transformed cell line. Detection of virus DNA in transformants and characterization of the active genome sequences is critical to understanding the role of the virus in transformation. The use of DNA–DNA and DNA–cRNA hybridization techniques are highly sensitive methods for this purpose.

EBV DNA has now been demonstrated in all cell lines derived from Burkitt lymphoma, nasopharyngeal carcinoma, and to a lesser extent in lines derived from patients with infectious mononucleosis. Using DNA–cRNA hybridization techniques, it has been estimated that Burkitt lymphoma cells contain from 40 to 100 EBV genome equivalents per cell (Nonoyama and Pagano, 1971; zur Hausen *et al.*, 1972). In contrast, earlier DNA–DNA hybridization studies suggested that the number of genome equivalents per cell ranged from 2 to 5 (zur Hausen and Schulte-Holthausen, 1970; zur Hausen *et al.*, 1970). The most widely accepted estimate to date is about 50 genome equivalents per cell (Nonoyama and Pagano, 1973), a figure obtained by precise DNA–DNA reassociation kinetics. It is of interest to note that noninducible "virus-free" lines of Burkitt lymphoma cells contain as many genome equivalents per cell as do some virus-producing lines (zur Hausen and Schulte-Holthausen, 1970; Nonoyama and Pagano, 1971).

Roizman and Frenkel (1973) have demonstrated the presence of a fragment of HSV-2 DNA covalently linked to cell DNA and representing 10% of the virus genome within cervical carcinoma cells from a single tumor. The transcripts of this DNA represent 5% of the HSV-2 genome and contain both early and late sequences. Recently, Collard *et al.* (1973a) have reported that hamster cells transformed by ultraviolet light inactivated HSV-2 (Duff and Rapp, 1971a,b) transcribed 10 to

13% of the virus sequences normally found in productive infection.

Collard *et al.* (1973b) have also demonstrated the presence of mRNA in "summer" tumor cells from frogs with renal adenocarcinoma, further support for a herpesvirus etiology of this neoplasia. These techniques for the detection of virus nucleic acids have not yet been applied to other herpesvirus–neoplasia systems, but one could predict that such studies will be similarly fruitful.

C. Control of Virus Expression

The immunological response of the host to an infecting virus may determine what type of virus–host interaction can develop. Malignancies, either proven or suspected to be herpesvirus induced, have been reported in some, but not all, natural hosts of oncogenic herpesviruses. Chickens, for example, harbor Marek's disease virus and succumb to the lymphoproliferative disorder induced by this agent. However, as previously discussed (Section II,B), vaccination of 1-day-old chicks with attenuated MDV or with a related herpesvirus of turkeys prevents the oncogenic expression of wild-type virus, even though both viruses establish persistent infections. It is likely that a heightened immune response enables the host to counter the oncogenic potential of MDV (Nazerian, 1973a).

Marek's disease has strong parallels with the Burkitt lymphoma–infectious mononucleosis complex associated with EBV. Notably, EBV is highly cell-associated and induces lymphoproliferative disorders in its natural host (man), the severity of which might reflect the immunological competence of the host (Burkitt, 1969). Similarly, *Herpesvirus sylvilagus* of rabbits appears to be harbored only by the wild cottontail rabbit and may be infectious only for that species.

In direct contrast, *Herpesvirus saimiri* and *Herpesvirus ateles*, although highly oncogenic in related species, do not appear to cause neoplasia in their natural hosts. Equine herpesvirus 3 (EH3), which is capable of morphological transformation of embryonic hamster cells *in vitro* and can apparently induce lymphoma in hamsters (Karpas and Samso, 1972), is also in this category.

Understanding the factors that determine whether a virus will be oncogenic in its natural host is crucial to the eventual control of malignancy in man. This has been probed for Marek's disease with chickens bred for genetic resistance and susceptibility to the virus. Resistant birds, although still susceptible to infection, are less likely to develop the lymphoma characteristic of acute Marek's disease than are susceptible chickens (Nazerian, 1973b).

The degree of virus expression, and hence antigenicity, in herpesvirus-transformed cells is likewise critical to the host's ability to counter neoplastic proliferation. Herpesviruses are characteristically cell-associated and may exist latently in cells without expressing virus antigens. For Marek's disease, it is possible to demonstrate virus antigens in cells derived from the bursa of Fabricius but not to recover infectious virus. MDV-induced tumor cells contain complete virus genomes, as evidenced by transmission of the virus to permissive cells in culture (Section II,B), but do not express detectable amounts of virus antigen. Such a semi- or nonpermissive state is typical of several herpesvirus–cell systems.

Factors that limit herpesvirus expression are being investigated in several laboratories. One common goal of this work is to overcome host inhibitory mechanisms and to obtain complete expression of the virus genome, a goal which if realized would probably result in destruction of the cell.

For the EBV system this work has progressed rapidly following the demonstration by zur Hausen and Schulte-Holthausen (1970) and Nonoyama and Pagano (1971) that all cell lines derived from Burkitt lymphoma, as well as cells from patients with infectious mononucleosis, contain EBV genomes. Having established that virus genomes are present, it was reasonable to expect that they could be induced under appropriate conditions.

D. Induction of Virus Synthesis

The Lucké frog adenocarcinoma provides a unique system to study the effect of exogenous factors on virus production. Expression of this virus is temperature-dependent and, as previously discussed (Section II,A), can be induced in "virus-free" summer tumor cells maintained at low temperature for prolonged periods (Mizell *et al.*, 1968; Breidenbach *et al.*, 1971). Identification of the temperature-dependent molecular mechanisms critical to Lucké virus replication and cell transformation would offer a major contribution to current understanding of herpesvirus control mechanisms.

A second herpesvirus that may be activated by exogenous factors is herpes simplex virus. Recurrent facial herpetic infections caused by HSV-1 are known to be activated by sunlight, hormonal changes, and stress. Work by Stevens and Cook (as reviewed, 1973) has shown that HSV is harbored in a latent form within ganglion cells of mice and rabbits between clinical recurrences. This has now been directly demonstrated in man (Bastian *et al.*, 1972). Since recurrence is common with

genital herpes, and such recurrence often precedes cervical dysplasia (Naib, 1966; Naib *et al.*, 1969), it would not be surprising to find that HSV-2 is similarly retained in a latent form within nerve tissue.

Induction of virus synthesis has been achieved in the laboratory with the halogenated pyrimidines 5-bromodeoxyuridine (BUdR) and 5-iododeoxyuridine (IUdR). These thymidine analogues alter natural virus–cell interactions, inducing an increased production of EBV-specific antigens and virus particles. Arginine deprivation and growth of cells at reduced temperature have also been reported to increase the yield of EBV (Henle and Henle, 1968). However, these methods, while useful, would not elicit production of virus if transformation had been effected by a defective virus, either artificially or naturally produced. Thus, negative results cannot be interpreted to indicate absence of inducible virus genetic information.

VI. Future Expectations

In the past years, scientists have come to view the herpesvirus group with new respect. Previously related to a number of infectious disease entities, these agents are now known to be capable of transforming a variety of cells, given the appropriate conditions. In addition, they possess the awesome property of lying sequestered in the latent state throughout the greater portion of the life of the host.

It can be predicted with some confidence that additional herpesviruses will be isolated with transforming potential. These viruses are likely to be less cytopathic than the prototype herpes simplex virus strains. Characterization of the early antigens induced by these viruses and the nucleic acids coding for them will lead to immunologic and epidemiologic studies which are necessary preliminaries for the association of these viruses to human neoplasia. Molecular events controlling virus expression, host cell controls, and control of the tumor cells by the organism will have to be resolved before effective steps can be recommended for control of disease due to these well-adapted viruses.

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CHAPTER 9

Replication and Integration of the Genome of Oncornaviruses

PHILIPPE VIGIER

I. Introduction

Oncornaviruses are typical moderate viruses, as defined by Dulbecco (1955). They can multiply in infected cells, transformed or untransformed, without impairing the cell's capacity to multiply, and they can also persist in cells without being produced, even though the viral genome is not defective (cf. Vigier, 1970; Temin, 1971a). In some cases, the viral genome can be, or becomes, defective, and the virus then requires a helper virus for its replication. But defective viral genomes can persist in cells, notably in transformed cells, without any helper

virus and can be rescued by other related viruses after many cell divisions.

Some oncornaviruses, which may be the ancestors of the other ones, also appear to be carried and transmitted from one host generation to the next as cellular genes. These cryptic viruses can act as helpers for defective viruses and recombine with exogenous viruses. They can also become activated, either spontaneously or by treatment of cells with physical and chemical agents which are either carcinogens or inducers of bacteriophages in lysogenized bacteria.

These biological features of oncornaviruses may be related to the fact that although the genome of the free virus consists of RNA, this RNA is replicated and maintained in the cell by the intermediary of complementary DNA which might be integrated with the cellular genome. The possibility of such an unorthodox "detour" that reverses the normal flow of information was suggested about 10 years ago by Temin (cf. Temin, 1971a) on the basis of various studies, including our own, which showed that replication of avian oncornaviruses requires a transient DNA synthesis in the early hours of infection and DNA-dependent RNA synthesis even after the cells have become established virus producers. Similar requirements were found later on in the case of murine oncornaviruses. However, what may now be called the "Temin model" has been established only in the last 3 years notably by the finding that oncornaviruses all carry an RNA-instructed DNA polymerase able to synthesize *in vitro* DNA complementary to the viral RNA, and by the recovery of infectious DNA from both permissive and nonpermissive cells transformed by avian oncornaviruses.

Another feature of the viral genome that appears of importance for its replication is that although the viral RNA is very large—its molecular weight is about 10^7 daltons—it consists of an aggregate of 3 or 4 subunits that seem to be replicated separately and become linked only after the budding of the mature virions. It is not yet known how the viral genetic information is distributed between these subunits, but the high incidence of mutation and recombination encountered with some strains of avian viruses suggest that at least some viral genes may be carried by different subunits.

My purpose will be to review briefly and discuss the data pertinent to the structure and replication of the viral genome in permissive and nonpermissive cells, with special attention being devoted to those that suggest that the viral genome, i.e., the DNA "provirus" of Temin, is integrated with the cellular genome, as that of oncodnaviruses and temperate bacteriophages.

II. The Viral Genome

The genome of oncornaviruses consists of high molecular weight RNA with a sedimentation constant of 60 S–70 S, corresponding to a molecular weight of about 10^7 daltons (cf. Robinson *et al.*, 1967). Virions also contain RNA's of lower molecular weight, some of which appear to be of cellular origin, and low molecular weight DNA, the function of which, if any, is unknown (cf. Temin, 1971a).

The 60 S–70 S RNA can be dissociated, under denaturing conditions, such as heat and dimethylsulfoxide, into RNA sedimenting at 30 S–40 S, that is, of 3 to 4×10^6 daltons (Duesberg, 1968; Erikson, 1969; Montagnier *et al.*, 1969). This suggests that the 60 S–70 S RNA is an aggregate of 3 or 4 subunits of similar size held together by hydrogen bonds. These subunits, as well as the large viral RNA, can be seen on electron micrographs of the RNA of avian myeloblastosis virus (AMV) prepared by the Kleinschmidt technique, following dilution in 8 *M* urea which presumably acts in the same way as heat or DMSO (Granboulan *et al.*, 1966).

The 30 S–40 S subunits of avian sarcoma (focus-forming) viruses can be resolved electrophoretically into a slower migrating a subunit and a faster migrating b subunit, whereas only the b subunit is found in non-focus-forming avian leukosis viruses, including nontransforming mutants of sarcoma viruses (Duesberg and Vogt, 1970). This difference suggests that only the a subunit bears the transformation markers of avian sarcoma viruses, whereas the b subunit(s) bears the information for the replication of viral components. The existence of the a and b subunits also provides an explanation for the high rate of recombination (close to 50% in some cases) between the transformation markers of some avian sarcoma viruses and the host-range marker (i.e., the viral envelope glycoproteins) of some avian leukosis viruses (Vogt, 1971b; Kawai and Hanafusa, 1972). The genetic mixing could be a reassortment of independent subunits from each parental virus, the a subunit of the sarcoma virus reassorting with the b subunits of the leukosis virus (Vogt, 1971b). However, more recent data (Duesberg *et al.*, 1973) have shown that cloned sarcoma viruses contain only the a subunit, suggesting that avian tumor viruses may contain 3 or 4 copies of only one subunit. If this is so, the a and b subunits would have common genes for replicative and structural properties, and genetic recombination between transforming genes of sarcoma viruses and host-range genes of leukosis viruses would represent true genetic recombination. The possible mech-

anisms of this recombination will be discussed later on (see Section IV).

It is not possible presently to decide whether the viral genetic information is distributed between different subunits, as suggested by the high rate of recombination encountered with some strains of avian viruses, or carried by one subunit present in triplicate or quadruplicate in virions, as suggested by the data of Duesberg *et al.* Arguments exist in favor of each model: (a) A single subunit of 3 or 4×10^6 daltons can accommodate the information for all the viral proteins presently known and identified, i.e., the reverse transcriptase, five internal proteins, and two envelope proteins, for avian viruses (Fleissner, 1971), or less, for murine viruses (Nowinski *et al.*, 1971; Moroni, 1972). In the case of avian viruses, the added molecular weights of these proteins amount to less than 3×10^5 daltons, and in that of mouse mammary tumor virus (MTV) to about 3.4×10^5 daltons, assuming that the molecular weight of reverse transcriptase is 7×10^4 (see below). In addition, sarcoma viruses must accommodate the information that controls transformation, but this may correspond to only one or two small proteins (cf. Table I).

TABLE I

Information Carried by the Viral Genome (Avian Oncornaviruses)[a]

Viral proteins	MW[b] GuHCl[c]	MW[b] PAGE[c]
Envelope (type-specific, glycoproteins)		
m_1	100 k	32 k
m_2	70 k	70 k
Internal		
Group-specific		
gs_1	27 k	25 k
gs_2	19 k	21 k
gs_3	15 k	12 k
gs_4	12 k	14 k
Nonantigenic		
gs_5	10 k	12 k
Reverse transcriptase α	65 k	
(+RNase H) $\alpha\beta$	170 k	
Nonviral proteins		
Transforming protein(s)	?	

[a] For the viral envelope and internal proteins, the data are those of Fleissner (1971), and, for reverse transcriptase, those of Grandgenett *et al.* (1973).

[b] $k = 10^3$ daltons.

[c] GuHCl, guanidine HCl column; PAGE, polyacrylamide gel electrophoresis.

However, it has been reported that Rous sarcoma virus (RSV) can contain 23 proteins with a total molecular weight near 7×10^5 daltons (Cheung *et al.*, 1972), and reverse transcriptase of AMV has been shown to contain a subunit of about 10^5 daltons, in addition to that carrying the enzymatic activity (see below). If all these proteins are actually coded for by the virus, at least two subunits would be necessary to accommodate all the corresponding information. (b) Avian tumor viruses are inactivated by UV light following one-hit kinetics (Levinson and Rubin, 1966) and so are murine leukemia and sarcoma viruses (Yoshikura, 1971). This is against the existence of redundant subunits of viral RNA. However, inactivation of RSV by X irradiation may follow multiple-hit kinetics (Levinson and Rubin, 1966). (c) A high incidence of nontransforming virions is observed in some clones of avian sarcoma viruses, i.e., up to 17% (Vogt, 1971a). It is difficult to imagine that, in this case, three or four a subunits could all have lost their transformation markers together and become b subunits.

Whatever the distribution of the viral information between the RNA subunits, these subunits appear to be replicated separately, at least at the stage of transcription of the DNA provirus, as suggested by two sets of data.

First, the largest virus-specific RNA's detected in virus-producing cells by hybridization with homologous DNA made *in vitro* by the viral reverse transcriptase (see below) have the size of the viral RNA subunits, and not the full viral RNA (Tsuchida *et al.*, 1972).

Second, virions of RSV harvested within 5 minutes of their release do not contain 60 S–70 S RNA, but only 30 S–40 S RNA. This RNA is subsequently converted into 60 S–70 S RNA inside the virions (Canaani *et al.*, 1973). Similar, although less conclusive, data have been reported by Cheung *et al.* (1972).

However, this does not rule out that incoming viral RNA might be transcribed as a single piece into DNA, which might only subsequently be transcribed into smaller size RNA in the second step of the replication of the viral genome. This possibility is suggested by the finding that following infection of cells with [^{3}H]uridine-labeled RSV, the labeled 60 S RNA is transported in less than 10 minutes into the cell nucleus without being degraded (Dales and Hanafusa, 1972).

III. The DNA "Provirus"

Several sets of evidence show that replication of the viral RNA is mediated by a DNA "provirus" that may integrate with the cellular DNA (see Table II).

TABLE II

Evidence for a DNA Intermediate (Provirus) in the Replication of Oncornaviruses

1. Viral replication requires DNA synthesis early after infection and DNA-dependent RNA synthesis at any time
2. In cultures of stationary cells infected with RSV, then treated with 5-bromodeoxyuridine (BUdR), exposure to light inactivates the capacity of infected cells to give rise to virus and Rous cells
3. Noninfectious virus and a high incidence (10^{-1}) of virus mutants are produced in B-RSV-infected cells treated with BUdR early after infection
4. Cells infected with exogenous viruses, or carrying a cryptic virus, contain DNA homologous to the viral RNA
5. Oncornaviruses carry an RNA-directed DNA polymerase (reverse transcriptase) that makes DNA complementary to viral RNA, *in vitro*
6. Permissive cells infected with avian oncornaviruses and nonpermissive cells transformed by RSV contain DNA that can infect permissive cells: the virus recovered from the latter cells is phenotypically similar to that in the donor cells

A. The Action of Analogues and Inhibitors of Macromolecular Synthesis on Viral Replication

As already observed ten years ago (cf. Bader and Bader, 1970; Vigier, 1970) viral replication requires DNA synthesis early after infection and DNA-dependent RNA synthesis even after the cells have become established virus producers. However, this DNA synthesis could be that of cellular DNA, and the DNA-dependent RNA synthesis could correspond to the transcription of cellular genes.

In cultures of stationary cells infected with Rous sarcoma virus (RSV) and then treated with 5-bromodeoxyuridine (BUdR), exposure to light inactivates the capacity of infected cells to give rise to virus and transformed cells (Rous cells) without impairing their capacity to subsequently multiply (Balduzzi and Morgan, 1970; Boettiger and Temin, 1970). Hence, the effect of light is presumably on new DNA synthesized after infection, and not on cellular DNA.

Noninfectious virus and a high incidence (about 10^{-1}) of virus mutants that are defective for transformation at high temperature are produced by cells infected with RSV and treated with BUdR early after infection (Bader and Bader, 1970; Bader and Brown, 1971). This suggests that alteration of the DNA synthesized after infection results in alteration of the viral RNA.

B. The Presence of Virus-Specific DNA in Infected and Transformed Cells and in Cells Carrying Cryptic Viruses

Cells infected with avian and murine viruses contain DNA that hybridizes with the viral 60 S–70 S RNA. In these investigations, labeled

viral RNA was hybridized with the DNA from infected cells (transformed and untransformed) and from uninfected controls. Discussable results were obtained initially (cf. Vigier, 1970; Temin, 1971a). However, a higher degree of hybridization of viral RNA with the DNA from infected cells was reported later by some authors in the case of avian tumor viruses (Rosenthal *et al.*, 1971; Baluda, 1972). Yet, a high background of hybridization of viral RNA with the DNA from uninfected chicken cells was always observed. This is presumably due to the fact that all chicken cells carry cryptic oncornaviruses integrated in the form of DNA with the cellular genome (see below). Indeed, no background of hybridization was observed with the DNA from normal mouse or rat cells, whereas the DNA from the same cells transformed by RSV (but nonpermissive for viral replication) hybridizes with the viral RNA. On the other hand, the high melting temperature of the hybrids suggests that they are specific.

More demonstrative results have been reported recently by Neiman (1972, 1973) using a large excess of cellular DNA. This allows the kinetics of hybridization to be studied, and estimates more precisely the number of viral genome equivalents (DNA copies) present in the cells. It was found that chicken cells transformed by RSV contain DNA that hybridizes with most of the viral RNA, but in small amount (no more than 3 copies per cell). However, about one-third of the viral RNA appears to be complementary to DNA present in much larger amount (50–100 copies per cell), which suggests the possibility of an amplification of the synthesis of DNA copies of one part of the viral genome. DNA hybridizing with most of the viral RNA was also found in low amount (2–4 copies per cell) in leukosis-free chicken embryos, as well as in embryos from a line carrying the cryptic virus RAV-O (see below). But no amplification was evident in these cases (Neiman, 1973).

For comparison, Baluda (1972) has found that all apparently normal chicken embryos or adult chickens tested contain virus-specific DNA in amounts ranging from 1.7 to 4.6 viral genome equivalents per cell, whereas cells infected and transformed with RSV or AMV contain 4 to 13 viral genome equivalents.

The reassociation of denatured DNA probes made *in vitro* by the viral reverse transcriptase (see below) is accelerated in the presence of cellular DNA from virus-carrying cells. This acceleration is due to the presence of virus-specific DNA in the cellular DNA and is a function of the number of viral genome equivalents. It was thus found that both mouse and rat cells infected with murine leukemia virus (MuLV) or murine sarcoma virus (MSV, associated with MuLV) and normal cells (presumably containing cryptic viruses) contain 20–50 viral genome equivalents (Gelb *et al.*, 1971), whereas RSV-infected and normal chicken cells contain 10–20 (with an RSV DNA probe) (Varmus *et al.*,

1972a), and cells producing or carrying MTV contain as much as 90 (Varmus *et al.*, 1972b).

However, the method presents a complication, due to the fact that the *in vitro* synthesized DNA probe consists of 85–90% rapidly reannealing (RR) DNA, which represents only 25% of the viral genome, and 10–15% of slowly reannealing (SR) DNA, which represents most of the viral genome (Gelb *et al.*, 1971; Varmus *et al.*, 1971). Thus, the DNA copies could consist only of a minor part of the viral genome. Yet, the same number of viral genome equivalents have been found in RSV-infected and normal chicken cells using only the SR DNA probe (Varmus *et al.*, 1972a), and about 13 viral genome equivalents have been found in normal mouse cells (carrying a cryptic virus), using SR DNA of MuLV (Gelb *et al.*, 1973). Moreover, in the latter work, the virus-specific DNA appeared to be covalently linked with high molecular weight cellular DNA.

In the same way, about 2 copies of RSV-specific DNA were detected in transformed mouse and rat cells (four lines), but not in normal controls. This DNA was also included in pieces of cellular DNA of high molecular weight (over 3×10^7) (Varmus *et al.*, 1973).

C. The Presence of an RNA-Instructed DNA Polymerase (Reverse Transcriptase) in All Oncornaviruses

This polymerase, first found by Baltimore (1970) and Mizutani and Temin (1970), synthesizes *in vitro* DNA that is homologous to the viral RNA and is present inside the virions of all oncornaviruses (Baltimore, 1970; Mizutani and Temin, 1970; Spiegelman *et al.*, 1970; Green *et al.*, 1970; Duesberg and Canaani, 1970). Its main characteristics are the following.

The polymerase is presumably coded for by the virus (Linial and Mason, 1973). It transcribes the whole viral RNA into double-stranded DNA (Duesberg and Canaani, 1970) by a two-step reaction, in which the enzyme first makes an RNA–DNA hybrid and then double-stranded DNA of small size (4 S–8 S). The first step of the reaction requires an RNA primer in addition to the RNA template, since the enzyme, as other DNA polymerases, does not initiate the synthesis of new chains of DNA but incorporates deoxynucleotides onto the 3′-OH end of the primer strands (Verma *et al.*, 1971; Leis and Hurwitz, 1972). The enzyme also prefers the viral 70 S RNA for a template to other viral, or virus-associated, RNA's (Garapin *et al.*, 1973). On the other hand, most of the DNA made *in vitro* corresponds to only a minor fraction of the viral RNA, which may be particular to the enzyme *in vitro*.

The purified enzyme from AMV, and presumably also other avian viruses, consists of two subunits of 65,000–70,000 daltons (α subunit) and 105,000–110,000 daltons (β subunit) (Kacian *et al.*, 1971; Grandgenett *et al.*, 1973). Of these subunits, only the smallest (α) carries the enzymatic activity, plus an enzyme (RNase H) that specifically degrades the RNA moiety of RNA–DNA hybrids (Mölling *et al.*, 1971; Grandgenett *et al.*, 1973). The polymerase of MuLV consists only of an α subunit (Ross *et al.*, 1971). This suggests that the β subunit of the avian enzyme might be coded for by the cell and not by the virus (Grandgenett *et al.*, 1973).

Reverse transcriptases from oncornaviruses of different animal species also differ antigenically and can thus be characterized immunologically (Scolnick *et al.*, 1972).

It may also be noted that nonpermissive cells transformed by oncornaviruses may not contain any detectable amount of reverse transcriptase, even though other viral proteins are synthesized (Livingston *et al.*, 1972). This suggests that the reverse transcriptase(s) might not be the most reliable marker in the search for cryptic oncornaviruses.

D. The Recovery of Infectious DNA from Permissive and Nonpermissive Cells, Transformed and Untransformed, Infected with Avian Oncornaviruses

This appears to be the most demonstrative proof that the RNA of oncogenic viruses is replicated by the intermediary of a DNA provirus, and is also maintained in the form of DNA in transformed non-virus-producing (TNP) cells.

As was first shown by Hill and Hillova (1971, 1972a), treatment of chick embryo cells (CE cells) with DNA from the XC rat sarcoma cells gives rise to RSV of the same phenotype (Prague strain RSV-C) as the virus that initially transformed the donor cells, whose genome persists in these cells (Svoboda, 1964). Virus with the same phenotype as the virus that transformed the donor cells was also recovered in the same way from CE cells treated with DNA from hamster BHK-21 cells transformed *in vitro* by a conditional mutant of Schmidt-Ruppin strain RSV, which is defective for transformation at high temperature (see below) (Hill and Hillova, 1972b). In both cases, only a few cells gave rise to virus and Rous cells following the DNA treatment, and subculturing of treated cells was required for virus recovery. Successful infection also required pretreatment of test cells with the polycation DEAE–dextran, which presumably favors penetration of the foreign DNA inside the cells.

Similar results have been reported by Svoboda *et al.* (1972) and Hlozanek and Svoboda (1972) with XC cells and hamster RSCH cells transformed by SR-RSV.

We (Montagnier and Vigier, 1972, 1973) have similarly recovered SR-RSV-D and the ts mutant FU-19 of this virus, defective for transformation at high temperature (Biquard and Vigier, 1972), from CE cells treated with DNA from CE cells infected productively and transformed by these viruses. The results of our experiments can be summarized as follows (cf. also Table III).

1. The DNA from the virus-producing transformed CE cells induced foci of transformed virus-producing cells in treated cultures of CE cells (pretreated with DEAE-dextran) after two or three passages of the treated cultures, i.e., 9–25 days after treatment.
2. The virus produced in treated cultures was phenotypically undistinguishable from that in the donor cells. Notably the ts transformation marker of FU-19 was present in the virus recovered from cultures treated with the DNA from FU-CE cells.
3. Activity of the DNA was destroyed by DNase, but not by RNase or pronase.
4. The infectious DNA was present in purified cell nuclei and presumably associated with chromosomal DNA, since it was always present also in the 1 *M* NaCl precipitate of DNA prepared by the method of Hirt (1967), and never in the supernatant fraction which contains episomal and plasmidal DNA's.
5. The density of infectious DNA was the same as that of cellular DNA in CsCl density gradients.
6. The sedimentation constant of infectious DNA sedimented in sucrose gradients varied from 20 S to 35 S, which corresponds to molecular weights of 6×10^6 to 4×10^7 daltons. The latter figure suggests that the infectious DNA may be covalently integrated with chromosomal

TABLE III

Characteristics of Infectious DNA from RSV-CE Cells

1. Induces foci of transformed, virus-producing cells
2. The virus produced is identical to that used for infection of donor cells
3. Activity is destroyed by DNase, not destroyed by RNase or pronase
4. Present in the cell nucleus and presumably associated with chromosomal DNA (always in precipitate of Hirt extract)
5. Density in CsCl that of cellular DNA
6. Sedimentation in sucrose between 20 S and 35 S (6 to 40 $\times$ 10^6 daltons)
7. Infects gs^- cells as well as gs^+ cells

DNA, since the size of virus-specific DNA complementary to the whole 60 S–70 S viral RNA is only 2×10^7 daltons. However, it is also possible that high molecular weight infectious DNA may correspond to tandem copies of the viral genome.

On the other hand, the fact that DNA molecules of only 6×10^6 daltons can also be infectious suggests that the whole viral genetic information may be carried by DNA of this size, which corresponds to one viral RNA subunit. Yet, small size infectious DNA might also carry only one part of the viral genes, notably those genes required for transformation and for the synthesis of viral envelope proteins (since the recovered virus always displayed the same antigenicity as the virus of origin). A cryptic virus present in the test CE cells might supply the missing genes, by recombination. This possibility is supported by the fact that the test cells contained, in some cases, the viral group-specific (gs) antigen, and, therefore, presumably carried a cryptic helper virus (cf. T. Hanafusa *et al.*, 1970). However, experiments carried out with gs^- embryos from the Reaseheath C line, which are helper negative (although they also carry a cryptic virus), also gave positive results. This does not rule out the possibility that recombination might occur in gs^+ cells, but it suggests that infectious DNA may carry the full viral genetic information. It may be noted, from this standpoint, that the molecular weight of DNA able to infect C line cells was at least 2×10^7. However, more experiments are necessary to know the minimal size of infectious DNA assayed on helper-negative cells.

From the quantitative viewpoint, the assay of infectious DNA appears to be very inefficient since the minimal infectious dose was about 10 μg, i.e., the DNA content of about 5×10^6 donor cells. On the other hand, no linear relationship has been found between the infectivity and the dose of DNA, which might be due to interference of nonviral DNA with the uptake of viral DNA or some subsequent step leading to virus production (Vigier and Montagnier, 1973).

Another possible cause for the low efficiency of infection with DNA and the late recovery of virus from treated cultures might be that viral replication requires integration of the viral DNA and that this integration is favored in virus-infected cells, but not in DNA-treated cells, by some virus-associated enzymes, such as the endonuclease and the ligase found in RSV by Mizutani *et al.* (1970, 1971).

We have also, in preliminary experiments, recovered virus phenotypically undistinguishable from that produced by donor cells following treatment of CE cells with DNA from untransformed CE cells infected productively with two different avian lymphomatosis viruses (RAV1 and RAV50). Similarly, Lacour *et al.* (1972) have recovered AMV from CE

cells treated with DNA from AMV-producing cells. The CE cells were implanted into chickens after treatment, which provided an agrandizer system, but may also have provided a helper virus.

In any case, it now seems established that part, and probably all, of the genetic information of avian oncornaviruses can be transmitted by DNA associated with that of virus-producing or nonproducing transformed cells.

IV. The Cryptic Viruses

A. Avian Viruses

In 1966, Dougherty and Di Stefano reported that liver homogenates from normal chick embryos, from which leukosis virus could not be isolated, reacted in the complement fixation test with serum from hamsters bearing RSV-induced tumors. As is known (Huebner, 1965), this COFAL test detects the internal group-specific (gs) proteins common to all oncornaviruses, which can be found as well in nonproducer transformed cells, such as RSV-transformed mammalian cells, as in virus-producing cells. The identity of the normal liver antigen(s) detected with that associated with avian tumor viruses was confirmed by means of immunodiffusion tests (Dougherty *et al.*, 1967). The gs antigen was similarly found in whole embryo extracts and in extracts of cultured chick embryo fibroblasts, provided that the cell concentration was sufficiently high (Payne *et al.*, 1968).

Subsequent investigations have shown that "natural" gs antigen is present only in some embryos and that its presence is determined genetically. All embryos of some inbred lines of fowl are gs^+, whereas all embryos of other lines are gs^-, and, following crosses between these lines, the *gs* marker segregates according to expectation if expression of the antigen was controlled by a single autosomal gene.

The natural gs antigen is widely distributed in the tissues of embryos of gs^+ lines (Payne and Chubb, 1968), but it can also be absent in embryos and appear only at the time of hatching and in only some tissues (liver) (Payne, 1972). This suggests that it depends on genetic information that can be repressed, and derepressed by aging.

Further investigations have shown that the presence of natural gs antigen in chick embryos depends on a cryptic virus that presumably codes for this antigen and is transmitted in chickens as a cellular gene. This cryptic virus, or viruses, can act as a helper for the replication of defective Bryan strain RSV(O), following solitary infection, and it confers its envelope characteristics (notably its unusual host range) to the virus produced with its help.

Weiss (1969) first observed that embryo cells from an inbred gs^+ line produced infectious RSV(O) following single infection, whereas cells from another inbred gs^- line produced noninfectious virus. Presence of gs antigen and ability to replicate infectious RSV(O) segregated together in crosses between the two lines (Weiss and Payne, 1971). The helper factor was also recovered from X-irradiated gs^+ cells in infectious form, since extracts of the irradiated cells enhanced production of infectious RSV(O) by transformed quail cells (which normally produce noninfectious virus since they are always gs^-).

H. Hanafusa *et al.* (1970) observed independently that certain chick embryos contain genetic material, termed chf (chick cell-associated helper factor), which is indispensable for the production of infectious RSV(O). All embryos containing chf were also found to be gs^+. Moreover, after infection of chf^+ cells with avian leukosis or Rous sarcoma virus, chf was recovered by incorporation into infectious virus, called RAV-60, which shared the properties of oncornaviruses. Hence, superinfection activated the production of a cryptic helper virus that formerly behaved as a cellular gene. On the other hand, RSV(O) produced by chf^+ cells shared its envelope characteristics with RAV-60, which suggests that RSV(O) is defective for some envelope protein(s) that it can obtain, by phenotypic mixing, from activated chf. Among the properties shared, is the unusual host range of the virus (which cannot infect most chick embryo cells, including cells with the same phenotype as the chf-carrier cells, but can infect quail cells).

Vogt and Friis (1971) similarly observed that cells of most gs^+ embryos of a certain phenotype could produce infectious RSV(O) following single infection, whereas quail embryo cells yield noninfectious progeny. Moreover, occasional gs^+ embryos were found to spontaneously release a virus, named RAV-O, which was also shown to possess the same envelope specificity as RSV(O). This suggests that there may exist diverse degrees of expression of cryptic avian oncornaviruses carried by gs^+ cells, the ultimate degree being virus production.

This spontaneous virus production further led to investigations, as in the case of temperate phages in lysogenized bacteria, of whether physical agents (X rays, UV light) or chemical carcinogens and mutagens might activate gs^+ cells to release infectious virus [as was already suggested by the data of Weiss and Payne (1971)] and, also, whether these same agents might activate virus production in gs^- cells, if these cells carried repressed cryptic viruses.

Indeed, as shown by Weiss *et al.* (1971), induction of virus able to infect pheasant cells (which are susceptible to infection by cryptic viruses) and help in the production of infectious RSV(O) was observed following treatment of cells of the gs^+ lines of Weiss and Payne and of Vogt

with X rays (1000 R), UV light, mitomycin C (1 mg/ml for 18 hour), urethane, 20-methylcholantrene, and 4-nitroquinoline-1-oxide. In the cells of Weiss, no spontaneous virus induction was observed, and, in those of Vogt, spontaneous induction was much less frequent than induction by the agents tested. Moreover, virus induction was similarly observed following treatment with methylcholantrene or nitroquinoline oxide, in the cells of embryos of red jungle fowl obtained from Malaya, which suggests that cryptic oncornaviruses are carried by wild fowl as well.

On the other hand, virus induction was also observed in cultures of embryos of the gs^- line of Weiss and Payne, following treatment with X rays or methylcholantrene. The incidence of induction by X rays in gs^- cells was less than that in gs^+ cells, but the difference observed is of doubtful significance, since the probability of induction in gs^+ cells was approximately 5×10^{-6} per cell, against 2×10^{-6} in gs^- cells. In the experiments with methylcholantrene, it was also observed that most, if not all, treated cultures of gs^- cells acquired the gs antigen. These results show that gs^- cells can also carry a cryptic virus and that, therefore, the chromosomal locus that controls the presence of natural gs antigen in chicken cells does not represent the viral genome itself, but regulates its expression. Results of induction experiments with gs^+ and gs^- cells are summarized in Table IV.

Confirmation that gs^- cells can also carry a cryptic virus has been brought by Hanafusa *et al.* (1972) who have shown that RAV-60 can be recovered from cultures of gs^- cells by superinfection with a leukosis virus in the presence of quail embryo cells (which serve as an agrandizer system).

Weiss *et al.* (1973) have further shown that in gs^+ cells chf can give a high incidence of genetic recombinants with superinfecting nondefective strains of RSV. These recombinants, as in the case of double infection with RSV and other avian leukosis viruses, have the transformation markers of RSV and the host-range marker of the cryptic virus. They have not been found following infection of gs^- cells, nor after infection of gs^+ cells with Bryan strain RSV. This latter observation suggests that the perpetuation of defective strains of avian tumor viruses depends on their inability to undergo genetic recombination.

The fact that gs^- embryos cells give rise to no recombinants, although they also carry chf, may be due to the fact that no viral RNA is produced in these cells (as detected by hybridization with virus-specific DNA made *in vitro* by reverse transcriptase) (Leong *et al.*, 1972; Hayward and Hanafusa, 1973), whereas 50–80% of the cryptic viral genome is transcribed in gs^+ cells (Hayward and Hanafusa, 1973; Bishop *et al.*,

TABLE IV

Induction of Cryptic Viruses in Fowl and Mouse Cells[a]

Cells	Inducing agents[b]									
	None	X ray	UV	Mit. C	Urethane	MC	DMBA	4-NQ-1-O	IUdR	BUdR
CEF										
gs^+	+	+	+	+	+	+		+	(+)	(+)
gs^-	−	+				+				
MEF										
AKR	+	+		+		+	+		+	+
BALB/c	+		−	−					+	+

[a] Data taken from Weiss *et al.* (1971), for CEF; from Rowe *et al.* (1972a), for MEF AKR^-; and from Aaronson *et al.* (1971) and Aaronson (1971) for BALB/c. CEF, chick embryo fibroblasts; MEF, mouse embryo fibroblasts.

[b] UV, ultraviolet light: Mit.C, mitomycin C; MC, methylcholanthrene; DMBA, dimethylbenzanthracene; 4-NQ-1-O, 4-Nitroquinoline-1-oxide; IUdR, 4-iododeoxyuridine; BUdR, 4-bromodeoxyuridine. In cells in which spontaneous induction is observed, inducing agents increase to various degrees the level of this induction.

1973). Indeed, as suggested by Weiss *et al.* (1973), the initial event in genotypic mixing could be the formation of "heterozygous" particles including nontransforming RNA subunits of chf (or leukosis virus, in double infection) and transforming subunits of RSV. During the next replicative cycle, the proviral DNA subunits transcribed by the reverse transcriptase could undergo crossing-over, if they contain homologous sequences, either before or during integration with the host genome, which is itself presumably a cross-over event. High-frequency recombination could also result from copy choice during RNA-directed DNA synthesis in a heterozygote particle. Thus, genetic mixing of avian tumor viruses would result both from reassortment of the RNA subunits of the viral genome and from true genetic recombination.

B. Murine Viruses

A large body of information has been accumulated to indicate that genetic determinants of murine leukemia viruses (MLV) are present in all strains of mice, and probably all individual mice (Todaro and Huebner, 1972; Gilden and Orozlan, 1972; Rowe *et al.*, 1972a). In some inbred lines of mice, such as AKR, this can be demonstrated by tests for infectious virus (Hartley *et al.*, 1969; Rowe and Pincus, 1972) and by tests for viral antigens (Aoki *et al.*, 1968); in other strains, such as the NIH Swiss strain, no infectious virus could ever be found, but the gs antigen of the MuLV group was regularly demonstrated at concentrations equivalent to, and even greater than, those found in other strains of mice where infectious viruses were demonstrated (Gilden and Orozlan, 1972). In C3H/He mice, virus has occasionally been found in embryos and was detected in 40% of adult spleens, whereas in BALB/c mice, no virus has been recovered from fetuses or weanlings, but the spleens of more than half the mice over 6 months have been found to yield virus (Hartley *et al.*, 1969). These last observations suggest that the virus may exist in cryptic form in the embryos and become activated only later in life. However, this does not demonstrate that the cryptic virus is carried as a cellular gene, as is chf. Notably, in reciprocal matings between AKR and BALB/c mice, virus recovery appeared to be maternally determined (Hartley *et al.*, 1969), which is classically interpreted as evidence for a cytoplasmic factor. From this viewpoint, it may be recalled that avian leukosis viruses are also transmitted by the egg, but not by the sperm (Rubin *et al.*, 1962).

More demonstrative evidence that murine leukemia viruses may be carried as cellular genes has been provided by studies of cloned lines of mouse cells producing no virus, but from which virus could be recovered

by treatment with physical and chemical agents in the same way as cryptic avian viruses.

Rowe *et al.*, (1971, 1972a) have isolated three virus-negative lines of AKR embryo cells and single-cell clones from each of two of these lines and shown that all these lines and their clonal derivatives retained the capacity to initiate synthesis of MuLV. In some of the clonal lines, spontaneous virus production was observed with a frequency of the order of 10^{-8} to 10^{-9}. In addition, a number of procedures were found to increase notably the frequency of virus activation. Thus, a level of activation of 10^{-5} to 10^{-6} was observed after transformation with SV40, X irradiation, and treatment with mitomycin C, 3-methylcholantrene, and 7,12-dimethylbenzanthracene. A much higher level of activation (10^{-2} to 10^{-3}) was found after exposure of the cells for 24 to 30 hours to the halogenated pyrimidines, 5-iododeoxyuridine (IUdR), 5-bromodeoxyuridine (BUdR), and 4-iododeoxycytidine. All the clonal and subclonal AKR lines have been induced by these drugs.

The number of AKR cells in which the viral gene expression was activated by IUdR or BUdR was found, furthermore, to be much larger than that of the virus-producing cells, since up to 5% of the treated cells showed virus antigens by immunofluorescent staining on the third day after addition of the drug. This might be due to the fact that cells are damaged by the drug, or that induction is abortive in most cells. Another possibility raised by the authors is that production of viral proteins may be a prerequisite for the activation of infectious virus, as in the case of induction of prophages (Gottesman and Weissberg, 1971), and that the induction step is inefficient.

Subsequent investigations (Teich *et al.*, 1973) have shown that incorporation of IUdR or BUdR into cellular DNA plays a vital role in the activation process. If DNA synthesis is blocked or decreased by cytosine arabinoside or serum depletion during the halogenated pyrimidine treatment, the proportion of cells activated is decreased. When analogue incorporation is blocked by thymidine, no induction occurs. However, when analogue incorporation is increased by FUdR the proportion of induced cells is increased. Induction is also enhanced when cells containing analogue-substituted DNA are irradiated with visible light or X rays, which produce breaks in the DNA.

Aaronson *et al.* (1971) similarly recovered MuLV from 10 non-virus-producing clones and subclones of BALB/c mouse embryo cells, untransformed or transformed by SV40 or X irradiation, following exposure to BUdR. In one subclone, no virus production was previously detected for over 500 cell generations. In most cases, however, virus production declined after an initial peak. This is presumably due to the fact that the induced virus poorly infected BALB/c cells, whereas it ef-

ficiently infected NIH/3T3 cells. This inability of the induced virus to infect cells of the same phenotype as the carrier cells relates the induced MuLV from BALB/c cells to chf.

In the same way, murine sarcoma virus (MSV) was recovered from 2 clones of MSV-transformed BALB/c cells and one clone of MSV-transformed rat cells (Aaronson *et al.*, 1971; Aaronson, 1971). In these experiments, induction was observed following treatment with BUdR, IUdR and iododeoxycytidine, but not following treatment with UV, mitomycin C, or various analogues (8-azaguanine, nitrisoguanidine, and 6-thioguanine). In all cases, activation of MSV production was related to the activation of a cryptic murine or rat leukemia virus. Klement *et al.* (1971, 1972) have also reported the rescue of MSV from MSV-transformed rat cells following activation of an endogenous rat virus by BUdR.

Genetic investigations, by Rowe *et al.* (1972b) have shown that the ability of the cells of AKR mice to synthesize infectious virus (MuLV), spontaneously or after induction with IUdR, depends on two independently segregating loci that may be integrated viral genetic determinants. One of these loci has been shown to be in linkage group I (corresponding to the chromosome 7).

More recently, Aaronson and Stephenson (1973) have shown, similarly, that two independent genetic loci control the induction of C type viruses in BALB/c cells. However, whereas the two loci in AKR cells appear to control the synthesis of the same virus, the loci of the BALB/c cells each control the production of a distinct virus, since the viruses corresponding to each locus can be distinguished biologically by their host range: One grows preferentially in NIH Swiss embryo cells, whereas the other one grows poorly in NIH Swiss or BALB/c cells, but replicates well in rat cells. In this case, also, it was assumed that the loci may be viral genetic determinants integrated with the DNA of the carrier cell.

Induction of a cryptic oncornavirus in hamster cells by IUdR has also been reported (Todaro and Huebner, 1972).

On the other hand, C type particles containing RNA hybridizing specifically with DNA made *in vitro* by the reverse transcriptase of the avian sarcoma virus B77 have been recovered from nonpermissive rat cells transformed by this virus, following cultivation in arginine-less medium (Kotler *et al.*, 1972). However the viral RNA was only 35 S RNA, which could be due to some defect in its structure.

Another particular group of murine viruses that also appears to exist in proviral form in the germ line, and, therefore, in all the somatic cells of the host, is that of murine mammary tumor viruses (MTV's). Integrated and biological studies carried out chiefly by Bentvelzen *et al.*

(1972) suggest that each mouse contains genetic information for some MTV in its genome, i.e., a proviral DNA. This provirus is usually not transcribed, but can be switched on by physical or chemical carcinogens, such as X rays or urethane. The induced derepression is usually incomplete, so that oncogenes can be transcribed and translated without concomitant virus production. Switching on of the genes that control the synthesis of viral coat protein and permit virus production appears to be triggered by age.

On the other hand, the MTV's are transmitted genetically only in those mouse strains to which they are indigenous. Introduction into other strains leads to transfer by the mother's milk.

Table IV summarizes the main induction data obtained with normal chicken and mouse embryo fibroblasts, MTV's excluded.

V. Discussion

As can be seen, an impressive body of evidence has accumulated these last years in favor of the Temin model for the replication and persistence of the genome of oncornaviruses in permissive and nonpermissive cells infected with exogenous viruses. In parallel, data have accumulated showing that in some vertebrate species, and maybe in all species, cryptic oncornaviruses can be carried as genes in the germ cells and, therefore, in all the cells of the host.

Thus, one may distinguish two classes of oncornaviruses. The first comprises viruses that are apparently not transmitted by the germ line and whose genome, therefore, integrates only with the genome of infected somatic cells, transformed or untransformed. Most viruses of this class appear to be pathogenic, and its best known representatives are the avian sarcoma and leukosis viruses that have no known mode of natural transmission, or are transmitted only horizontally or maternally. Some murine sarcoma and leukosis viruses also appear to belong to this class, as well as some feline sarcoma and leukemia viruses. The second class comprises viruses that are carried as genes in the germ line and are transmitted to the offspring by both parents. Some of these viruses, notably the MTV's and certain murine leukemia viruses, have been shown to be pathogenic, but a number produce no known disease and are classified among oncornaviruses only on taxonomic grounds.

However, the two classes presumably overlap, since, as suggested by the data of Bentvelzen *et al.*, viruses of the second class can become members of the first when they leave their original host strain. This also suggests that members of the first class may all have originated from the second class. Conversely it is plausible that members of the first class

may become members of the second class if they become integrated in germ line cells.

The three major lines of evidence for the replication of the genome of oncornaviruses by the intermediary of a DNA provirus and for the persistence of the genome in the form of this provirus in both permissive and nonpermissive cells are (1) the hybridization data, which show that virus-specific DNA covalently linked to cellular DNA of high molecular weight is present in virus-infected cells; (2) the finding of virus-associated reverse transcriptase in all oncornaviruses, except a few noninfectious mutants; and (3) the finding of infectious viral DNA in cells infected and transformed with avian oncornaviruses, and the association of this DNA with fractions of cellular DNA of high molecular weight.

The mechanism by which the viral DNA copies made after infection become integrated with cellular DNA remains unknown. However, recombination observed between chf and superinfecting avian oncornaviruses suggests that, as in the case of temperate bacteriophages (cf. Hayes, 1968; Borek and Ryan, 1973), integration of the viral DNA may be a recombinational event occurring at sites of the cell DNA which are homologous to some site of the viral DNA. There may be a limited number of such sites (one to three), as in most bacterial systems, but it is also possible that all chromosomes may carry one or more redundant sites.

If integration of the DNA provirus of oncornaviruses takes place by a mechanism similar to that of temperate phages, it is also plausible that the viral DNA copies become circular before integrating, as in the Campbell model that presumably applies to papovaviruses as well as to temperate phages. Therefore, the search for circular virus-specific DNA appearing in infected cells in the early period of infection might be rewarding.

Another feature of the integration of temperate phages is the production of a virus-coded protein repressor that switches off the transcription of all the phage genes, except that which codes for the repressor, and insures subsequent immunity to superinfection with homologous or closely related phages. This repressor, which presumably acts on the operator sites that control the transcription of the viral structural genes, may also exist in nonpermissive cells transformed by papovaviruses (Cassingena and Tournier, 1968; Cramer, 1969).

However, there is certainly no repressor in the case of oncornaviruses integrated in permissive cells which continuously produce a large number of virions, and the only situations in which a repressor might exist are the nonpermissive relationship or the cryptic relationship. In the case of the nonpermissive relationship, encountered in the case of

mammalian cells infected with nondefective avian sarcoma viruses, it is also possible that activation of virus production, which is observed generally only after fusion with a permissive (chicken) cell, requires an "antirepressor" present in the permissive cells, as suggested by the data of Cassingena *et al.* (1969) in the case of nonpermissive cells transformed by SV40. Indeed, studies with inhibitors of macromolecular synthesis (Vigier, 1973) suggest that, in nonpermissive hamster cells transformed by nondefective RSV, activation of virus production after fusion of the nonpermissive cell with a permissive (chicken) cell depends on unblocking of DNA transcription, which fits with a repressor–antirepressor model. Yet, other possibilities exist for explaining nonpermissiveness: for instance, that some virus component is coded for by the cell, and present only in the permissive cells.

Moreover, even assuming that no viral component is coded for by the host cell, the existence of a repressor is not a must for the integration of the provirus in nonpermissive cells. Indeed, here again, the panoply of phage–bacteria relationships provides other alternatives. Notably, integration of the provirus might be stable in the absence of a repressor, in the same way as in cryptic lysogens found in various bacterial systems (cf. Krizsanovich, 1973). As is known, cryptic lysogens produce no phage, either because the prophage carries a deletion or because it is integrated in some particular way. In the first case, the rescue of the defective cryptic phage requires recombination, but in the second, spontaneous induction is observed.

This raises the question of the induction of cryptic viruses in normal cells and in nonpermissive cells transformed by exogenous viruses. In the case of cryptic oncornaviruses, the analogies with the induction of prophages are impressive. First, there exists a spontaneous low level of induction of cryptic viruses in some, but not all, clones of carrier cells. Second, most of the agents that induce cryptic oncornaviruses also induce prophages (cf. Borek and Ryan, 1973). This is notably the case for UV light, X rays, and mitomycin C, or chemical carcinogens, but it is also the case for halogenated pyrimidines, since IUdR induces bacterial lysogens (Price *et al.*, 1964). IUdR is also a potent inducer of cryptic Epstein-Barr virus (Gerber, 1972; Hampar *et al.*, 1972) and increases the rescue of SV40 from transformed nonpermissive cells fused with permissive cells (Watkins, 1970). In the latter case, interference of the analogue with repressor synthesis or formation of a less active mutant protein have been suggested.

Arginine deprivation has not, to our knowledge, been shown to induce bacterial lysogens; but it has been found to induce the production of Epstein-Barr virus in cultured Burkitt cells (Henle and Henle, 1969). Here

again, it was suggested that induction might be raised by reduction of some intracellular inhibitor(s).

However, the mechanism of induction of oncornaviruses and of temperate phages might not be identical. Indeed, induction of prophages, and presumably also of oncodnaviruses, appears to be a two-step process involving, first, the derepression of the transcription of the provirus and, second, the excision and the replication of viral DNA, whereas induction of oncornaviruses may only be a one-step process, since only transcription of the viral DNA is required for virus production. In favor of this view is the observation that induction of RSV from nonpermissive hamster cells fused with permissive chicken cells requires no replication of DNA, but only the synthesis of DNA-dependent RNA and protein synthesis (Vigier, 1973).

Moreover, it is possible that the mechanism of virus activation in transformed nonpermissive cells and cells carrying cryptic viruses are not the same. This is suggested, first, by the fact that RSV-transformed hamster cells could not be activated by inducers of cryptic chicken oncornaviruses, i.e., X rays, UV light, mitomycin C, and BUdR (Vigier, 1972, 1973), and, second, by the finding that the percentage of cells that can be induced to produce virus is much higher in the case of the most inducible clones of RSV-transformed cells (it can even attain 100%) (Machala *et al.*, 1970; Vigier, 1973) than in that of cells carrying cryptic viruses.

Thus, many problems remain to be solved to complete the picture of the relationship of oncornaviruses with permissive and nonpermissive cells. Before this is done, it does not appear urgent to decide which of the theories, if any, proposed to explain their origin (Huebner and Todaro, 1969; Todaro and Huebner, 1972; Temin, 1971a,b) is correct.

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CHAPTER 10

Comparison and Evolution of RNA Tumor Virus Components

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I. Introduction

In seeking to understand how the current relationships in any virus family are a reflection of their past history, it is most important to have techniques available that can indicate with some precision rankings of relatedness. It is thus possible to compare such rankings with known host-species relatedness, obviously known with much more assurance, and then draw inferences regarding the coevolution or lack of coevolution of virus and host. The type C viruses provide a relatively unique opportunity to study this question because of several important biological features. Most important are observations indicating that, at least for chickens and mice, the viral genome is inherited as part of the normal

cellular genome (Huebner and Todaro, 1969; Todaro and Huebner, 1972). Along with evidence of viral structural proteins in embryonic tissues in the absence of infectious virus (Huebner *et al.*, 1970; Payne and Chubb, 1968), current evidence includes induction of virus from cells by halogenated pyrimidines (Aaronson *et al.*, 1971; Klement *et al.*, 1971; Lowy *et al.*, 1971; Rowe *et al.*, 1972b), viral nucleic acid sequences (both RNA and DNA) in noninfected cells (Baluda and Nayak, 1970; Gelb *et al.*, 1971; Harel *et al.*, 1967; Hayward and Hanafusa, 1973; Nieman, 1972; Parks *et al.*, 1973a; Varmus *et al.*, 1972; Wilson and Bauer, 1968), and most impressively the ability to map the mouse viral genome in specific linkage groups (Rowe, 1972; Rowe and Hartley, 1972; Rowe *et al.*, 1972a). In the AKR mouse, at least two such viral gene linkages have been reported (Rowe, 1972; Rowe and Hartley, 1972; Taylor *et al.*, 1971). If this is true of type C viruses in other species, one could theoretically treat the evolution of their genetic material exactly as any other gene complex. Thus, measurements of relatedness could be done on selected gene products, i.e., structural proteins, or on the nucleic acids themselves by interviral hybridization. Such studies, while yielding interesting and provocative data, are still in early stages, and current conclusions may require future modification. As they proceed, more precise information regarding virus–host relationships are becoming available. These have a strong bearing on interpretation of evolutionary studies, and for this reason this chapter should be considered as a basis for future refinements.

II. Materials and Methods

A. Viruses

The following viruses, all obtained from supernatant fluid of chronically infected cultures were used: four mouse strains, Rauscher (RLV), AKR, New Zealand black (NZB) (Lerner *et al.*, 1972), and wild mouse (Gardner *et al.*, 1971); three rat virus strains, the virus originally termed MSV(O) (Ting, 1968), now MSV(RaLV) (Oroszlan *et al.*, 1972a), and two endogenous rat viruses generously provided by Dr. V. Klement (Children's Hospital, Los Angeles; 1973); three cat strains, the Theilen feline lymphoma virus (Theilen *et al.*, 1969), a helper virus derived from the Gardner strain of feline sarcoma virus (Gardner *et al.*, 1970; Sarma *et al.*, 1971), and the RD 114 virus (McAllister *et al.*, 1972), now known to be of cat origin; two primate viruses obtained from a woolly monkey (Theilen *et al.*, 1971) and gibbon ape (Kawakami *et al.*, 1972). The

mouse viruses were all grown in mouse cells, the rat viruses in rat cells, the cat viruses in cat and human cells, and the primate viruses in human cells; the latter generously provided by Parks and Scolnick (Parks *et al.*, 1973b).

The culture supernatants were filtered through Millipore filter RA (1.2 μm) and chilled in ice. Viruses were harvested from the filtrates by a continuous flow isopycnic centrifugation either with the model K, Mark II centrifuge (Electro-Nucleonics), or with the CF-32 rotor in a Spinco L-350 centrifuge. The virus band was localized by absorbance at 260 nm and complement-fixation tests for the appropriate virus group-specific antigen. The pooled virus fractions were diluted with 0.01*M* Tris-HCl, pH 7.4, 0.1*M* NaCl, 0.001*M* EDTA (TSE) to less than 20% sucrose, and rebanded in a Ti-15 zonal rotor (Beckman Instruments) through 25% sucrose layer onto 50% sucrose cushion in TSE for 16 hours at 4°C. The virus band was collected and used either directly or after repelleting by a centrifugation to remove sucrose. When necessary, the virus was further banded through a linear sucrose gradient of 20 to 60% sucrose in TSE in a Spinco SW 25-1 head.

B. Virus Group-Specific Antigens

The immunochemical studies described herein have focused on one major viral structural protein commonly known as the group-specific (gs) antigen. Because there are several proteins that could be classified as gs antigens, we will refer to this protein as p30, based on molecular weight estimates from either polyacrylamide gels or agarose gel columns developed by guanidine-HCl. All type C viruses show a p30 ($\pm$20%), which accounts for approximately 30% of the virion protein mass (Oroszlan *et al.*, 1970).

The p30's were purified by isoelectric focusing according to the technique of Vesterberg and Swensson (1966). Sucrose gradient purified virus was disrupted by combined Tween 80-ether treatment or more recently with sodium dodecyl sulfate (SDS). In the latter case, SDS was removed prior to focusing by passage through a Dowex 1×2 ion-exchange resin. Tween-ether disrupted virus was treated with RNase and DNase, and clarified at 100,000 *g* for 1 hour before focusing. Columns of 110 ml were made with either sucrose or urea gradients to afford stabilization of the gradient. Individual fractions from gradients (selected on the basis of pI values from preliminary runs) were tested for purity using polyacrylamide gel electrophoresis and antigenic reactivity with appropriate antisera. Purified materials were used to prepare specific antisera, mainly in guinea pigs using a sequence of inoculations with Freund's complete adjuvant.

Sera were tested in gel diffusion using microplates coated with 0.8% agarose gel in 0.01 *M* Tris-HCl buffer with 0.9% NaCl and Merthiolate (1/10,000) as a preservative. The microtechnique of complement fixation (Sever, 1962) was also used to evaluate reagents. The various reagents used in this particular study were available from previous work summarized in Gilden and Oroszlan (1972), Oroszlan *et al.* (1972b), and Gilden *et al.* (1973).

Quantitative microcomplement-fixation (C'F) tests were made according to the technique of Wasserman and Levine (1961), with an adjustment of final volume to 3 ml. Optical densities were determined on the Beckman acta spectrophotometer at 413 nm. In brief, a series of antigen dilutions was mixed with graded antibody concentrations and 1.1–1.2 50% C' units at 4°C for 18 hours. Sensitized red blood cells were added and the tubes incubated for a further 30 minutes at 37°C before clarification at 2,000 rpm (clinical centrifuge) for 10 minutes. Controls included incubations with antibody + C', antigen + C', and both reactants in the absence of C'. In general, 100% lysis gave A_{413} readings of ~2.0, and controls contributed minimal absorbing material (<0.1 units) at the dilutions used (absence of C'); neither antigens nor antisera showed significant anti-complementary effects. The percent fixation at the peak of the C'F curves obtained at varying antibody concentrations was plotted against the log serum dilution to obtain curves describing the homologous and heterologous reactions (Sarich and Wilson, 1966). All cross-reacting systems gave parallel slopes (based on statistical analysis), thus permitting calculations of the relationships between the antigens compared. The term "index of dissimilarity" is used to indicate the factor by which the serum concentration must be raised to give equivalent C'F curves with homologous and heterologous antigens. In practice, this factor was calculated from the 50% points on the percent maximum fixation versus log serum concentration plots. The immunological distance is defined as 100 times log of the index of dissimilarity, which is a most useful calculation because of direct relationship to sequence differences in well-studied proteins (Prager and Wilson, 1971; Hanke *et al.*, 1973). The approximation of five immunological distance units equals 1% sequence difference is assumed to hold for the p30's based on the above quoted references.

C. Viral RNA

The high molecular weight 70 S RNA was prepared from the purified virus (1–5 mg/ml protein) by lysing with 1% SDS and sedimenting

through a linear sucrose gradient of 15 to 30% (w/v) in TSE containing 0.01% SDS at 40,000 rpm for 3 hours in a Beckman SW 41 rotor. The 70 S region was pooled, deproteinized with 80% phenol (pH 5.2) three times at room temperature, and precipitated repeatedly with 2 volumes of ethanol in 0.2 *M* NaCl. The concentration was estimated by the absorbance at 260 nm.

D. Viral DNA Probes

In order to obtain quantitative hybridization, it was found necessary to use single-stranded DNA probes purified from the viral 70 S RNA hybrids in the endogenous reaction mixture. The endogenous polymerase reaction mixture consisted of 100 m*M* glycine buffer, pH 8.3, 5 m*M* $MgCl_2$, 50 m*M* NaCl, 10 m*M* DTT, 50 μM each of deoxyribonucleoside triphosphates, one ^{3}H-labeled deoxyribonucleoside triphosphate at 10 μM (usually 45–50 Ci/mmole [^{3}H]TTP), 0.01% NP-40, and purified virus (0.2–1 mg/ml protein). The mixture was incubated at 37°C for 60 minutes, and the reaction was stopped by the addition of 1% SDS. The mixture was directly layered on a 10-ml column of 15–30% (w/v) linear sucrose gradient in TSE containing 0.01% SDS, and centrifuged at 40,000 rpm for 3 hours in a SW 41 rotor. Fractions of about 0.6 ml were collected from the bottom, and aliquots were removed to measure acid precipitable counts. The 70 S regions were pooled, made to 0.5% SDS and 0.01 *M* EDTA, and then extracted with an equal volume of 80% phenol, pH 7.8, for 5 minutes at room temperature, followed by chloroform:isoamyl alcohol (24:1) for an additional 5 minutes. The organic phase was discarded after a centrifugation, and the extraction was repeated two more times with chloroform. The aqueous phase was finally removed, treated with 0.2 *N* NaOH at 80°C for 20 minutes to destroy RNA, chilled in ice, and neutralized with 1 *N* HCl. The DNA was precipitated with two volumes of ethanol in the presence of 100 μg of yeast RNA repeatedly, and finally dissolved in 0.1 × SSC. The concentration of DNA was estimated from the acid-precipitable counts of the preparation, on the basis of the specific activity of ^{3}H-labeled substrate.

When such products were analyzed for complexity, they gave results similar to that obtained using single-stranded probes prepared in the presence of actinomycin D. The latter preparations represented (in our experiments) at least 60% of the viral genome based on protection of RNA from RNase digestion. Specific activity of the DNA products averaged 85×10^3 dpm/ng.

E. Single-Stranded Specific Nuclease

The S-1 enzyme from *Aspergillus oryzae* was prepared from α-amylase, according to the method of Sutton (1971). The specific activity was 69,300 U/A_{260} unit. The enzyme preparation was stored frozen at −20°C, and diluted 10-fold before use with 25% glycerol in 0.01 *M* sodium phosphate, pH 6.8.

F. Hybridization

Hybridization was carried out in 100 μl of 0.3 *M* NaCl, 0.02 *M* Tris-HCl, pH 7.2, 0.001 *M* EDTA, and 0.1% SDS. The mixture of a given amount of RNA and 500 to 1000 cpm of [^{3}H]DNA probe in 0.1 × SSC was boiled for 3 minutes, chilled in ice, and brought to 0.3 *M* NaCl; the mixtures were then incubated at 67°C for a given time.

G. Assay by S1 Enzyme

Hybridized mixtures (usually 100 μl) were diluted to 2.5 ml to give final concentrations of 0.034 *M* sodium acetate, pH 4.5, 0.14 m*M* $ZnCl_2$, 0.18 *M* NaCl, and 10 μg/ml of denatured calf thymus DNA. Twenty-five microliters of enzyme was added (26×10^3 U/ml) and the mixtures were incubated at 45°C for 120 minutes. DNA resistant to nuclease digestion was precipitated with cold 10% TCA in the presence of 50 μg calf thymus DNA, collected onto HA Millipore filters, washed with 6% TCA, and counted in Liquifluor-toluene using a Beckman LS-250 liquid scintillation system.

III. Results

In considering any familial grouping, the key assumption is common ancestry. While for viruses this is inferred from common morphology and biological behavior, there are certain groupings in which precise relationships must at present be made on faith. For example, the relationship of viruses such as Visna, Mason-Pfizer, and mouse MTV to type C particles cannot be assessed in quantitative terms, yet the presence of reverse transcriptase and approximate morphologic similarity of virions presents a strong case for common ancestry however remote.

Thus, our present discussion is of necessity restricted to viruses whose familial relationship is based on clear evidence of genetic homology. This is approached first by the demonstration that the ultrastructural morphology and polypeptide composition of type C particles are similar (Gilden and Oroszlan, 1972; Oroszlan *et al.*, 1971a; Nowinski *et al.*, 1972); secondly by the demonstration of a common antigenic determinant designated gs-3 (Geering *et al.*, 1970) associated with the p30 virion structural protein (Gilden and Oroszlan, 1972; Gilden *et al.*, 1971; Oroszlan *et al.*, 1971b; Parks and Scolnick, 1972); and, most definitively, assumed from the immunological results, amino acid sequence homology of this particular protein (Oroszlan *et al.*, 1972c, 1973). The viruses listed in Table I fulfill these criteria and thus permit attempts at quantitative comparison. Specifically omitted from this discussion are type C particles described for cattle (Ferrer *et al.*, 1971) and guinea pigs (Hsiung, 1972). Polypeptide patterns of these viruses and determinants cross-reactive with the viruses of Table I have not yet been presented or demonstrated; thus there is insufficient data at present to include these viruses in any meaningful comparison. If indeed type C viruses are found that lack cross-reactive determinants with the viruses of Table I, this would only prove the fallibility of grouping based on morphology alone. We should stress here that groupings such as "type C" are man-made abstractions, and arguments of differences are only in-

TABLE I

Relationship of Type C Viruses to Their Species of Origin

Virus designation[a]	Species of isolation	Induction[b]	V-RNA[c]	V-DNA[d]
MuLV	Mouse	+	+	+
RaLV	Rat	+	+	+
FeLV	Cat	(−)	−	+
RD 114	Cat	+	+	+
HaLV	Hamster	N.D.[e]	N.D.	N.D.
GaLV	Gibbon ape	(−)	?	−
WoLV	Woolly monkey	(−)	?	−

[a] The designation "LV" does not necessarily indicate pathogenicity.

[b] From cells of the homologous species using halogenated pyrimidines.

[c] Virus-specific RNA in normal cells using DNA product of reverse transcriptase.

[d] Virus-specific DNA in cellular DNA or normal cells using DNA product of reverse transcriptase.

[e] Not determined.

dicators of variability that are difficult for men to accommodate in simple classification schemes. Yet, we should insist that positive or negative assertions be based on use of appropriate reagents and test systems which permit definitive conclusions.

Once the ability to make comparisons is granted, a second major problem of critical significance to any attempt to discuss evolutionary relationships arises. Simply stated this is, how do we know that the viruses chosen for analysis are representative of the species from which they were isolated? Until recently this seemed to pose no difficulties. Multiple isolates were available from four mammalian species: mice, rats, cats, and hamsters. All isolates from within a species shared a common group-specific antigenic reactivity associated with the major internal protein of the virion (Figs. 1 and 2). This protein is referred to as "p30" based on approximate molecular weight of 30,000 in the various viruses. The p30's from viruses of these four types also shared a common determinant originally referred to as gs-3 (Figs. 2 and 3); thus, it seemed logical that this pattern would be followed for new isolates

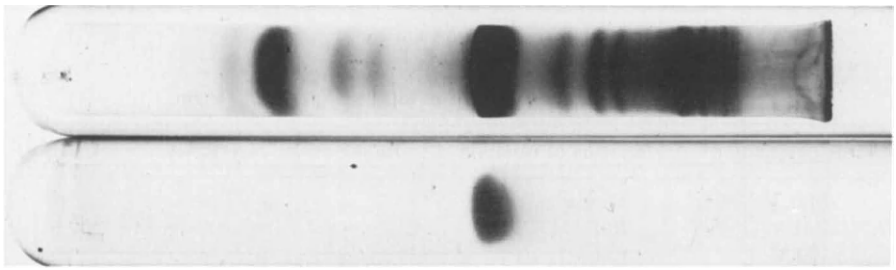

FIG. 1. Polyacrylamide gel electrophoresis of mouse virus and its major group-specific antigen purified by isoelectric focusing. The isoelectric focusing technique permits the isolation of the homologous protein from each virus, although with distinct isoelectric points. Such purified material was used for antiserum production and the various assays described in this report. Disrupted virus and purified gs protein were analyzed in SDS-polyacrylamide gels (10% acrylamide–0.1% SDS–0.1 *M* sodium phosphate buffer, pH 7.0). Before electrophoresis, the virus and gs protein were treated with SDS (1%), urea (6 *M*), and 2-mercaptoethanol at 37°C for 2 hours. The samples were then dialyzed against 0.01 *M* phosphate buffer (pH 7.0) containing 0.1% SDS–0.5 *M* urea–0.1% 2-mercaptoethanol. Electrophoresis (from right to left) was for 5 hours at 8 mA per gel; gels were stained with Coomassie blue. The purified gs protein (lower pattern) has a calculated molecular weight of 31,000, and is thus p30. From Gilden and Oroszlan (1972).

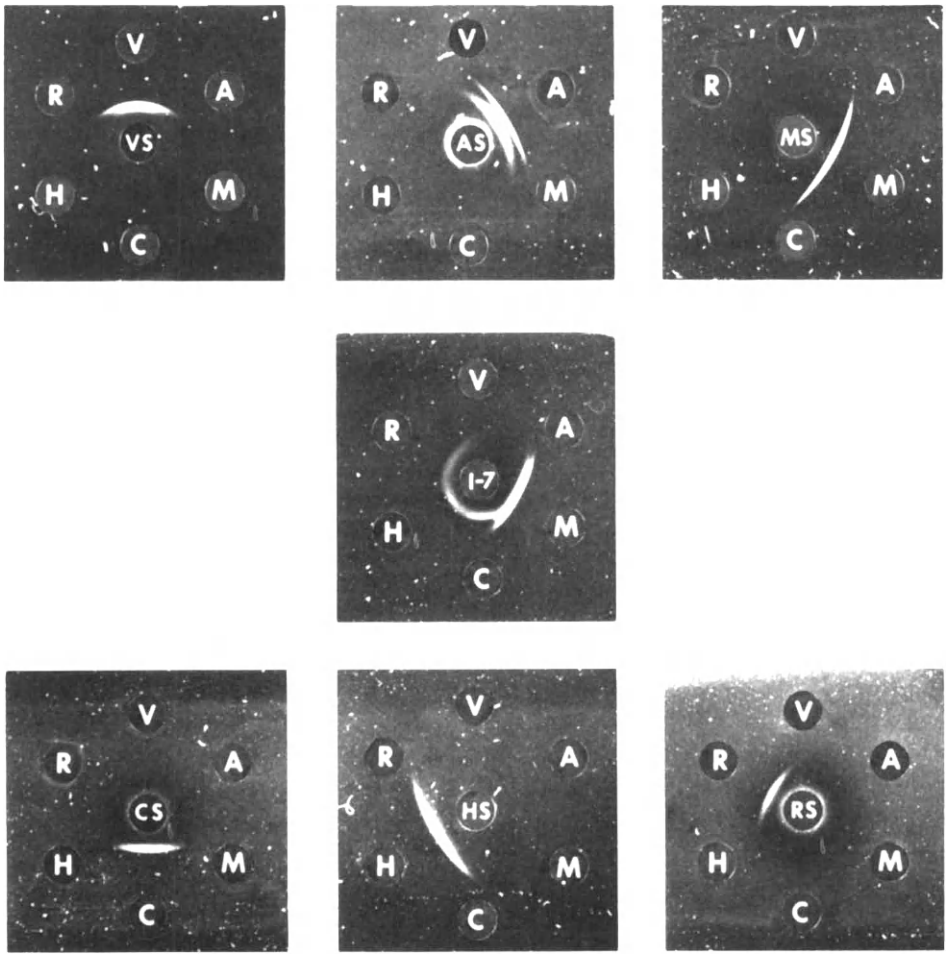

FIG. 2. Gel diffusion analyses of C type viruses from six species. Outer wells contain C type viral concentrates disrupted by Tween 80-ether treatment. V, viper; A, avian; M, mouse; C, cat; H, hamster; R, rat. Inner wells contain immune serum prepared against purified gs protein (p30) in guinea pigs with two exceptions: AS is hamster serum from animals bearing tumors induced by Rous sarcoma virus; 1–7 is sera from rats immunized with rat tumors induced by and containing murine sarcoma virus. The various guinea pig antisera (VS, MS, CS, HS, and RS) are reactive only with the homologous virus giving a single precipitin band. The hamster anti-avian serum detects species-specific determinants located on two separable polypeptides, while 1–7 detects the gs-3 determinant shared by mammalian C type viruses, in addition to MuLV species-specific determinants. From Gilden and Oroszlan (1972).

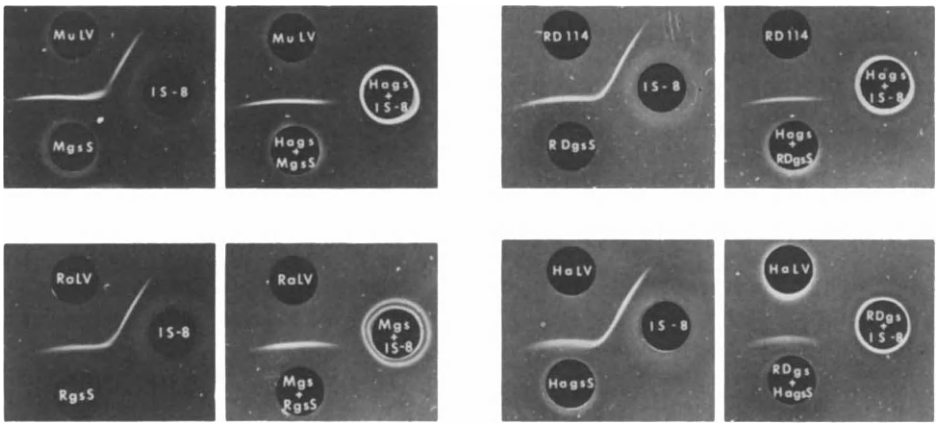

FIG. 3. Coexistence of species-specific and cross-reactive determinants on the same molecule. Purified p30's are placed in the upper well, guinea pig antisera to the homologous p30 in the lower well, and a goat anti-FeLV gs serum (1S-8) as indicated. The left-hand panel of each set shows a line of identity formed between 1S-8 and specific guinea pig sera. The right-hand panel shows the same pattern; however, the sera are first absorbed (*in situ*) with heterologous p30. As shown the 1S-8 reaction is completely eliminated by each p30 used while the guinea pig sera are unaffected. Thus two different reactions must be involved and the identity reaction depends on both being present on the same molecule. This figure also shows the presence of gs-3 determinants on RD 114 p30. As shown (upper right) the hamster p30 removes the 1S-8 reaction (which is not accomplished when tested against FeLV p30) with RD 114, but does not affect the guinea pig serum reaction with RD 114. This serum does not react in CF or gel assays with FeLV.

within this family. More recently, three new prototype viruses have been studied in detail. Findings with these viruses have raised many questions and provided important insights into the question raised above. The first case considered, that of the virus designated RD 114, is particularly instructive. This virus appeared in a human cell line after passage through a fetal cat (McAllister *et al.*, 1972). The virus differs from the well-studied feline type C viruses (generally abbreviated FeLV or FeSV) in host range, envelope antigen, p30 antigens, amino acid sequence of p30 (McAllister *et al.*, 1972; Oroszlan *et al.*, 1972c, 1973), and antigenic reactivities associated with other structural proteins, including the virion reverse transcriptase (Scolnick *et al.*, 1972; Long *et al.*, 1973). This virus could be included in the type C family based on the decisive criteria of polypeptide pattern, gs-3 reactivity (Fig. 3), and p30 homologous sequences. This raised the possibility that the virus could have been derived from human cells. The possibility that RD 114 was represent-

ative of a second type C cat family was also raised (McAllister *et al.*, 1972), although this would obviously have broken the pattern of one p30 group per species. The issue was decisively resolved by the following experiments. A virus immunologically similar to RD 114 was induced in virus-free cat cells by treatment with IUdR (Livingston and Todaro, 1973; Sarma *et al.*, 1973). This virus, as does RD 114, replicates preferentially in human or other primate cells. RNA homologous to RD 114 DNA, produced by reverse transcriptase reactions, was detected in each of a small series of normal and tumored cat cells and tissues (Okabe *et al.*, 1973a,c). The DNA probe was nonreactive with cellular RNA from all other species tested (Table II). RD 114 DNA copies were detected in similar fashion in cellular DNA only from cats (Baluda and Roy-Burman, 1973; Ruprecht *et al.*, 1973). In a limited series of normal and tumor tissues from cats, an equivalent number of DNA copies were found in each sample despite wide variability in RNA copies (H. Okabe, R. V. Gilden, and M. Hatanaka, unpublished data). The above three types of evidence give proof that RD 114 is a genetically inherited virus of cats which ordinarily is not expressed at the protein level in cat cells. In similar experiments, FeLV RNA was only detected in FeLV producing cells, however, preliminary findings also indicate the presence of DNA copies in cat cell DNA (H. Okabe and M. Hatanaka, unpublished data). Thus, there may be two inherited type C viruses in cats. The manner of the isolation of RD 114, in one sense a rare experimental situation, thus opens the question of multiple type C virus p30 groups in other species. Studies with this virus also point up important criteria for identifying species of origin of new virus isolates (induction, hybridization). The extent of current information using this set of criteria is also shown in Table I, and indicates that the known viruses of mice and rats fulfill the criteria of inherited viruses, while the hamster viruses have not yet been adequately tested. Current results also indicate that the viruses isolated from a gibbon ape and a woolly monkey, respectively, may fail to meet the important criteria of being represented in cellular DNA Scolnick *et al.*, 1974). This is of obvious significance in attempting to derive evolutionary relationships in host species and in the quest for human type C viral genetic information. The above discussion has emphasized difficulties for the evolutionist which were not suspected one year ago. From this perspective, any current conclusions are necessarily tentative.

Obviously, an unbiased view of interviral relationships can be made without *a priori* knowledge of the precise origin of the viruses. These data can then be tested against the hypothesis of coevolution of host and viral genomes (Gilden and Oroszlan, 1972) predictable from the inheri-

TABLE II

Hybridization of Various Cellular RNA's with the DNA Product of RD 114 Virus[a]

Cell	Origin	Type of cell	Virus	Amount of RNA hybridization[b]		
				μg	cpm	%
RD 114	Human	Rhabdomyosarcoma	RD 114	33.3	498	90.7
RD	Human	Rhabdomyosarcoma		229	0	0
HeLa	Human	Carcinoma		238	0	0
Raji	Human	Lymphoblastoid	(EBV)	286	0	0
SV-80	Human	Fibroblast	Gibbon type C	201	0	0
AGM	Monkey	Normal liver		197	0	0
Rhesus	Monkey	Normal liver		147	0	0
Calf	Cattle	Normal spleen		605	0	0
Crandell	Feline	Normal kidney cell		140	476	86.7
(CC)	Feline	Normal kidney cell		104	340	73.4
Cat Embryo	Feline	Normal fibroblast		74.3	285	61.6
FL-74	Feline	Leukemic cell	FeLV	103	519	94.5
Cat	Feline	Normal liver		275	251	45.7
Cat	Feline	Normal spleen		209	226	41.2
Cat	Feline	Normal kidney		193	200	36.4
Cat tumor	Feline	Hepatoma (69024)		124	447	81.4
Cat tumor	Feline	Hepatoma (69203)		143	463	84.3
XC	Rat	RSV-transformed	RSV-NP	118	2	0.4
Rat	Rat	Normal kidney		441	0	0
C3H	Mouse	Normal fibroblast		68.4	1	0.2
C3H(MSV)	Mouse	MSV-transformed	MSV/RLV	84.0	0	0
Mouse	Mouse	Normal kidney		276	0	0
HT-1	Hamster	MSV-transformed	(MSV)-NP	150	1	0.2
Rat tRNA				75.0	3	0.5
Poly(A)				20.0	0	0
Poly(G)				20.0	0	0
RD 114, 70 S viral RNA				0.09	549	100

[a] From Okabe *et al.* (1973c).

[b] Each RNA preparation was hybridized with the [^{3}H]DNA product of RD 114 (660 cpm). Hybridized counts were obtained as TCA-precipitable counts after S-1 nuclease digestion, and given as a net cpm after subtracting the background (59 cpm). The percent hybridization was normalized, taking the cpm hybridized with RD 114 70 S RNA as 100%.

tance theory. Our studies have focused on techniques of immunochemistry and molecular hybridization, both of which allow ranking according to relatedness. Using the quantitative complement-fixation test, which has provided valuable information of relationships of a variety of proteins, we have compared the p30's of the viruses listed in Table I. Of initial interest were the two primate viruses isolated from species with an estimated divergence time of 40×10^6 years. Using two sera prepared against purified p30 of WoLV (Fig. 4), a series of C'F

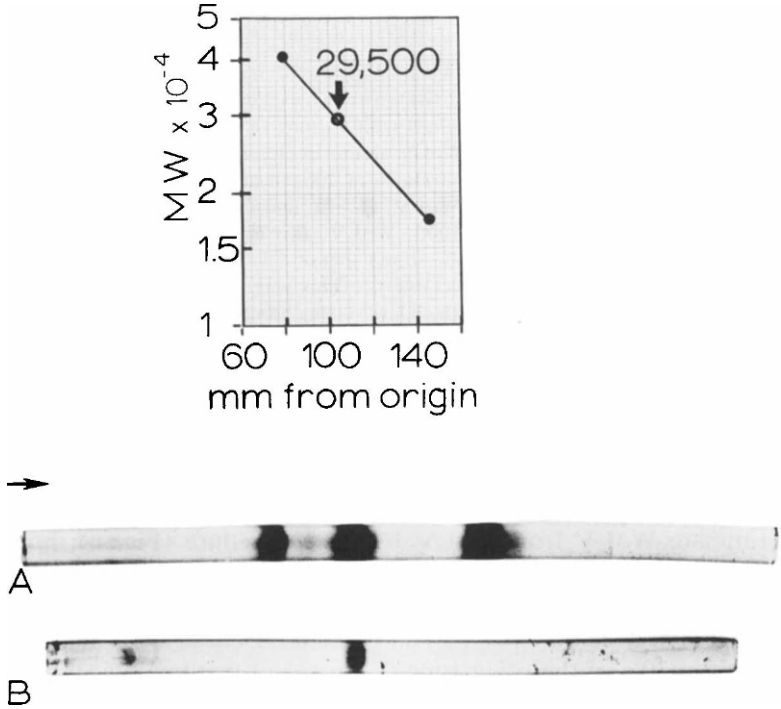

FIG. 4. Polyacrylamide gel electrophoresis of purified woolly monkey type C virus gs protein. Woolly gs protein purified by isoelectric focusing was analyzed in the presence (A) and absence (B) of alcohol dehydrogenase (MW 41,000), and myoglobin (MW 17,200) using neutral sodium dodecyl sulfate-polyacrylamide gels. Electrophoresis in 10% acrylamide gels was for 5 hours at 8 mA per gel, after which gels were stained with Coomassie blue. The molecular weight (~29,500) was calculated from measurement from the origin to the center of each stained zone using a greater enlargement than shown in the figure. The plot is log MW $\times 10^{-4}$ versus distance moved from the origin. The arrow indicates direction of migration. Differences in lengths of the gels are from variable shrinkage during destaining.

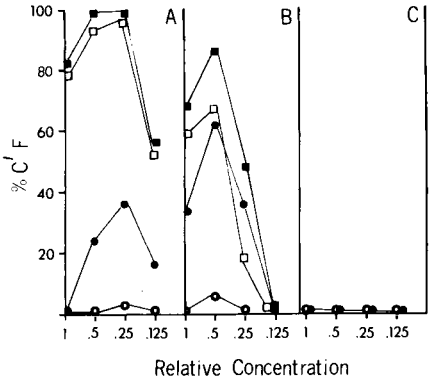

FIG. 5. Quantitative C'F curves of anti-WoLV p30 versus WoLV and GaLV p30's. Reactions of serum 1 (A) and serum 2 (B) are shown at 2 dilutions in each case. These are representative of multiple curves obtained with varying serum concentrations. The higher dilution indicates the ability of anti-WoLV serum to clearly differentiate between WoLV and GaLV p30. The absolute concentrations of antigen at maximal fixation was ~0.016 μg/ml. Serum dilutions: (A) 1:300: WoLV, ■—■, and GaLV, □—□; 1:5000: WoLV, ●—●, and GaLV, ○—○. (B) 1:1000: WoLV, ■—■, and GaLV, □—□; 1:2000: WoLV, ●—●, and GaLV, ○—○. (C) shows that at a 1:100 dilution of serum 1 neither MuLV nor RD 114 p30's fixed detectable C'. This was true of p30 concentrations extending 10-fold higher than shown in the figure. Using homologous serum both of the latter proteins were readtive in a concentration range similar to WoLV and GaLV.

curves were prepared (Fig. 5). When the peak of the C'F curves were plotted versus the log of the serum dilution, parallel slopes were obtained as expected (Sarich and Wilson, 1966). It was clearly possible to discriminate WoLV from GaLV by this procedure (Fig. 6); however, the average immunological distance between these proteins (defined in Section II) was calculated to be 12, leading to an estimate of ~97–98% relatedness between the two p30's (Table III). These same sera failed to react with p30's of the other type C viruses at immunological distances of 170 (Fig. 5), thus giving estimates of <65% relatedness from primate to nonprimate viruses. This high degree of relationship among primate viruses is supported by hybridization studies (Okabe *et al.*, 1973b), showing ~70–80% relatedness (Fig. 7), results of antigenic assays for the virion reverse transcriptase (Scolnick *et al.*, 1972), and alternate methods of assay of p30 (Fig. 8). The guinea pig antisera used in this study were prepared in the same fashion as for other species p30, which have always been highly species-specific (Fig. 2). Thus, in reciprocal micro-C'F tests, guinea pig sera for other p30's gave no reaction with WoLV and GaLV.

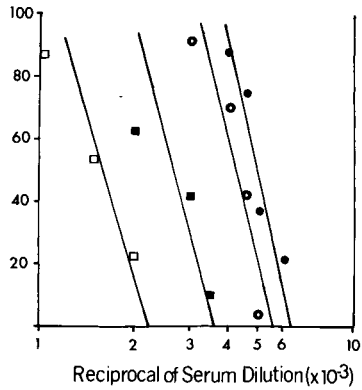

FIG. 6. Quantitative differences between WoLV and GaLV p30 determined by quantitative C'F. The data of Figure 4 (and similar data) were plotted as indicated. The curves were drawn by eye; however, they fit statistical analysis and showed no difference in calculated slopes. The 50% C'F points were used for the calculations presented in Table III.

In intra-MuLV comparisons (Okabe *et al.*, 1973b), again both hybridization (Fig. 7) and micro-C'F permit discrimination among virus strains with relatedness estimates of ~98% for p30. In this case, NH_2-terminal sequence analysis has shown one residue difference out of 25 compared between a wild mouse isolate and the Rauscher laboratory strain. Pep-

TABLE III

Estimated Relationship between WoLV and GaLV by Quantitative C'F

Sera	Serum dilution	Index of dissimilarity[a]	Immunological distance[b]	Estimated % sequence difference[c]
Anti-VoLV No. 1				
WoLV	4900			
GaLV	4200	1.17	6.8	1.4
MuLV		>49	>169	>34
RD 114		>49	>169	>34
Anti-WoLV No. 2				
WoLV	2700			
GaLV	1700	1.58	20	4
Sera 1 and 2 pooled according to titer		1.32	12	2.4

[a] Ratio serum dilutions giving 50% C'F.

[b] 100 × log index of dissimilarity.

[c] Five immunological distance units = 1% sequence difference.

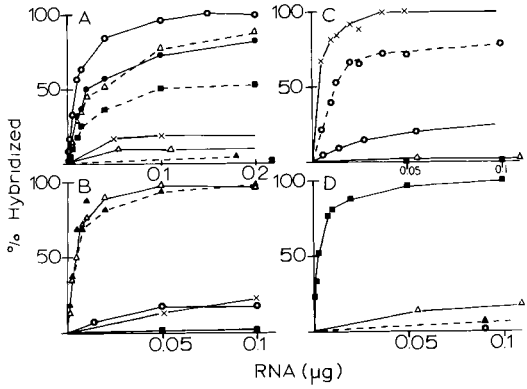

FIG. 7. Hybridization between [^{3}H]DNA probes and 70 S RNA from various viruses. [^{3}H]DNA probes of RLV (A), FeLV (B), gibbon virus (C), and RD 114 (D) purified as 70 S complexes from the endogenous reaction mixture of each virus were hybridized with increasing amounts of various 70 S RNA preparations in 0.3 *M* NaCl, 0.02 *M* Tris-HCl, pH 7.2, 0.001 *M* EDTA, 0.1% SDS (total 100 μl) at 67°C for 16 to 20 hours. The radioactivity in the hybrids formed was determined by precipitation with 10% TCA after S-1 nuclease digestion and plotted relative to the hybridization attained with each homologous DNA probe at saturation. The homologous ratios of hybridized to input counts were RLV, 713/1349 (0.72); gibbon, 1390/1638 (0.84); RD 114, 1077/1256 (0.86). The background obtained without added RNA was subtracted before each calculation (54, 53, 66, and 94 cpm, respectively). The 70 S RNA's used were RLV, ○—○; FeLV, △—△; gibbon, ×—×; RD 114, ■—■; NZB, ●—●; WML, △---△; AKR, ■---■; FeSV, ▲---▲; and woolly, ○---○. The data in this figure show cross-reactivity but distinction between MuLV strains (A) and FeLV strains (B), complete distinction of conventional FeLV's and RD 114 in reciprocal assays (B,D), and the close relationship between WoLV and GaLV (C). From Okabe *et al.* (1973b).

tide mapping has shown only one to two differences out of approximately 35 spots, thus it is reasonable to conclude that the estimates of MuLV p30 relationship of greater than 95% are firm. When tested with heterologous p30's, the guinea pig anti-MuLV gs serum were nonreactive; thus, it was not possible to provide species ranking with such sera. In sensitive radioimmunoassays it is known that such sera will show cross-reactions; however, here again with titers 20- to 100-fold less than homologous titers. Thus, in attempts at virus ranking, the guinea pig sera point up the striking resemblance between WoLV and GaLV and, in turn, the lack of any close relationship between the two most closely related species with known endogenous viruses, the mouse and rat. This is emphasized by the results of hybridization experiments in which $<5\%$ cross-reactivity was obtained between MuLV and RaLV (Table IV). This lack of cross-hybridization has made it possible to provide evidence

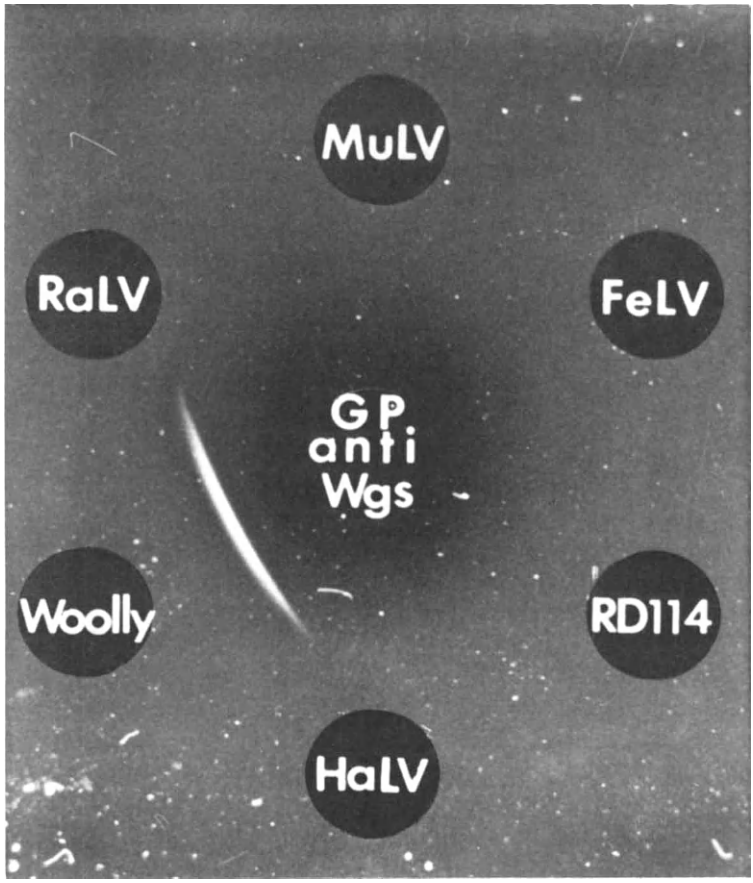

FIG. 8. Specificity of guinea pig anti-WoLV p30. Purified p30's placed in circumferential wells and antiserum in the center well. In keeping with C'F results, the anti-WoLV serum is nonreactive with other p30's with the exception of GaLV (not shown) (Gilden *et al.*, 1973). Thus this serum with the exception of GaLV behaves as the guinea pig antisera shown in Figs. 2 and 3.

for the hybrid nature of a rat-specific virus isolated from a rat tumor induced by murine sarcoma virus (Okabe *et al.*, 1973b).

In contrast to the guinea pig antisera, which are highly "species"-specific, certain goat anti-p30 sera have proven highly cross-reactive in immunoassays. One such anti-MuLV serum was used in attempts to rank heterologous p30's. This serum clearly distinguishes MuLV p30 from the others, and at the same time indicates approximately equal im-

TABLE IV

Specificity of Mouse and Rat Viruses Determined by Molecular Hybridization

Viral RNA	Viral [^{3}H]DNA transcripts		
	M-MSV	NRK-9	RPL
AKR	50[a]	<5	<5
RLV	75	<5	<5
M-MSV	100	<5	<5
NRK-9	<5	100	
RPL	<5		100

[a] Percent hybridization at saturation concentrations of viral RNA normalized to 100% based on the homologous virus. Actual values of homologous reactions were 80–85% of input radioactivity. NRK-9 and RPL are rat viruses from the O.M. and Lewis strains, while AKR, RLV, and M-MSV are mouse laboratory strains.

munological distances from MuLV to the other proteins with calculated sequence relatedness of approximately 90%. The goat serum reaction was clearly demonstrable in gel diffusion assays where spurring among heterologous p30's was not seen. Thus, when using cross-reactive or specific antisera prepared against MuLV p30, there was no indication of preferential reactions with RaLV p30 compared to p30 from any other virus. A second goat antiserum prepared against FeLV p30 indicates certain qualitative differences among p30's indicative of intrafamilial relationships. Thus, in gel diffusion, this particular serum is nonreactive with the two "primate" virus p30's (it is, however, positive in more sensitive radioimmunoassays) (Gilden *et al.*, 1974), suggesting that these viruses have diverged from the others prior to their own divergence. In radioimmunoassays using the FeLV p30 serum and ^{125}I-labeled MuLV p30, complete inhibition of precipitation can be obtained with unlabeled MuLV, RaLV, HaLV, and FeLV p30's. The p30 of primate viruses and RD 114 gave only partial inhibition under the same conditions (Parks and Scolnick, 1972). The possibility of a close relationship between RD 114 and the primate viruses is negated by lack of cross-reactivity of p30's using guinea pig antisera and the distinction of these viruses by reciprocal reverse transcriptase antisera. In such assays, three subfamilies are indicated in a manner consistent with the p30 results taken collectively (Scolnick *et al.*, 1972). In this view, RD 114 has also diverged from the other subprimate viruses prior to their own divergence.

Initial sequence data on p30's (Oroszlan *et al.*, 1973) do show several interesting features (Fig. 9) that can perhaps explain the difference in

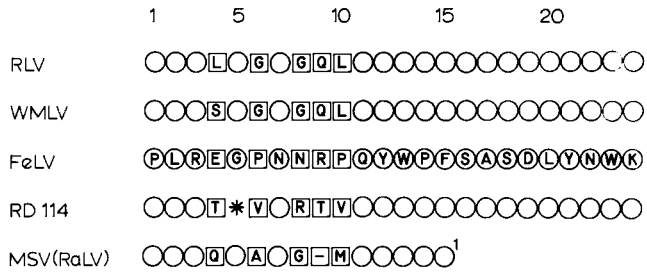

FIG. 9. NH_2-terminal sequences of p30's from several type C viruses. The first 24 positions of FeLV are indicated within the circles. Identical residues are found for the other p30's where no residue is indicated. The two mouse p30's (RLV and WMLV) differ only at position 4. The asterisk indicates the gap placed at position 5 of RD 114 to maintain homology with all other p30's. The rat sequence is completed only to position 15, and position 9 in this sequence is also uncertain at present. The key features of these results are the obvious overall sequence homology, a hypervariable region (residues 4–10), and an invariant region (residues 11–24). All residues were determined by gas chromatography thin layer chromatography, and amino acid analysis after degradation using a Beckman automated sequencer.

relatedness estimates from guinea pig and goat antisera. First, there is obvious homology among all the p30's examined, giving unqualified support to a common ancestry hypothesis. The number of differences estimated between any two nonrelated p30's appears to be approximately 5/25 residues (Table V), thus (if this were maintained) permitting a

TABLE V

Relatedness among Type C Viral p30's Based on NH_2 Terminal Sequence Analysis

Viruses	No. residues compared	No. of differences[a]	No. of base changes
AKR versus RLV	15	0	
RLV versus WMLV	19	1	1
MuLV versus FeLV	24	5	8
MuLV versus RD 114[b]	25	5	6–7
FeLV versus RD 114[b]	25	5	9
MuLV versus RaLV[c]	13	3	3–4
FeLV versus RaLV	13	4	6
RD 114 versus RaLV	13	4	5

[a] All differences thus far, occur after residue 3 and before residue 11.

[b] A gap is necessary in the RD 114 sequence, assigned to position 5, to maintain positional homology. This is not employed in the calculation.

[c] The rat comparisons suffer from uncertainty at position 9, which is a variable in other viruses.

calculation of ~80% relatedness. However, examination of the sequence indicates a large measure of uncertainty in this calculation. There is an obvious hypermutable region between positions 3 and 11, where five differences, many requiring two base changes, are recorded. From positions 11 through 25, complete homology is maintained. The sequence data thus seem to support the C'F data, showing no striking closeness between MuLV and RaLV and equidistance between MuLV and two cat viruses, FeLV and RD 114. It seems possible that the guinea pig sera are directed to variable regions of the p30 molecules, while the goat antisera may recognize more of the molecule including regions of extensive homology. The guinea pig response could thus possibly be influenced by relative immunologic unresponsiveness to shared sequences with the p30 of the currently hypothetical guinea pig type C virus. The two cat viruses also appear equidistant from one another as predicted from C'F and lack of interviral hybridization (Fig. 7). One important feature of the RD 114 sequence is the necessity of a gap, tentatively at position 5, to maintain homology. Such events are relatively rare compared to replacements and thus serve to indicate a major event in the evolution of this virus.

IV. Discussion

If one were to draw an evolutionary tree from the data currently in hand, without regard to the knowledge of species of origin, it would show WoLV and GaLV with the same relatedness as strains of MuLV; both of these viruses are more distantly related to the subprimate viruses based on polymerase and gs-3 differences, and the RD 114 virus is somewhat removed from the others based on the sequence gap (Fig. 10). This event could have occurred postdivergence in a single locus or predivergence in one or multiple structural loci. A multiple locus hypothesis would be required if FeLV was an inherited virus based on extreme difference between viruses; however, since FeLV may not be an inherited virus in cats and thus a recent epigenetic arrival in the species (from where?), this cannot be decided. In view of the close relationship of MuLV strains, the inheritance theory would certainly predict a close relationship with RaLV. At present, this is not seen, and the complete lack of cross-hybridization is striking. Is it reasonable for two species, separated by 40×10^6 years, to have almost identical viruses (woolly and gibbon), and two species separated by 10×10^6 years to have completely distinct viruses? The current evidence that WoLV and GaLV apparently are not inherited viruses in primates helps us out of this dilemma and explains our current inability to find homologous nucleic

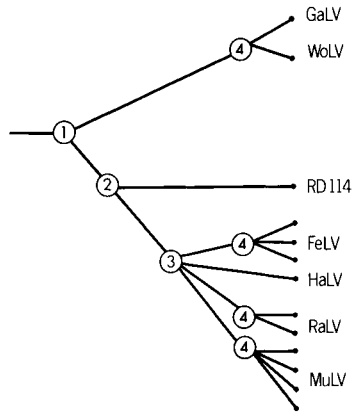

FIG. 10. Approximate relationships among mammalian type C viruses deduced from immunologic and molecular hybridization data. (1) Divergence of 3 major groups of mammalian type C viruses all of which contain a common determinant associated with p30 and show sequence homology of p30. All p30's react with a reference goat anti-MuLV in gel diffusion. (2) All p30's react in gel diffusion with goat anti-FeLV p30, which does not react with the primate virus p30's in gel diffusion. RD 114 is distinct based on antigenicity of polymerase and partial inhibition of gs-3 reaction between goat anti-FeLV and MuLV in RIA. Also gap at position 5 in p30 sequence. (3) Viruses show polymerase cross-reactivities, "identity" of gs-3 determinant in gel diffusion and RIA. (4) All viruses show specific p30 reactivities and extensive cross-hybridization between members attached to a common origin.

acid sequences in human cellular DNA. This certainly would have been required based on the inheritance theory, since gibbon and man are much more closely related than gibbon and woolly monkey. However, the question of their origin and that of FeLV becomes of paramount importance with obvious concerns for possible disease implications. Thus, a new virus with no growth restrictions may be accidently introduced in a new species, perhaps by vaccine, and these become epigenetic as opposed to a rarely seen endogenous virus. Possibilities of recombinants are thus raised as described above, which could have an extended or newly acquired oncogenic potential.

The above analysis points out the large extent of uncertainty concerning the evolution of RNA tumor viruses in mammals and virtually predicts many new findings of import not conceived at this writing.

ACKNOWLEDGMENTS

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CHAPTER 11

Genetics and Biology of Murine Leukemia Virus

J. A. McCARTER, P. K. Y. WONG, AND J. K. BALL

I. Introduction

Recently, RNA-containing viruses (Gross, 1970) have been studied intensively because they have been found to cause leukemia in animals and possibly in man. It is important to study the nature of the genomes of these viruses, the expression of the genetic information that they contain, and the regulation of its expression in cells.

There is little published information on the genetics of murine leukemia viruses and for that reason this chapter may be written prematurely. However, it is appropriate at the outset to consider the evidence and the concepts that influence the direction of this work. In this paper, we shall review our studies that have been made in the murine system

and discuss the findings in relation to those of similar studies of the avian RNA tumor viruses. The genetic evidence will be considered in terms of models based on the concept that the genome consists of segments of single-stranded RNA. We shall review the biochemical evidence on which this concept is based and conclude that biological problems of these viruses make the interpretation of data from molecular biological studies uncertain. We state our opinion that progress on the biological side of the problems is a prerequisite to progress on the molecular side. In particular, the answers to many questions concerning the nature of the genomes and their functions can come only from genetic studies of these viruses.

Studies of the genetics of the RNA-containing tumor viruses became possible with the isolation of mutants of helper-independent strains of Rous sarcoma virus (RSV) that are temperature sensitive (ts) with respect to functions required for the maintenance of the transformed state (Toyoshima and Vogt, 1969; Goldé, 1970; Martin, 1970; Biquard and Vigier, 1970; Kawai and Hanafusa, 1971; Bader, 1972; Wyke, 1973). Mutants have been described that are defective in replication as well as transformation (Friis *et al.*, 1971; Wyke and Linial, 1973), and other mutants are defective in replication only (Friis and Hunter, 1973). The availability of ts mutants of the avian sarcoma viruses has allowed genetic analysis of these viruses to begin (Vogt, 1972), and the results of these studies will be discussed later in this chapter.

In the murine system, ts mutants of murine leukemia viruses (MuLV) have been isolated for the Kirsten strain (Stephenson *et al.*, 1972), the Rauscher strain (J. R. Stephenson, personal communication) and the Moloney strain (Wong *et al.*, 1973). Scolnick *et al.* (1972) have also reported the isolation of ts mutants of the Kirsten strain of murine sarcoma virus (MuSV). Genetic studies are just beginning.

II. Isolation of ts Mutants of MuLV

These murine leukemia viruses, unlike the murine sarcoma virus, replicate in cells without inducing alterations in cellular growth or morphology (for an exception, see Hackett and Sylvester, 1972). It is therefore necessary to use markers other than transformation to look for mutants of MuLV.

Stephenson *et al.* (1972) isolated a large number of clones of MuLV from mutagenized stocks. For this purpose a specially designed microtiter system was employed. The ts properties of the clones of virus were assessed at permissive and nonpermissive temperatures using a modifi-

cation of the XC-cell assay (Klement *et al.*, 1969) or an assay based on measurement of the activity of the enzyme, reverse transcriptase (Scolnick *et al.*, 1970). More than 3000 individual cultures were screened for the production of virus, and 1000 were found to be producers. From these, 9 clones of ts leukemia viruses were isolated.

We (Wong *et al.*, 1973) have described the isolation of spontaneous ts mutants of Moloney murine leukemia virus (M-MuLV) using a selective procedure and ordinary equipment available in any tissue culture laboratory. The selection procedure uses S+L− cells (Bassin *et al.*, 1970), which are transformed murine cells that carry the genome of the murine sarcoma virus (S+) but not, apparently, that of murine leukemia virus (L−). The cells produce noninfectious particles (Bassin *et al.*, 1971) but on infection of S+L− cells with MuLV, they produce MuLV and MuSV of the S+L− type (Peebles *et al.*, 1971). Although these cells produce a mixture of viruses, infectious MuLV particles are readily separated by cloning procedures as described below.

The usefulness of S+L− cells in the selective procedures for ts mutants of MuLV depends on the fact that S+L− cells infected with wild-type (wt) MuLV become rounded up and are easily dislodged from the culture dish by shaking, whereas uninfected cells, or those infected with ts MuLV and kept at the nonpermissive temperature (39°C), remain firmly attached. Cells that are infected and can be shaken off the dish kept at 39°C are discarded. When the dish is then placed at 32°C, further rounding up of cells occurs, virus is released, and this virus is found to be enriched with respect to ts mutants. The selective procedure can be repeated further to enrich the stock, but the ratio of titers of virus assayed at 32° and 39°C reaches a limit of approximately 10. The explanation for this is probably that most of the mutants are very leaky.

The clonal isolation of the mutants is accomplished in the following way: Cells of an established culture of mouse fibroblasts (Wright *et al.*, 1967) are infected in single cell suspension with leukemia virus, are plated sparsely in petri dishes and grown at the permissive temperature, 32°C. When the cells have grown into small colonies, an excess of XC cells (Klement *et al.*, 1969) is added to the dish. After a further period of incubation, the clones that are infected with MuLV are identified: those infected with MuLV have syncytia of XC cells adjacent to them (XC^+), whereas uninfected colonies do not (XC^-). The proportion infected adheres very closely to Poisson's distribution over a wide range of values, and this permits one to calculate the titer of infectious particles in the preparation that was used to infect the mouse cells. This fact also means that an infected colony can arise from a single infectious unit infecting a single cell. It is thus possible to isolate the progeny of single, infectious viral units, either by picking medium above an infected colony

or by picking the chronically infected cells of the colony. Fortunately cross-infection between colonies does not present serious problems for three reasons: (1) Cells are infected at low multiplicities of infection so that double infections will be rare; (2) low numbers of cells are plated so that the colonies will be well separated; (3) in the absence of polyanions (DEAE-dextran) mouse cells are resistant to infection by MuLV (Vogt, 1967). Furthermore, the medium can be replaced after the addition of XC cells by medium containing agar, and this further ensures that the material picked from one XC^+ clone will not be contaminated with material from another.

Two procedures are used to isolate infectious viruses. In the first, medium solidified with agar is picked above an XC^+ clone and is used to infect fresh mouse cells to raise a stock at 32°C. The viral titer in the medium reaches a maximum at a time coinciding with the development of confluency. The titer is then determined by the XC assay described above—performed at 32° and at 39°C. The ratio of titers for wt virus is close to 1. Of 42 clones of virus picked from a nonenriched stock, none had 32°C:39°C ratios significantly different from 1. Of 100 clones picked from S+L− enriched stock, 29 had 32°C:39°C ratios $\geqq 5$. Of these 29, 23 were too leaky to be worthy of further study. Three had 32°C:39°C ratios of 100 or more. Our genetic studies have so far been confined to these three mutants designated M-MuLV ts1, ts2, and ts3.

In the second procedure, the cells of an XC^+ clone are picked and grown. XC cells are picked as well as the mouse cells, so a mixed culture develops. When the cultures have grown enough to be divided, some cells are placed at 32°C, others at 39°C, and the cultures are examined for the presence of syncytia. Cells chronically infected with ts MuLV produce syncytia at 32°C but not at 39°C. Of course, there is a chance that the observed ts defect is cellular rather than viral, but the distinction can be made by harvesting virus from the chronically infected cells and examining its properties. It might be thought that virus defective in some transiently required early function would not be detected by this cell cloning procedure, but ts1, which we now know is defective in an early function, can be isolated by this procedure because it is defective in inducing XC syncytia at 39°C.

One of the benefits derived from the cell-cloning method is that the virus is isolated after a single infection. When virus is isolated from a plaque or a focus of infection, or is grown up by infecting an excess of fresh cells, opportunities are presented for there to be multiple rounds of infection from cell to cell and multiple infections of individual cells by different viral particles. Thus alterations in the genome may be favored. This has been clearly demonstrated by us in our studies of a helper-in-

dependent form of the murine sarcoma virus (Ball *et al.*, 1973). When attempts were made to grow up this competent MuSV by infecting an excess of mouse cells, the result was a mixture consisting almost exclusively of MuLV and defective MuSV. On the other hand, if the competent MuSV was clone-purified after a single infection, and the resulting infected cells were grown up as a culture of chronically infected cells, the integrity of the competent MuSV was retained. Such cultures can be used for the production of the virus on a large scale, and segregation does not take place until new cells are infected.

The behavior of this form of murine sarcoma virus suggests that it is a single infectious entity and that it is the murine counterpart of the helper-independent avian sarcoma viruses. Vogt (1971a) has reported that the latter viruses segregate nontransforming ("transformation-defective") viruses on passage through chick embryo cells. It has been suggested that the transformation-defective viruses arise by the deletion of a portion of the genome of the sarcoma viruses. To explain the behavior of the murine sarcoma virus, we suggest that in each complete replicative cycle involving formation of DNA provirus there is a finite probability that a defective virion will be produced, either replication-defective (defective MuSV) or transformation-defective (MuLV). It is important, therefore, to minimize the generation of defective viruses by minimizing the number of complete replicative cycles of the virus. The importance of avoiding cycles of infection in the purification of these viruses may not have been recognized previously. Work in progress should answer the question whether heterozygotes (mixed genotypes of different viruses) can also be preserved in this way so that they can be studied.

III. Complementation by ts Mutants of MuLV

Complementation is recognized to have occurred when two viruses, mutant in different cistrons and infecting the same cell, assist one another to replicate, i.e. the amount of virus produced by a culture with a mixed infection at the nonpermissive temperature is greater than that produced by the single infections. Unfortunately, the ts mutants with which we have to work produce some virus at the nonpermissive temperature, and this leakiness increases with increased multiplicity of infection. It may, therefore, be difficult to distinguish real complementation from apparent complementation resulting from the nonlinear dependence on multiplicity of infection. For this reason we have calculated complementation values by comparing the amount of virus produced by

the culture with a mixed infection with that of the *greater* of the singly infected controls (Wong *et al.*, 1972).

For the three ts mutants mentioned above, mixed infection with ts1 and ts2 did not give yields of virus higher than the single infections, i.e., the complementation value was not significantly different from 1. On the other hand, mixed infection with ts1 and ts3, or with ts2 and ts3, gave complementation values of approximately 3, significantly different from 1. Although the complementation values were low, they did allow ts1 and ts2 to be put, tentatively, in one group and ts3 in another (Wong and McCarter, 1973). As shown below, the biological properties of ts1 and ts2 show that they are defective in a different function than ts3.

IV. Characterization of ts Mutants of MuLV

Growth curves for ts1, ts2, and ts3 in freshly infected cells kept at 32°C were similar to the growth curve for wt virus, i.e., viral titer reached a plateau at about 10^8 infectious units per ml at 4–5 days after infection. The viral titers reached by wt virus at 39°C were the same as at 32°C, but for ts1 and ts2 the titers were reduced about 40-fold and for ts3 about 500-fold.

When such cultures were shifted from 32°C to 39°C, a culture of ts1 or ts2 continued to produce virus at about the same level as it was producing at 32°C at the time of shift. In contrast, the production of ts3 was markedly inhibited within 1 hour after shifting the culture from 32°C to 39°C.

A probable explanation for the behavior of ts1 and ts2 is as follows: The experiments with freshly infected cultures involved a situation in which, at the multiplicities employed, not all cells were infected at the start of the experiment. (It will be remembered that there is a multiplicity-dependent leakiness that forces us to use low multiplicities of infection to get large differences in titers.) Thus, as the cells grew, there were new rounds of infection. For mutants defective in an early function having to do with the establishment of the infection, an early and perhaps transient period of permissiveness would be required. If the culture should be shifted to the nonpermissive temperature during this period, then only those cells in which the infection has become established would continue to produce virus after the shift. Mutants ts1 and ts2 are probably defective in some function that is required prior to the establishment of a permanent infection. The defect in ts3, however, is in a late function, hence shifting the temperature from 32°C to 39°C caused an inhibition of the production of virus.

The difficulty that not all cells were infected at the start of the experi-

ment was overcome by using cloned cells chronically infected with ts viruses. After an initial period of 8 days at 32°C, XC^+ clones were picked and grown at 32°C and at 39°C. For ts1 the production of virus at 39°C was 0.7 of that at 32°C, whereas the corresponding figure for ts3 was 0.005. That is, after an initial period of permissiveness, the ts1 defect was largely overcome, but for ts3 it was not. These data also suggest that the ts1 defect is in an early function, while that of ts3 is in a late function.

Cell cultures infected with ts1 and ts3 were maintained at 39°C, then shifted to 32°C. Cells infected with ts3 released large quantities of virus almost immediately after down shift (100-fold increase in titer in 2 hours), whereas the production of ts1 continued more or less unchanged. Furthermore, the release of ts3 for the first hour or so occurred unabated even in the presence of actinomycin D and of cycloheximide at concentrations that inhibited the release of wild type and ts1. The ts3 defect, therefore, is in some process in viral replication that comes *after* the requirement for synthesis of RNA and protein, whereas that of ts1 is much earlier in the replicative cycle.

Electron micrographs of cells infected with ts3 and held at 39°C show (Fig. 1A) virus presumably arrested in the process of budding, having very unusual morphology. At 32°C, or some hours after shifting a culture from 39°C to 32°C, the morphology of the budding virus is normal (Fig. 1B). The nature of the biochemical defect of ts3 is unknown, but it is clearly in a function that plays some part in budding. These are the only ts mutants that we have so far studied with regard to function. All of the experiments described in this section are reported in detail by Wong and McCarter (1974).

V. Genetic Recombination in MuLV

We have shown that genetically stable wild-type recombinants are produced with high frequency (8%) in pairwise crosses between ts mutants belonging to two different complementation groups (Wong and McCarter, 1973). The recombination frequencies are 200 to 800 times greater than the frequencies of spontaneous reversion of these ts mutants to wt. For mutants in the same complementation group, the recombination frequency was too low to be detected; however, ts1 and ts2 might be independent isolates of one mutant because they were isolated from the same stock.

To perform these experiments, cells were infected with the ts mutants in all pairwise crosses and the cells were grown at 32°C. The viral har-

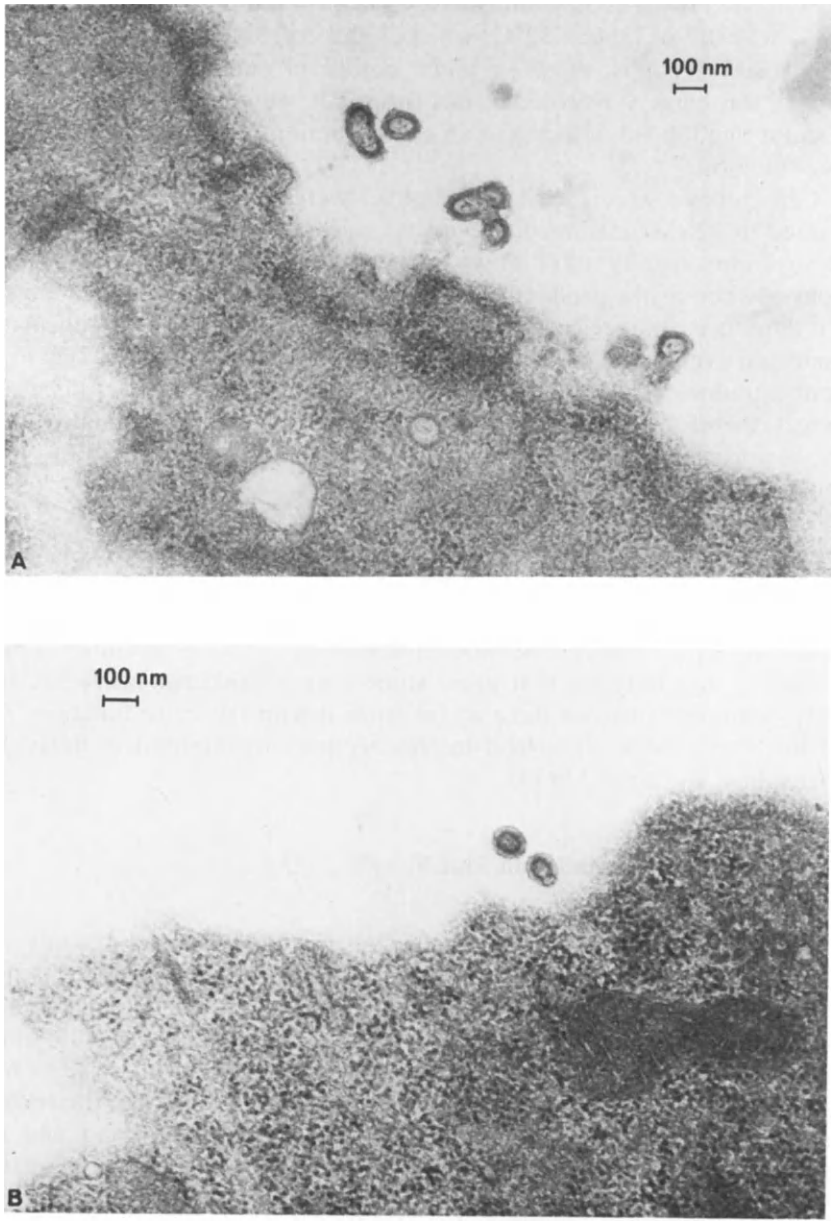

FIG. 1. Electron micrograph of mouse cell chronically infected with M-MuLV-ts3. (A) Kept at 39°C, (B) 2 hours after shifting from 39°C to 32°C.

vests from these cultures and the parental cultures were then assayed using the XC assay at 32°C and at 39°C. The data allowed the recombination frequencies for the production of wt virus to be calculated. However, identification of virions of the wild type rests not only on such calculations but also on the clonal isolation of the viral progeny of a cross as described below (Wong and McCarter, 1973).

Seventeen 32°XC$^+$ clones were obtained from a cross between ts1 and ts3. The virus from these clones when assayed at 32°C and at 39°C showed 15 of the clones to be producing ts virus (32°C:39°C = 100), 1 to be wild type (32°C:39°C = 1), and 1 to be neither truly wild type nor ts (32°C:39°C = 3–10 assayed on different occasions). The incidence of wild-type virus agreed with that obtained from the titration data (Fig. 2A).

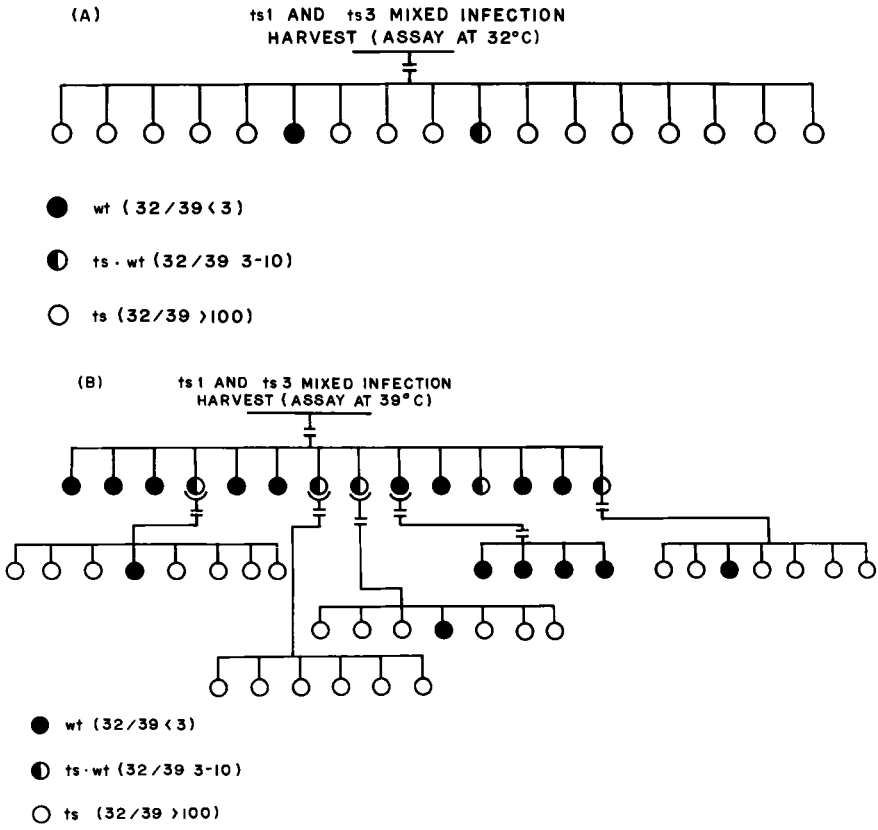

FIG. 2. Nature of viral progeny from a cross between M-MuLV-ts1 × M-MuLV-ts3. (A) 32°C XC$^+$ clones, (B) 39°C XC$^+$ clones. ●, wild type, i.e., ratio of titers 32°C:39°C ~ 1; ◐, intermediate type, i.e., 32°C:39°C = 3–10; ○, ts, i.e., 32°C:39°C ≧ 100.

Fourteen 39°XC$^+$ clones also from a cross between ts1 and ts3 gave 9 clones producing wt virus and 5 producing virus with 32°C:39°C = 3–10. No clones were producing ts virus alone at this nonpermissive temperature. The virus obtained from one wt clone remained wt on subsequent passage, but the virus from 4 of the 5 clones producing progeny having 32°C:39°C = 3–10, did not breed true. The fifth clone was lost. The viral progeny from these 4 clones, when examined by further clonal purification and assay were found to be either ts or a mixture of ts and wt virus: for one clone 6/6 subclones produced ts virus only (32°C:39°C $\geqq$ 100); for another the ratio of wt to ts was 1/8; for another 1/7; and for the fourth also 1/7 (Fig. 2B).

The virus that gave rise to these 4 clones was derived from a cross between ts1 and ts3 and contained not only these two viral species but also wt to the extent of about 8% of the infectious virus. There was therefore a possibility that the cells that gave rise to the 4 clones were infected with both ts1 and ts3, which can complement one another, or with both ts mutant and wt. Presumably, the wt would be dominant in the cell with a mixed infection. In either case, the infected colony should have been XC$^+$. Another possibility is that the virions infecting the cells that gave rise to the 4 clones were heterozygotes or heteropolyploid virions, i.e., virions containing mixed genotypes of the parental strains; or possibly they were aggregates of virions. We do not have an adequate means of distinguishing these possibilities from one another, especially where viruses of these intermediate phenotypes are concerned. What distinguishes these genetically mixed situations from the wild-type and true ts mutants is the genetic stability of wt and true ts viruses. As far as we know, heterozygotes or heteropolyploids or aggregates segregate into their constituent genetic units on passage through cells. Presumably in this respect they differ from true molecular recombinants. However, one has to be aware of the finding by Vogt (1971a) that nontransforming viruses segregate from helper-independent strains of the Rous sarcoma virus which are thought to be homozygotes. The truth is we do not know enough about the genomes of either the avian or murine oncornaviruses to understand their behavior.

The spontaneous forward rate of mutation of wt virus to ts-MuLV is perhaps as high as $1:10^3$ or $1:10^4$, and the spontaneous rate of reversion of ts mutants has been estimated by us to be of the order of $1:10^4$. However, through many *clonal* passages of wt M-MuLV and ts1 and ts3, they have retained their characteristic 32°C:39°C ratios: their genomes are, apparently, quite stable.

The frequency with which wild-type virus was produced in a cross between ts1 and ts3 was about 8%. A similarly high frequency has also

been reported for the avian tumor viruses by Kawai and Hanafusa (1972). These authors suggested that the high frequency of recombination might be accounted for by assortment of subgenomic fragments in analogy to the situations in influenza virus and reovirus (Hirst, 1962; Simpson and Hirst, 1968; MacKenzie, 1970; Fields and Joklik, 1969; Fields, 1971; Sugiura *et al.*, 1972). Vogt (1971b) showed that frequencies of genetically stable combinant forms between avian tumor viruses may be as high as 50%, but not all avian tumor viruses were able to interact genetically with each other. A value of 50% suggested (Vogt, 1972) a genetic equilibrium between unlinked markers, as if the corresponding genes were on independently replicating segments of the viral genome. In a more recent study (Weiss *et al.*, 1973), a selective technique was used to obtain recombinants between the genome of the chick helper factor (chf) and helper-independent strains of RSV. The majority of progeny isolated possessed the host range of chf and of the RSV, but were unstable and segregated into parental or stable recombinant genotypes. It was suggested that genetic reassortment of independent segments of the genome can occur, resulting in the production of heterozygotes. Ordinarily on subsequent passage, heterozygotes should segregate into the constituent units, but occasionally, a true molecular recombination might occur. Weiss *et al.* (1973) also suggested that genetic reassortment takes place at the level of RNA, i.e., between positive strands of viral RNA transcribed, presumably, from the viral genome integrated into the cellular DNA.

VI. Models of the Genome of MuLV

In what follows, we make certain assumptions. First, we assume that the ts mutants we have isolated do not contain more than one mutant site in the genome. This may not be so. We do not know whether these mutants can be back-crossed to wt virus to allow the segregation and purification of single mutants. Second, we assume that the genome is segmented. As pointed out below, this may not be so. The high frequencies of recombination that have been observed and the weight of biochemical evidence reviewed below favor this hypothesis. Third, we assume that the segments can replicate independently. The findings of Vogt (1971b), Kawai and Hanafusa (1972), Weiss *et al.* (1973), and Wong and McCarter (1973) are most readily explained by this hypothesis, but other explanations are not ruled out. It is helpful to consider several possible models of the genome, as has been done by Vogt

(1973). Martin and Weiss (1974) have discussed the current information coming from studies of the avian sarcoma viruses in the light of these models.

A. All Segments Are the Same

In this model, it is supposed that there are three segments of size 30 S to 40 S and the segments have the same genetic composition, i.e., *x,x,x*. If one of the ts mutants under consideration is represented by *x'*, i.e., there is an altered base sequence in the RNA of one of the segments, then the genome could be *x'xx*, *x'x'x*, or *x'x'x'*. It is likely that all three genomes exist, but the procedure for selection of the ts mutants favors the isolation of *x'x'x'*. The reasons for this are (a) that the virions having the genetic composition *x'xx* or *x'x'x* should have minimal ts characteristics due to dominance of the wt segments, and (b) if the segments can replicate independently, then infection of cells by *x'xx* or *x'x'x* should yield progeny having the compositions *x'x'x'*, *x'x'x*, *x'xx*, and *xxx*. The last genome is wt. We would expect therefore that wt virus should be produced with high frequency, perhaps 1:3 or 2:3 if the genomes were *x'xx* or *x'x'x*, respectively. We have estimated (Wong and McCarter, 1973) that the rate of reversion of ts1, ts2, and ts3 to wt virus is of the order of 10^{-4}. These arguments, on the basis of the assumptions stated above, would lead us to suppose that if the genome is polyploid then the ts mutants that we have isolated must bear the marker on each segment, i.e., *x'x'x'*.

On the basis of the above reasoning we suppose that the structure of ts1, might be *x'x'x'* and of ts3 might be *x"x"x"*. We have seen that a cross between ts1 or ts2 and ts3 produces wt progeny with a frequency of 8%. A cross between *x'x'x'* and *x"x"x"* cannot produce *xxx* by genetic reassortment. The progeny that have the composition *x'x"x"* or *x'x'x"* might appear to have wt characteristics because ts1 and ts3 have been shown to be complementary mutants (Wong and McCarter, 1973), but it would be expected that on passage, the parental mutant types should be regenerated. As noted above, the wt characteristics were retained on passage of virus from XC^+ clones produced in a cross between ts1 and ts3. Only the viral progeny having a ratio 32°C:39°C = 3–10 segregated into ts viruses having 32°C:39°C $\geqq$ 100 and wt virus.

It might be possible that wt virus could be produced by molecular recombination involving crossing over between segments *x'* and *x"*, but we do not know whether the frequency could be as high as 8% or not.

B. Two Segments Identical: Third Different

This model supposes the structure xxy where x is one sort of strand and y is another having a different genetic composition. In that event, and by the same arguments as those used for the model where all strands are the same, the structure of a stable mutant should be $x'x'y$ or xxy'. Crossing $x'x'y$ with another mutant $x''x''y$ should produce no stable wild-type progeny by genetic reassortment alone, although unstable heterozygotes capable of internal complementation might be produced. Wild-type viruses might be produced by crossing over—as argued above.

However, if one mutant (say ts1) has the composition $x'x'y$ and the second (say ts3) has xxy', then a cross should produce by genetic reassortment: xxy (wt), $x'xy$, and $xx'y$ (unstable heterozygotes that might have phenotypes intermediate between ts and wt), $x'x'y$ (ts1), $x'x'y'$ (a double mutant) and xxy' (ts3). This sort of model can be used to explain our findings (Wong and McCarter, 1973) reviewed above. We have isolated, from a cross between ts1 and ts3 a stable virus that might be a double mutant: It does not appear to be complemented either by ts1 or ts3, and it does not appear to recombine with either mutant. These are the properties to be expected of a virus containing mutant segments from ts1 and ts3, but proof that this viral particle is a double mutant has yet to be obtained. In addition, we have no data on the frequency with which double mutants might be obtained, i.e., if the frequency of production of wt is 8%, is the frequency of production of a double mutant also 8%?

C. All Segments Different

This model supposes that the genome consists of segments xyz, each having a unique genetic composition. Mutant ts1 might be $x'yz$, and mutant ts3 might be $xy'z$. The progeny of a cross between such viruses would give $x'y'z$ (double mutant), xyz (wt), and the parental types. Viruses of the haploid genotype having intermediate phenotypes (having 32°C:39°C ratios greater than 1 but less than the ratios for the parental ts mutants) cannot result from such a cross by genetic reassortment.

We have not considered the case where the mutations are on the same segment, e.g., $x'yz$ for one mutant and $x''yz$ for the other. In this instance, wild-type virus could result only from crossing over and not from genetic reassortment.

These arguments would lead us to favor the model with the structure *xxy* because it can account for our observations, but the problem of the structure of the genomes of these viruses is not resolved. We have considered the models because they are at present those that are under active consideration by other workers in the field. Speculations based on hypothetical models are only of use if they suggest experiments, but experiments in this field are difficult to do in a way that leads to the unequivocal interpretation of results. Arguments based on the various models have overlooked a number of problems. For example, it has been assumed that the subunits can replicate independently and their genetic information can be expressed independently also. This is not necessarily so. Furthermore, we have ignored until now the fact that particles produced from cells with a mixed infection may be heteropolyploid, i.e., contain both parental genomes in one virion.

According to Simon (1972) multiploid viruses have been found for Newcastle disease virus, M13, and respiratory syncytial virus. Their existence in the murine leukemia viruses has not been demonstrated, but the particles we have described having a phenotype intermediate between wt and ts might be of this type. At this time we cannot be certain that the observations can be accounted for by one explanation and not another. Clearly, more data are needed, not more hypotheses.

It is also necessary to be aware that the high frequency of genetic recombination may arise because of the unique method of replication involving the formation of DNA copies of the RNA (Temin and Baltimore, 1972).

There is one further complication that underlies all of this work, and it is unique to the RNA-containing tumor viruses: All cells in which one has to propagate the viruses themselves contain endogenous viral information. Weiss *et al.* (1973) have provided the first evidence with defined genetic markers that recombination takes place between avian sarcoma viruses and endogenous genetic elements of the host cell. The extent of genetic interaction between exogenous murine leukemia viruses and endogenous cellular information is not known.

These deficiencies in our knowledge of the biology and genetics of the RNA tumor viruses lead us also to ask how good are the conclusions coming from molecular biological studies of the genomes of these viruses?

VII. Evidence That the RNA of the Virion is Segmented

It is known that the virion consists of a core containing ribonucleic acid (RNA) and protein surrounded by a lipoprotein envelope acquired

from the plasma membrane of the infected cell. In this and other respects, the murine leukemia viruses are very similar to the RNA-containing avian leukosis and sarcoma viruses.

A problem in studies of the physical nature of the RNA of the virion is that the infectivity of the virion diminishes spontaneously at 37°C with a half-life of 2–5 hours (Dougherty, 1961; Hanafusa *et al.*, 1964; Pluznik and Sachs, 1964). It has been suggested (Bader and Steck, 1969) that the loss of infectivity is due to the scission of the molecule of viral RNA. It has been common practice to harvest tissue culture medium at intervals constituting several half-lives, e.g., after a period of 10–24 hours; therefore, it is likely that the medium contains a considerable quantity of inactivated virus. Data on the ratio of physical particles/infectious units are scarce in the literature. Crawford and Crawford (1961) found a value of 750 for a 4-hour tissue culture harvest of the defective strain of RSV. After correcting for the presence of RAV and inefficiency of the focus assay Vogt (1966) estimated that the ratio might be 5:1 to 15:1 physical particles per infectious unit. Bader and Bader (1970) found, for a 42-hour collection, a ratio of 2600 to 5000 for physical particles to focus forming units. Applying the corrections noted above, the ratio of physical particles to infectious units might be 25:1 to 50:1, however, in neither of these published studies were there measurements of the actual titer of RAV, so these corrected ratios are only estimates. During the purification and isolation of the virus particles, there is opportunity for further loss of infectivity. The RNA that is isolated from tissue culture harvests after a period of labeling of several hours may therefore be largely from noninfectious virions. Bader and Steck (1969) recognized the importance of collecting virus at short intervals (hourly harvests) to minimize the production of artifacts during the isolation of the RNA.

When RNA is isolated using procedures that should preserve the native state, i.e., rapid purification of virus followed by extraction of the RNA with sodium dodecyl sulfate and phenol, the nucleic acid consists largely of single-stranded RNA with a sedimentation coefficient of approximately 60 S to 70 S. This value corresponds to a molecular weight of about 10^7 daltons (Robinson *et al.*, 1965; Robinson and Baluda, 1965; Duesberg and Robinson, 1966; Bader and Steck, 1969). Treatment of the high molecular weight RNA with heat or dimethylsulfoxide results in the production of smaller RNA molecules with sedimentation coefficients approximately 30 S to 40 S and 4 S to 12 S (Duesberg, 1968; Erikson and Erikson, 1971; McCain *et al.*, 1973). The major 30 S to 40 S component had a molecular weight approximately 3×10^6 daltons. It appears that the RNA of the virion must consist of 3

or 4 subunits rather than a single polynucleotide. According to McCain *et al.* (1973) the 69 S RNA of murine sarcoma-leukemia virus is composed of at least three physicochemically distinct subunits. It has to be kept in mind, however, that the murine sarcoma-leukemia virus is a mixture of at least two viruses. Duesberg *et al.* (1973) have reported that the RNA of the Kirsten strain of murine sarcoma virus is significantly smaller than that of the Kirsten murine leukemia virus. In fact, the purity of a viral stock in terms of whether or not it consists of a single infectious viral entity is seldom, if ever, known, and in general not enough care is taken to use clonally purified stocks of virus for molecular biological studies. For example, Duesberg and Vogt (1970) reported the RNA of avian-transforming viruses contains two electrophoretically separable subunit classes a and b. In subsequent work, Duesberg *et al.* (1973) prepared stocks of avian sarcoma viruses from clones of singly infected cells and found that the RNA of the sarcoma viruses grown in this way contained only the a subunit, whereas stocks grown by repeated cycles of infection contained both a and b subunits.

It has been shown that Rous sarcoma virus (Prague) harvested at 3-minute intervals contains a minor amount of 60 S to 70 S RNA, major amounts of 30 S to 40 S RNA, and variable amounts of 4 S to 12 S RNA (Canaani *et al.*, 1973). Upon incubation of the virus in cell-growth medium or Tris-saline buffer, most of the 30 S to 40 S RNA was converted to 60 S to 70 S RNA. Cheung *et al.* (1973) showed that RSV (Prague) harvested at 5-minute intervals contained very little 68 S RNA. The principal native component had a sedimentation coefficient of 55 S to 60 S, and in the denatured state the RNA consisted of approximately equal parts of very homogeneous 36 S RNA, RNA which sedimented heterogeneously between 36 S and 4 S, and 4 S RNA. By contrast, the native RNA of mature (24 hour) virus consisted of 68 S RNA which, on denaturation, gave heterogeneous RNA of 36 S to 4 S.

These observations argue for 30 S to 40 S RNA as precursor of the 60 S to 70 S RNA of Rous sarcoma virus. The techniques of nucleic acid hybridization using DNA probes made on 70 S viral RNA template have been used to examine the size of intracellular RNA in infected cells. Leong *et al.* (1972) showed that virus-specific RNA is present in both nuclei and cytoplasm of Rous sarcoma virus-infected chick cells. The RNA was very heterogeneous in size and varied from preparation to preparation. Much of the *native* virus-specific RNA in some preparations of cytoplasm from infected cells appeared to be 70 S RNA. There was also a species of 90 S to 95 S RNA, presumably from the nuclei, along with smaller species of RNA. When the RNA was denatured, the 70 S and 90 S to 95 S species were lost and the RNA sedimented heterogenously from 50 S to 4 S. Leong *et al.* (1972) attributed inconsis-

tencies from preparation to preparation to the presence of varying amounts of budding virions not removed from cell surfaces by trypsinization. Tsuchida *et al.* (1972) found single-stranded 35 S and 20 S viral RNA species synthesized in virus-producing mouse and rat cells transformed by the murine sarcoma virus: once again it should be pointed out that the presence of a mixture of viruses (MuSV and MuLV) complicates the interpretation of such data.

It is evident that as yet there is no definite answer from molecular biological studies as to the size of the genome of the RNA-containing tumor viruses. Nor is it certain that the RNA is segmented. Despite the best efforts to isolate the RNA in intact form, there remains the possibility that large molecular weight RNA possesses a limited number of sites uniquely sensitive to the action of nucleases. The virions are known to possess an associated endonuclease (Quintrell *et al.*, 1971; Grandgenett *et al.*, 1972). Only two nicks would be sufficient to produce three fragments. To account for the observations that have been made, one would have to say that the nuclease-sensitive sites, if there are such, are located so that equal-sized pieces, 30 S to 40 S, are made, and for unknown reasons, these can associate with one another and with 4 S to 12 S RNA to form the RNA structure of the mature virion. While the conclusion must be left open, the weight of evidence favors the segmentation of the RNA of the virion.

VIII. Conclusions

In view of all these difficulties, problems and lack of factual data, further speculation is unjustified. We do not know how much RNA constitutes the genome, i.e., whether it consists of the entire complement of RNA of the virion or not. An answer should come from genetic studies now in progress which should reveal how many cistrons there are. Also likely to emerge from genetic studies will be an answer to the question of whether cistrons are linked, as would be predicted by a segmented genome, or not. It is important to have available mutant viruses that are defective in various viral functions, not only to learn what those functions are but also to learn about transcriptional and translational regulation of the expression of these functions in infected cells. Ways must be found to overcome the formidable obstacles that block unequivocal understanding of the genetics and biology of these viruses.

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CHAPTER 12

Comparative Biology of Murine and Avian Tumor Viruses

P. BENTVELZEN

Acquérir de l'expérience et s'appuyer sur l'observation est autre chose que faire des expériences et faire des observations.

Claude Bernard, Introduction à l'étude de la médecine expérimentale (1865)

L'expérience n'est au fond qu'une observation provoquée.

Claude Bernard, *ibid.*

I. Introduction

The student of carcinogenesis is continuously confronted with an overwhelming complexity of etiological and permissive factors and their various interactions. For that reason he must/will often focus his attention on a single aspect of cancer biology, or will resort to simplistic unitarian concepts of carcinogenesis, such as the somatic mutation (Boveri, 1914) or oncogene-activation (Huebner and Todaro, 1969) hypotheses.

With regard to the present assignment to review the comparative biology of avian and murine tumor viruses, I decided for similar reasons of simplicity to discuss only the oncogenic RNA viruses. The avian and murine RNA cancer viruses share so many structural, biochemical, and biological properties that they can be regarded as belonging to the same separate virus group, the oncornaviruses (Nowinski *et al.*, 1970). These viruses display several highly interesting interactions with the host genome (Bentvelzen, 1972a) as Lwoff (1953, 1960) had foreseen, although he may not have expected that RNA viruses would play a predominant role in naturally occurring viral carcinogenesis at that time.

The decision to refrain from reviewing oncogenic DNA viruses leaves out several viruses, which are highly interesting from a naturalist's point of view, such as the herpesvirus associated with Marek's disease in chicken (Churchill and Biggs, 1967; for a review, see Nazerian, 1973),

the murine polyoma virus (Gross, 1953; Stewart, 1953; for reviews, see Eddy, 1969; Sambrook, 1972), and a papovavirus associated with papillomas of chaffinches (Lina *et al.*, 1973). All these viruses display interesting features with regard to latency and transmission. Less to be regretted is the omission of the chicken embryo lethal orphan (CELO) virus. This adenovirus is oncogenic to hamsters (Sarma *et al.*, 1965; Anderson *et al.*, 1971), but as McAllister *et al.* (1972) discussed for those human adenoviruses, which are oncogenic to rodents, it is doubtful that this virus is oncogenic to its natural host.

The present chapter will focus on the interactions between host genome and oncornaviruses and will present a somewhat opinionated view on carcinogenesis. However, the multifactorial approach to cancer, so typical for the Mühlbock school of cancer research (Emmelot and Bentvelzen, 1972), will probably glimmer through. Finally, it must be mentioned that my personal experience is only with the murine oncornaviruses. The knowledge attained in this field has been applied to that of the avian viruses, of which I have been a passive observer. (See the citations of C. Bernard above.)

II. Delineation of the Oncornavirus Group

Oncogenic RNA viruses resemble myxo- and paramyxoviruses in that maturation and release of virions takes place through a budding process of the cell membranes. They have been regarded as being different in that they have no cytocidal effect (Hanafusa, 1970; Montagnier, 1970; Nowinski *et al.*, 1970). However, various notorious exceptions have been described since then for various RNA cancer viruses (Klement *et al.*, 1969a; Bassin *et al.*, 1971b; Kawai and Hanafusa, 1972; Graf, 1972; Smith and Bernstein, 1973; Reinisch and Bang, 1973).

The RNA tumor viruses can be distinguished morphologically from the myxo- and paramyxoviruses, since they have a spherical internal structure at a restricted site instead of a nucleocapsid with an obvious helical configuration, which is distributed throughout the interior of the virus (Nowinski *et al.*, 1970). It has been suggested to call these agents leukoviruses (Fenner, 1968) or Thylaxoviridae (sac-like viruses) by a group of senior electron microscopists studying those viruses [*J. Nat. Cancer Inst.* **37**, 395–397 (1966)]. However, these names do not refer to the most outstanding biological property, which is the induction of neoplasms in their natural hosts. It is for that reason that we go along with the unusual practice of renaming a virus group and will use the term "oncornaviruses" as Nowinski *et al.* (1970) have suggested.

The oncornaviruses display unique biochemical properties, such as the heavy weight single-stranded RNA (60 S–70 S), which, upon heating or treatment with dimethyl sulfoxide is disaggregated into subunits of approximately 36 S (for a review, see Duesberg, 1970). An even more interesting characteristic is the presence of an RNA-dependent DNA polymerase ("reverse transcriptase") as first discovered by Temin and Mizutani (1970) and Baltimore (1970) and subsequently found in a great variety of oncornaviruses (Spiegelman *et al.*, 1970a,b; Scolnick *et al.*, 1970). Implications of this finding will be briefly discussed below and at great length by Vigier in Chapter 9 of this volume. The reader is also referred to the review of Temin and Baltimore (1972).

The agents, which induce sarcomas or various forms of leukosis, belong to the avian oncornaviruses. Some of the avian leukosis virus strains can also induce renal carcinomas (Carr, 1956, 1960; Walter *et al.*, 1962), ovarian tumors (de Thé, 1964), or osteopetrosis (Burmester *et al.*, 1959; Frederickson *et al.*, 1964). The murine oncornaviruses comprise the sarcoma-leukemia virus complex and the mammary tumor virus.

The sarcoma and leukemia viruses of either animal species have the so-called C type electron-microscopic appearance (Bernhard, 1958). There are minute differences between the avian and murine viruses with regard to the size of the nucleoid and surface projections (Feller *et al.*, 1971). The murine mammary tumor virus differs from either C type virus in having the considerably smaller nucleoid eccentrically located. The virion, called B type particle by Bernhard (1958), has very prominent protrusions at its outer membrane.

Type B particles have been observed in pulmonary (Calafat, 1969) and brain tumors of mice (Moore *et al.*, 1969). Type C particles have been seen in murine pituitary and adrenal tumors (Mitchell *et al.*, 1971), hepatomas (Maca *et al.*, 1970), and also pulmonary adenomes (Rabotti, 1966; Brooks, 1970; Kimura *et al.*, 1972; Bucciarelli and Ribacchi, 1972). In all these cases an etiological role has not been established. It is very well possible that these particles represent passenger viruses only.

For an extensive historical account of the discovery of the oncornaviruses and their various strains see the book by Gross (1970). One must keep in mind that several of the virus isolates, such as the Rauscher leukemia virus, which have been intensively used in experimental studies, are laboratory artifacts, and also are often complicated mixtures (Graffi *et al.*, 1966; Smith and Moscovici, 1969; Ishizaki *et al.*, 1971). Although these isolates are valuable tools in cancer research, it is important to study naturally occurring tumor viruses as well (Andervont, 1952; Pope, 1963; Frederickson *et al.*, 1964; Weiss, 1972), as they may provide more accurate insight into the natural history of cancer.

III. Interactions between Host Genome and Oncornaviruses

At one time, when there was still little evidence for the viral etiology of several neoplasms, Lwoff (1953) suggested that lysogeny—integration of a phage's genetic material into that of a bacterium—could have its animal counterpart in carcinogenesis. Lwoff (1960) detailed this supposition in that neoplasia could arise either after the integration of the genome of administered virus into the host genome, or by the induction of genetically transmitted viruses following the administration of carcinogenic drugs.

A. Somatic Provirus

As far as the oncornaviruses are concerned, sufficient evidence is accumulating that upon infection with an exogenous virus a DNA copy of the viral genome will be made, which may become integrated into the host genome. This copy, called somatic provirus by Bentvelzen and Daams (1972), may serve as a template for virus replication and under certain curcumstances give rise to neoplastic conversion of its host cell.

Several oncornaviruses may replicate in various tissues without transforming them (Dougherty and Di Stefano, 1969). For the establishment of such infections DNA synthesis is also necessary, such as in the case of the transforming viruses (Duesberg and Vogt, 1969; Vigier, 1970), suggesting that also in the absence of neoplastic conversion a somatic provirus will be produced and integrated. Since such viruses may rapidly transform other tissues, it is obvious that epigenetic factors are highly important for the expression of the "oncogenes" of a somatic provirus. Other virus functions may be also strongly influenced by the differentiation status of a cell.

B. Germinal Provirus

I have suggested that in various inbred mouse strains endogenous mammary tumor viruses can be transmitted as a genetic factor of the host (Bentvelzen, 1968a, 1972; Bentvelzen *et al.*, 1968, 1970b, 1972a; Bentvelzen and Daams, 1969, 1973). It was envisaged that one of the chromosomes would carry genetic information (germinal provirus, Bentvelzen and Daams, 1972) for such a virus in the form of a DNA copy of the viral genome. This copy would be transcribed either spontaneously or following treatment with ionizing radiation or carcinogenic drugs, giving rise to virus release in the target cell and eventually to neoplastic

transformation. It was hypothesized that inhibition of virus release would be the normal state and would be achieved by classical repressors. Later on a genetical transmission has also been postulated for an endogenous murine leukemia virus (Rowe, 1972; Rowe and Hartley, 1972) and an endogenous avian C type virus (Weiss, 1972). It must be noted here that Huebner and Todaro (1969) presented a unitarian hypothesis, that a segment, oncogene, of a single genetically transmitted C type virus, whose germinal provirus is called virogene, can be held responsible for the development of most tumors, irrespective of the initial carcinogenic stimulus. The various carcinogenic treatments can activate the expression of the whole virogene, leading to the production of virions, or of only some segments such as the oncogene(s). This hypothesis has encountered vehement opposition, but also attracted zealous support from many cancer researchers.

C. Genetic Susceptibility

A more classical aspect of host–virus interactions is genetically controlled susceptibility to exogenous infections. Several genes have been detected, which contribute to the chance that an organism will develop a tumor after administration of an oncogenic virus. Such genes can control the penetration of the virus in a target cell, influence the rate of replication, and allow cell-surface alterations, which may lead to neoplastic conversion or determine the degree of immunological response to either the virus or virus-coded tumor-specific cell-surface antigens (Bentvelzen, 1972a). In addition, genes may influence various other humoral factors (e.g., hormone production, growth rate) that contribute to the development of a tumor (Heston, 1945). Some of those genes also influence tumorigenesis by endogenous viruses. The presumed genetic transmission of oncornaviruses and the sometimes predominant role of such genes, leads to the reemergence of the idea that cancer is a hereditary disease.

D. Viral Gene Action

A very important aspect of this host genome–virus interaction is whether the virus turns on certain cellular genes, which cause the cell to disobey growth-controlling stimuli (or switch off the genes that do the opposite), or whether unlimited growth is the consequence of direct action of viral genes. Unfortunately, no single experimental fact is available to have a sensible discussion of this problem.

IV. Technological Aspects of the Study of Oncornaviruses

Differences in the biology of tumor virus groups may be due to (a) different target organ, (b) differences in the biology of the target organ from one species to the other, or (c) an intrinsic property of the virus itself. Such differences may be artificial owing to a lack of patience, as seen in the case of researchers in the avian tumor virus field as compared to the mammary tumor virologists (Emmelot and Bentvelzen, 1972), or to a "technology gap" (Bentvelzen, 1972c). Techniques for detection and assaying various oncornaviruses are presented in Table I and are detailed below.

Rapid developments in virology may only be anticipated when the virus can be grown in tissue culture and can be quantitated effectively. It is preferable that the virus show a cytopathic effect *in vitro*, although this is not a prerequisite. Tumor viruses often induce morphological and physiological transformation of cells *in vitro*. This phenomenon has been extensively described and been discussed in relation to neoplasia by Macpherson (1970) and Pontén (1971).

A. Avian Sarcoma Viruses

Rous sarcoma virus (RSV) rapidly transforms chicken embryonic fibroblasts *in vitro* (Manaker and Groupé, 1956). This phenomenon has

TABLE I

Technological Aspects of the Assay of Oncornaviruses[a]

	ASV	ALV	MSV	MuLV	MTV
Transformation *in vitro*	+++	++	+++	+	−
Cytocidal effect *in vitro*	+	++	−	+++	−
Replication *in vitro*, detectable by immunoassay	+	+++	−	+++	+
Interference	−	+++	−	+++	−
Rescue	−	+++	−	+++	−
Tumor induction *in vivo*	+++	+++	+++	+++	+++
Quantitative assay of preneoplastic lesions	−	−	−	++	++
Antibody production test	+	+	+	+++	+
Immunobioassay	−	+	−	+	+++
Reverse transcriptase	+	+	+	+++	+++

[a] +++, routine method for all members of the group; ++, routine methods not applicable for each member of the group; +, good perspectives for the development of a routine method.

been utilized for an accurate quantitative assay of this virus (Temin and Rubin, 1958; 1959). So far only the MC29 strain of avian leukosis virus (ALV) is capable of transforming the same kind of cells (Langlois and Beard, 1967; Heine *et al.*, 1969). This effect is due to a defective principle (Ishizaki *et al.*, 1971) which is helped by an erythroblastic leukemia virus (Langlois *et al.*, 1971).

B. Avian Leukosis Viruses

The avian myeloblastosis virus (AMV) is capable of *in vitro* transformation of bone marrow cells, giving rise to continuous proliferation in shaker cultures (Beaudreau *et al.*, 1960). Baluda and Goetz (1961) observed in yolk sac cultures that within 1 week after exposure to the virus morphologically altered myeloblasts and osteoblasts appeared. Other cell types remained normal, but would produce virus. Various other ALV strains proved to be ineffective in this test (Baluda *et al.*, 1964). With some modifications of the culture technique Lacour *et al.* (1966) obtained foci of transformed cells within 4 days. The number of foci was proportional to the virus concentration in the medium. Thus far we have not noticed the use of this method on a routine basis in the literature.

Langlois *et al.* (1969) observed that the exposure of chicken bone marrow to the MC39 virus leads to a transient growth of myelocytes which does not take place in cultures of normal bone marrow. The cells look quite different from neoplastic myeloblasts transformed by AMV. This observation by Langlois *et al.* (1969) does not seem to be useful for a quantitative *in vitro* assay of the MC29 virus.

In vitro demonstration of an avian leukosis virus may be accomplished by one of the following: (a) the induction of resistance to morphological transformation by subsequent challenge with RSV (RIF test of Rubin, 1960a); (b) production of foci with virus-specific antigen, detectable by immunofluorescence (Vogt and Rubin, 1961); (c) the reproduction *in vitro* of a group-specific antigen, detectable by complement fixation (COFAL test of Sarma *et al.*, 1964) or radioimmunoassay (Weber and Yohn, 1972); (d) the rescue of "defective" Rous sarcoma virus from so-called nonproducer cells (Rispens and Long, 1970). Recently, a cytopathic effect has been observed with avian leukosis viruses in cells transformed by a temperature-sensitive mutant of RSV (Kawai and Hanafusa, 1972) or in normal cells (Graf, 1972; Smith and Bernstein, 1973). This effect is limited to viruses belonging to subgroups B and D.

C. Mouse Sarcoma Viruses

The mouse sarcoma virus (MSV) can transform *in vitro* mouse embryonic fibroblasts (Hartley and Rowe, 1966; Simons *et al.*, 1967b). Foci, which become apparent within 5 days, consist of either round or spindle-shaped cells. The kinetics of the production of these foci indicates that two infectious particles are necessary for the rapid development of such foci (Hartley and Rowe, 1966).

MSV can also readily transform morphological embryonic cells of other mammalian species (Ting, 1966; Bernard *et al.*, 1967; Simons *et al.*, 1967a). Kinetics of this phenomenon is single hit (Parkman *et al.*, 1970; Levy, 1971) and may be therefore better utilized for titration of MSV preparations.

D. Murine Leukemia Viruses in Vitro

Murine leukemia viruses (MuLV) have been reported to be able to transform embryonic fibroblasts *in vitro* (Tyndall *et al.*, 1966; Osato *et al.*, 1966; Dug-Nguyen *et al.*, 1967; Rhim *et al.*, 1969). Even when spontaneous transformation may be ruled out as a cause, virus-induced transforming events seldom seem to take place. Ishimoto *et al.* (1972) found that when spleen cells from 4-week-old C57BL mice are infected *in vitro* with the Rauscher leukemia virus, rapid cell proliferation took place 2 weeks after infection.

Hackett and Sylvester (1972) developed a peculiar subline of BALB/3T3 fibroblasts, which upon infection with a MuLV rapidly transforms morphologically. This cell line has some physiological attributes of transformed cells and seems to need only the MuLV to become morphologically altered. It is questionable whether the observed transformation has any relevance to the leukemogenic potential of the virus.

When obvious target cells (thymocytes) are mixed *in vitro* with GLV-passage A (Gross, 1957) or B/T-LV (Tennant, 1965), which predominantly induce thymic-dependent lymphosarcomas, transformation takes place only after several passages (Ioachim, 1967; Tennant, 1969).

Our laboratory is involved in the study of *in vitro* transformation of murine myeloid precursor cells by Rauscher leukemia virus (RLV). Normal bone marrow cells can produce colonies in a semisolid agar

culture system in the presence of a specific stimulating factor (derived from human urine, mouse embryo extracts, or conditioned medium of various kinds of cultures). This factor (CSF) has a concentration-dependent effect on the number and size of these colonies (Metcalf, 1970). We observed that bone marrow from BALB/c mice with RLV-induced myeloid leukemia produces considerably fewer colonies than control material following the administration of optimal amounts of CSF. At low levels of CSF, however, leukemic bone marrow produces several more clones than the controls. Even in the absence of CSF, leukemic cells were capable of producing several small colonies, whereas normal bone marrow produces virtually none (Bentvelzen *et al.*, 1974). The same has been found for well-established cell lines of murine myeloid leukemias (Pluznik, 1969; Metcalf *et al.*, 1969; Ichikawa, 1972). This finding could be interpreted to mean that the transformed cells have a more efficient utilization of CSF, as has been described for the "multiplication-stimulating activity" in the case of RSV- or MSV-transformed fibroblasts (Temin, 1969, 1970). However, the growth of leukemic clones is more pronounced when high number of cells are plated. This can be an indication that leukemic cells can produce, to some extent, their own proliferation-inducing factor (probably different from CSF), which can trigger some cells in the leukemic population to produce small colonies. This phenomenon would be comparable to the leakage of an "overgrowth factor" by RSV-transformed chicken embryonic fibroblasts (Rubin, 1970).

We found that for the establishment of an infection with RLV *in vitro*, as determined by virus reproduction, hemopoietic cells needed to be cultured in the presence of CSF. After 1 week of culture under optimal conditions, replating of the infected cells in a CSF-free system with a high cell number, leads to the production of several small colonies of immature myelocytes, whereas the control cultures only produced a few macrophage colonies (Bentvelzen *et al.*, 1974). Unfortunately, this system lacks constant reproducibility and is far from quantitative at the moment.

Murine leukemia viruses so far cannot satisfactorily be assayed *in vitro* for their transforming (oncogenic) potential, but several methods are available for the assessment of infectious entities. One of the fastest methods is by determining the number of antigen-positive foci by immunofluorescence (Osato *et al.*, 1964; Pinkel *et al.*, 1966; Woods *et al.*, 1970). All these authors study the appearance of a virus-specific antigen in the cytoplasm, but Nordenskjöld *et al.* (1970) investigated the appearance of a virus-coded cell membrane antigen, detectable by either immunofluorescence or immune adherence. The latter immunoassay proved

to be more sensitive with regard to virus titer, but it took several more weeks in culture before the antigen could be detected.

Another widely used immunological approach is the appearance of a virus-specific complement-fixing antigen, the so-called COMUL test by Hartley *et al.* (1965). The recent development of a radioimmunoassay for the group-specific antigen of MuLV (Parks and Scolnick, 1972) outdates the COMUL test.

Murine leukemia viruses have the ability to rescue defective mouse sarcoma virus from nonproducing transformed cells (Huebner *et al.*, 1966). This phenomenon can be employed in titrating murine leukemia viruses. A somewhat different approach is coinfection of a culture with a two-hit MSV preparation and a leukemia virus preparation. The eventual excess of helper virus would then give rise to a linear relationship between MSV dose and number of foci of transformed cells (Fischinger and O'Connor, 1968). Virus titer can be determined by the lowest dilution that still promotes focus formation by defective MSV. An interesting observation with this system is that the leukemia virus need not be capable of replication in the target cell by itself to provide helper activity (Fischinger and O'Connor, 1969b). Possibly, the defective sarcoma virus can complement defective leukemia viruses with regard to replication.

Preincubation with a leukemia virus can interfere with focus formation by MSV, as was described for the avian tumor virus system. This property can also be employed for titration of a leukemia virus (Sarma *et al.*, 1967).

Klement *et al.* (1969a) observed a cytopathic effect after cocultivation of MuLV-infected embryo cells and the XC cell line, derived from a RSV-induced rat tumor. This strange property has been utilized for the now widely used XC test by Rowe *et al.* (1970). Bassin *et al.* (1971b) observed that MuLV infection of a MSV-transformed mouse cell line (D56), which does not produce infectious virus, does not only lead to the rescue of the defective MSV genome but has a cytopathic effect as well. Grundner *et al.* (1972) compared this technique with the induction of a membrane antigen as described by Nordenskjöld *et al.* (1970). The plaque assay proved to be considerably faster and of a more quantitative nature. However, upon prolonged culture the immunological assay proved to be more sensitive.

E. Murine Leukemia Viruses in Vivo

In vivo assays often comprise long observation periods with a great variation in latency. Some fast-acting viruses can be assayed by estima-

tion of the average spleen weight within a fixed period (Rowe and Brodsky, 1959). This parameter correlates reasonably well with tenfold dilutions of the virus. Within 1 week, at the proper dilutions, erythroblastosis viruses produce conspicuous foci of erythroblasts at the surface of the spleen (Pluznik and Sachs, 1964; Axelrad and Steeves, 1964). This assay gives a different dose–response relationship than the spleen weight method. Depending upon the host and the proportion of the focus-producing principle and the helper virus, the response is either single or multi-hit (Lilly, 1970b; Steeves and Eckner, 1970; Bentvelzen *et al.*, 1972b).

The defectiveness of the erythroblastosis-inducing principle can be employed for the titration of helper virus activity. Mice in which the erythroblastosis virus gives a multi-hit pattern are infected with a mixture of a low dose of erythroblastosis virus and various doses of the preparation to be assayed for helping activity. The helping effect will become manifest in the augmentation of the number of foci (Steeves *et al.*, 1971).

For those viruses that do not have a short latency period the mouse antibody production (MAP test by Klein and Klein (1966) seems to be very worthwhile. Virus quantitation is based on the inverse relationship between virus doses and the latency period for cytotoxic antibodies against leukemic cells to appear. This tests requires 9 weeks for completion, which is relatively short as compared to the latency period of leukemogenesis of several virus strains. The sensitivity of the method corresponds well with titration on the basis of leukemogenicity (Fey and Pasternak, 1969), but one must keep in mind that attenuated forms of a leukemia virus may also be scored as positive in the MAP test.

F. Mouse Mammary Tumor Virus

The technology of the mouse mammary tumor virus (MTV) is still in a backward stage. The usual practice for demonstrating infectivity is the induction of tumors. Most investigators in this field keep the animals under observation during their whole life span, although this would not be necessary for virulent virus preparations. It seems that low dilutions of virus are only reflected in a lesser incidence of "early" arising tumors but not by a later appearance of malignancies. However, such patience has been rewarding because virus strains have been detected, which have latency periods in years (Hageman *et al.*, 1972). It would be worthwhile to look for such low-oncogenic strains in other tumor–virus systems, since they have great relevance to the natural history of "spontaneous" cancer.

Nandi (1963a,b) introduced a rapid bioassay for the detection of MTV. In this method mammary glands are inspected for the appearance of hyperplastic alveolar nodules, which are thought to be precursory stages of mammary adenocarcinomas. Even the recent improvements of this method (Nandi *et al.*, 1971) do not make it comparable to the spleen focus assay of murine erythroblastosis virus strains, because the virus concentration is not reflected so much in the average number of nodules per mouse but in the incidence of mice with nodules.

Charney *et al.* (1969) described an immunological bioassay for MTV in which the detection by immunodiffusion of the group-specific antigen in the milk is taken as evidence for infection. With the recent development of a complement fixation test (Parks *et al.*, 1972b) and of radioimmunoassay for MTV (Verstraeten *et al.*, 1973), which can also detect the presence of antigens in the serum of mice, this bioassay can be refined, but will always be semiquantitative in nature.

Links *et al.* (1972) described replication of MTV in cultures of baby mouse kidney cells as demonstrated by immunofluorescence method of Hilgers *et al.* (1971b, 1972a) and by electron microscopy. They also reported morphological transformation, but this seems to be a rarity similar to that described for MuLV in fibroblasts. The observed replication, however, opens up new avenues for the study of MTV.

G. Molecular Probes

Virus activity in various systems can also be assayed by the simultaneous detection of high molecular weight RNA and reverse transcriptase (RT) (Schlom and Spiegelman, 1971), and subsequent quantification of the enzyme (Kelloff *et al.*, 1972). The RT's of various oncornavirus groups display pronounced antigenic differences that can be used in determining the virus in a given sample (Aaronson *et al.*, 1971a; Oroszlan *et al.*, 1971; Scolnick *et al.*, 1972; Parks *et al.*, 1972a; Watson *et al.*, 1972; Nowinski *et al.*, 1972).

V. Some Biological Aspects of Various Oncornaviruses

> Despite the recent progress in techniques dealing with tumor virus infection at the cellular and the subcellular levels, questions of animal pathology remain of continued importance in research on avian tumor viruses. Many problems can be defined and solved only at the animal level, and insights gained by modern *in vitro* techniques must be implemented in the animal (P. K. Vogt, 1965a).

Before going into the comparison of the biology of the various oncornaviruses a brief resume of the most pronounced characteristics of every virus group will first be presented below.

A. Avian Sarcoma Viruses

Rous and associates (for a review, see Gross, 1970) succeeded in isolating sarcomatogenic viruses from several transplantable sarcomas. The kind of sarcoma induced by these agents differs according to the original source of the virus. Several other groups of investigators succeeded in isolating sarcoma viruses from field cases or even from chemically induced neoplasms. It seems that success of virus retrieval is associated with transplantability of the tumor. Since the malignancies were transplanted in random-bred flocks, it seems likely that the recurrence of the tumor in grafted animals is due to transfer of the virus, with subsequent transformation of host cells, rather than to proliferation of donor cells. With the aid of sex chromatin as a marker Pontén (1962) demonstrated that RSV-induced tumors have only limited transplantability. Pontén (1964) postulated that the growth of RSV tumor cells is due to both proliferation of donor cells and recruitment of host cells. In correspondence with this hypothesis is Pontén's observation (1970) that cultures of RSV-transformed chicken embryonic fibroblasts have a much more limited life span (10–14 passages) than normal cells (20–27 passages).

1. Defectiveness

An interesting aspect is the defectiveness of the sarcoma-inducing genome in several RSV preparations. The concept found its origin with the observation *in vitro* that at low multiplicities of infection the majority of the foci do not release virus, that is able to transform normal chicken embryonic cells (Temin, 1962; Hanafusa *et al.*, 1963). Infection of such nonproducer cells with an avian leukosis virus leads to the rescue of a transforming agent, which can be neutralized with an antiserum directed against the corresponding helper virus (Hanafusa *et al.*, 1964; Vogt, 1964). At low multiplicities of infection, the rescued virus again produces nonproducer foci, indicating that the retrieved RSV was not due to genetic recombination between the defective RSV genome and the superinfecting ALV, but to phenotypic mixing (Hanafusa *et al.*, 1964). It seems that the helper virus provides the outer coat of the defective RSV. The helper virus will probably also provide several enzymatic functions, which are associated with virus replication (Hanafusa and Hanafusa, 1971).

Later it was found that so-called nonproducing RSV-transformed cells often release C type particles, which are transforming but display another host range than the original preparation (Weiss, 1967; Vogt, 1967; Hanafusa and Hanafusa, 1968). The implications of this finding will be discussed in Section VIII, A.

2. *Transformation of Mammalian Cells*

Rous sarcoma virus can induce tumors in various mammalian species, ranging from mouse to monkey (for a review, see Zilber, 1965; Svoboda, 1966; Gross, 1970). The virus can transform *in vitro* cells from many various kinds of mammals. Transformed cells usually do not produce virus particles even when competent strains of the virus have been used. Some notorious exceptions have been described by Svoboda and Klement (1963), Altaner and Svec (1966), and Kryukova (1966). Nonproducing cells synthesize, however, the group-specific antigen of the avian tumor virus as detected by the complement-fixation test (Huebner *et al.*, 1964). Some C type virus particles, which are spontaneously released by so-called nonproducing RSV-transformed mouse or rat cells, do not contain the group-specific antigen of the avian tumor viruses despite its presence in the cytoplasm (Oda *et al.*, 1971; Klement *et al.*, 1972). These particles represent endogenous mouse or rat C type viruses.

The Rous sarcoma virus genome can be retrieved from nonproducing cells, transformed by competent RSV, by inoculation into chickens (Svoboda and Chyle, 1963), or by cocultivation *in vitro* with chicken cells (Simkovic *et al.*, 1962). The rescue of the RSV genome is achieved when intercellular bridges are formed between the mammalian and chicken cells, or better still when complete fusion between the cells takes place (for an extensive review, see Svoboda and Hlozánek, 1970). For the retrieval of defective RSV from mammalian cells, mixed cultures must be superinfected with avian leukosis virus (Sarma *et al.*, 1966; Vigier, 1966). Some cell lines from murine tumors, induced by nondefective RSV, are not virogenic. The cells carry a presumably virus-coded tumor-specific cell surface antigen but lack the internal group-specific antigen. It seems that in these cells only a fragment of the RSV genome is present (Svoboda, 1972).

Svoboda (1972) made the interesting observation that fusion or virogenic cells with chicken erythrocytes, macrophages, thymocytes, hepatocytes, and cells of the mesonephros does not give rise to infectious RSV or RSV coat antigen. Usually it is assumed that chicken cells, in contrast to mammalian fibroblasts, provide a factor that can lead to the full expression of the genome of competent RSV. This ability does seem to be limited to only one type of chicken cells.

Infection of rat cells with the B77 sarcoma virus produces, according

to Kotler (1971), many more virogenic than transformed cells. Focus formation *in vitro* by RSV on chicken embryonic fibroblasts is also strongly influenced by various external sources (Vogt, 1969). Synthesis of infectious virus is not automatically accompanied by morphological transformation (Rubin, 1960b; Trager and Rubin, 1964). Probably, the physiological status of a cell strongly influences the chance of neoplastic conversion following the integration of the sarcoma virus genome.

3. Multipotential Oncogenicity

An important question is whether avian sarcoma viruses have a pluripotency with regard to oncogenicity. The occurrence of lymphomatosis following infection with RSV (Burmester and Walter, 1961) can easily be explained by coinfection with a leukosis virus.

Altaner and Hlavayova (1973) were able to transform *in vitro* rat liver cells with B77 avian sarcoma virus. The transformed cells retained their epitheloid morphology. Biggs *et al.* (1973) tested two nontransforming mutants of cloned competent RSV for oncogenicity *in vivo*. In contrast to the parental RSV strains, the mutants did not induce sarcomas which otherwise would appear within 1 week. Both mutants evoked erythroblastosis and lymphoid leukosis. One mutant induced osteopetrosis and an osteochondrosarcoma as well. The most likely explanation is that the parental RSV has a multipotential oncogenicity, which is usually overshadowed by the sarcomatogenic property. The oncogenicity for, e.g., hemopoietic tissues, is coded by different genes than the ones for sarcomatogenic conversion, which have been lost or mutated in the two mutants.

4. Immunological Response

The amount of virus recovered from RSV-induced tumors is initially proportional to the quantity of virus in the inoculum (Bryan *et al.*, 1955). During subsequent growth of the tumor the amount of virus rapidly declined. However, there is ample evidence that cell-mediated immunity strongly reacts against virus-producing tumor cells (Rubin, 1962). The immune reaction seems to be mainly directed against the viral coat, since tolerance to the virus leads to progressive growth of virus-producing tumor cells. Antibodies against the virus do not seem to play a role in the elimination of a tumor.

The emerging virus-free tumors are probably derived from cells, which have been transformed after infection with a single defective RSV particle. Often such tumors will ultimately regress, indicating that they also evoke an immunological response, although weaker than the viral envelope.

By means of immuno-electron microscopy, Gelderblom *et al.* (1972) demonstrated that RSV-transformed cells have on their surface not only a viral envelope antigen but also an additional tumor-specific antigen. The same, or at least a cross-reactive, antigen can be found on RSV-transformed mammalian cells (Kurth and Bauer, 1972b; Gelderblom and Bauer, 1973). Antibodies to this antigen can be elicited in chickens by infection with avian leukosis viruses or nontransforming mutants of RSV (Kurth and Bauer, 1972a). This would indicate that this group-specific tumor antigen is also formed in other cell types.

B. Avian Leukosis Viruses

A great variety of chicken viruses have been isolated which are associated with tumors of the reticuloendothelial system (for reviews, see Gross, 1970; Burmester and Purchase, 1970). Viruses, which concur with RSV (Rous-associated viruses, RAV) and provide helper functions to the defective sarcomatogenic genome (Hanafusa, 1965) also belong to this group. Their leukemogenic activity still remains to be established in many cases. Well-established leukosis viruses also display the aforementioned helper activity (Vogt, 1964). Preinfection of chicken fibroblasts with a leukosis virus strongly inhibits focus formation by a subsequent challenge with RSV (Rubin, 1960a, 1961). The various RAV strains can induce a similar degree of resistance (Hanafusa, 1965; Vogt, 1965b).

The predominant reticuloendothelial malignancies in chickens are lymphoproliferative diseases. Visceral lymphomatosis, induced by oncornaviruses, may not be confused with the Marek's disease (neurolymphomatosis). The latter disease entity, which is caused by a herpesvirus (Churchill and Biggs, 1967) can easily be separated from visceral lymphomatosis following histological and cytological examination (Biggs and Payne, 1964).

1. Transmission and Immunity

Oncornaviruses, which induce visceral lymphomatosis, seem to occur in most commercial chicken flocks, and by sexual maturity the majority of the birds become infected. The incidence of clinical manifestations of infection is generally rather low (Burmester and Purchase, 1970). It is obvious that immunological factors play an important role in this. These viruses are horizontally transmitted by close contact (Burmester and Gentry, 1954) or "vertically" transmitted by the eggs (Burmester, 1962). Viremic roosters do not transmit the viruses, however (Rubin *et al.*, 1961). Horizontal transmission leads to humoral immunity to the virus, while "vertical" transmission leads to immunological "tolerance." The

immunological responders usually are not viremic and in most cases do not shed virus into their eggs (Rubin *et al.*, 1961, 1962).

2. *Oncogenic Spectrum*

Laboratory strains of avian leukosis virus often display a broad spectrum with regard to the type of induced neoplasms (Burmester and Purchase, 1970). The problem of whether these lesions are due to a single viral genotype or that the laboratory strains represent mixtures of viruses with a different oncogenic potential can usually be answered by the use of cloned virus only. The availability of plaque assays for avian leukosis viruses (Kawai and Hanafusa, 1972; Graf, 1972) makes it now possible to purify the various ALV strains.

The MC29 strain of ALV has a tremendously broad host response with regard to oncogenicity (Langlois *et al.*, 1970). The viral complex can transform chicken embryonic fibroblasts *in vitro* (Langlois and Beard, 1967), but sarcoma induction has rarely been observed for MC29. The transforming principle proves to be defective (Ishizaki *et al.*, 1971). A non-focus-forming agent has been isolated from MC29, by terminal dilution, which induces erythroblastosis, but not myeloid leukosis; renal adenomas; hepatocytomas; or mesetheliomas (Langlois *et al.*, 1971). Rescue of the transforming principle from nonproducing MC29-transformed cells by the associated erythroblastosis virus leads to complete restoration of the oncogenic spectrum of parental MC29 (Ishizaki *et al.*, 1971). This finding indicates that the defective transforming entity is multipotential as far as oncogenicity is concerned.

3. *Subgroups*

The avian leukosis viruses are momentarily divided into five subgroups (see Table II). The subgroups A and B were delineated by Vogt and Ishizaki (1965, 1966a,b) and Ishizaki and Vogt (1966) on the basis of reciprocal patterns of interference, genetic susceptibility, and immunological neutralization. Preinfection of cells with a virus from subgroup A only interferes with A pseudotypes of RSV, i.e., defective RSV that is phenotypically mixed with a virus of subgroup A. Chicken cell phenotypes were found which are resistant to subgroup A (C/A) but not to B and vice versa. Phenotypes (C/O) were detected which are resistant to neither subgroup. The pattern of genetic susceptibility coincided beautifully with serotypes and interference patterns.

The genetically controlled resistance to an ALV subgroup is explained by a lack of receptor for viruses of that serotype (Rubin, 1965). Interference, on the other hand, is assumed to be due to blocking of the

TABLE II

Classification of Avian Tumor Viruses on the Basis of Host Range (H), Serotype (S), and Interference (I)[a]

Tested against subgroup	Subgroup				
	A	B	C	D	E
A	$H^+I^+S^+$	$H^-I^-S^-$	$H^-I^-S^-$	H^-I^-S	$H^{+/-}I^-$
B		$H^+I^+S^+$	$H^-I^-S^-$	$H^{+/-}I^+S^{+/-}$	H^+I^+
C			$H^+I^+S^+$	$H^{+/-}I^-S^-$	H^+I^-
D				$H^+I^+S^+$	I^+
E					H^+I^+

[a] +, same host range, serotype, or mutual interference; +/−, some members have this property in common.

subgroup-specific receptor by the preinfecting virus (Steck and Rubin, 1966).

Later on Duff and Vogt (1969) recognized two additional subgroups, which displayed a somewhat more complicated pattern of host range. Some C/B phenotypes proved also to be resistant to viruses of the new subgroup C, whereas C/A are fully susceptible. Phenotypes, which are resistant to subgroup B, are semiresistant to subgroup D, irrespective of resistance to the A and C types. The B and D subgroups display some cross-reaction with regard to interference and neutralization.

Great differences in host range are displayed by the four subgroups when cultures of embryonic fibroblasts from different avian species, such as the turkey or ringneck pheasant, are employed (Vogt, 1970).

So-called nonproducing RSV-transformed cells sometimes release a transforming particle, with a different host range than the initial transformant (Weiss, 1967; 1969a,b; Vogt, 1967; Hanafusa and Hanafusa, 1968). Most phenotypes are resistant to this new type called E by T. Hanafusa *et al.* (1970b). Cells of several other avian species are quite susceptible to RSV with an E-type coat (Vogt, 1970). Viruses from subgroup B and D interfere with E.

C. Murine Sarcoma Viruses

Sarcomatogenic viruses have been isolated from mice infected with a murine leukemia virus (Harvey, 1964; Moloney, 1966; Kirsten and Mayer, 1967). Subsequent passage of one of the MSV strains (Moloney) in NB rats led to the isolation of an osteosarcoma virus (Soehner and

Dmochowski, 1969). An osteosarcoma virus has also been isolated from a spontaneous bone tumor in mice (Finkel *et al.*, 1966). Gazdar *et al.* (1971, 1972a,b) retrieved an interesting sarcoma virus from a spontaneous sarcoma in (NZW × NZB)F_1 mice. Some mouse strains have a fairly high incidence of spontaneous sarcoma (Mühlbock, in Bentvelzen, 1972b), and it seems worthwhile to attempt isolation of a sarcoma virus from them.

Ball *et al.* (1973) inoculated a nontransforming C type virus, isolated from murine JLS-V9 cells, into several mouse strains and obtained a rapid development of sarcomas. These tumors produced a sarcoma virus that is also transforming *in vitro*. These authors hypothesized that the JLS-V9 virus associates with an endogenous murine sarcoma virus by either phenotypic mixing or genetic recombination.

1. Pathogenic Spectrum

MSV preparations have been reported to be capable of inducing various types of tumors. The most remarkable finding in this respect is that MSV can transform cloned murine renal epithelial cells, and that upon transplantation, the transformed cells produced anaplastic medullary carcinomas with tubule formation (Ikawa *et al.*, 1970). Liver cells have been reported to be transformed by MSV while retaining their epitheloid morphology (Altaner and Hlavayova, 1973; Ikawa *et al.*, 1973). Upon transplantation the transformed cells produced hepatomas (Ikawa *et al.*, 1973).

Although the Harvey and Moloney strains of MSV have been derived from the same leukemia virus strain (Moloney), they display pronounced pathological differences. The sarcomas induced in three mouse strains are quite similar (Berman and Allison, 1969), although the Harvey strain-induced tumors display more endotheliomatous features. Simons and McCully (1970), however, observed in Prince Henry mice that the tumors induced by the Harvey strain (MSV-H) exhibit more angiosarcomatous features, while the Moloney strain (MSV-M) lesions are characterized by large, bizarre giant cells. Upon oronasal administration of equal amounts of virus, as titrated by focus formation *in vitro*, MSV-H induced vasoformative lesions in the lung and hemorrhages throughout the whole body in BALB/c mice. MSV-M only induces muscular tumors in addition to lymphatic leukemia (McCully *et al.*, 1971). Also *in vitro* the two MSV-strains display differences in the morphology of induced foci (Simons *et al.*, 1969; Simons, 1970).

Another pronounced difference between MSV-H and MSV-M is the rapid induction of erythroblastosis, characterized by splenomegaly, by the Harvey strain (Chesterman *et al.*, 1966). Attempts to separate the

sarcoma-inducing principle from the erythroblastosis-inducing component by means of terminal dilution have been unsuccessful, while a lymphocytic-inducing virus could be easily isolated in this way (Harvey, 1968). Infection of a nonproducing MSV-H induced tumor cell line with a lymphocytic leukemia virus leads to the release of a virus that induces both sarcomas and erythroblastosis (Bassin *et al.*, 1968). It seems that the MSV-H genome is capable of the induction of both lesions.

The Kirsten isolate of MSV is also associated with erythroblastosis (Kirsten and Mayer, 1967). The virus preparation contains an erythroblastosis virus as a helper (Somers and Kirsten, 1969), but the defective sarcoma genome may be erythroblastosis-inducing by itself (Kirsten, in Hirsch and Harvey, 1969).

Taylor *et al.* (1972) derived an erythroblastosis virus from cell cultures of MSV-M-induced rat rhabdomyosarcomas, which proved to be separate from the sarcomatogenic entity. It seems that the Moloney leukemia virus (MuLV-M) is a mixture of a lymphocytic leukemia virus and sarcomatogenic and erythroblastosis–inducing entities. The latter two are probably defective and helped by the lymphocytic component. The lymphatic leukemia virus usually overshadows the oncogenic activity of the two defective principles, although they may occasionally dominate. Varet *et al.* (1971) noted that treatment of MuLV-M-infected mice with antilymphocytic serum induced erythroblastosis! The Harvey isolate may contain a defective genome, which is a recombinant of the truly sarcomatogenic principle of MSV-M and the erythroblastosis virus.

2. *Pathogenesis of Sarcomas*

Extensive pathological descriptions of sarcoma formation by MSV have been given by Chesterman *et al.* (1966), Stanton *et al.* (1968), Berman and Allison (1969), and Simons and McCully (1970). The earliest change observed is the massive accumulation of edema in the connective tissue, followed by the formation of foci of fusiform cells in areas where the edema borders mesenchymal structures. It seems, at least in the early stages, that the tumor does not so much proceed from proliferation of transformed cells but from continuous recruitment of contiguous mesenchymal cells by the virus (Berman and Allison, 1969; Siegler, 1970b). Stanton *et al.* (1968), who regard these lesions as granulomatas, and Siegler (1970a,b) refuse to accept MSV tumors as being neoplastic. Siegler (1970a,b) is of the opinion that the mesenchyme proliferation for repair of virus-induced tissue injury. As long as the host's immune system is unable to keep the virus under control, the proliferation will proceed.

With some effort transplant lines can be developed from every tumor (Berman and Allison, 1969). It therefore seems that truely neoplastic cells are present in the lesion. In the initial phase mainly reactive proliferation is responsible for the growth of the tumor, but in the second phase neoplastic growth may be dominating.

3. Defectiveness

Defectiveness of MSV and its dependence upon murine leukemic virus is suggested by the following.

a. The two-hit titration pattern of focus formation by MSV in mouse cells, which is convertible to a linear pattern by coinfection with a murine leukemia virus (MuLV)
b. The observed inability of transformed cells to produce foci in agar
c. The suppression of focus development by antisera directed against the outer coat of the coinfecting leukemia virus (Hartley and Rowe, 1966; Huebner *et al.*, 1966; Huebner, 1967)

Levy and Rowe (1971) infected mouse embryo cells with low doses of MSV. Eighteen hours later the cells were treated with trypsin, which very effectively removes extracellular virus. They then added MuLV or plated the cells on embryo cells, which were infected with MuLV. The cells, which were exposed to MSV before, were capable of giving rise to foci following superinfection with MuLV. These results conform with the hypothesis that the MSV particle with a MuLV envelope penetrates the cell and causes its morphological transformation. Coinfection with MuLV would then give rise to the production of MSV particles with a MuLV coat and straight MuLV virions. The MSV particles can infect and transform neighboring cells which, if infected with the two classes of virions, can also reproduce MSV particles. The foci of transformed cells would then develop not so much from the proliferation of a single neoplastically converted cell but rather from the spread of virus (Fig. 1). This concept certainly would support Sieglers' (1970a,b) hypothesis that *in vivo* MSV tumors result only from reactive proliferations and not from true neoplastic cells.

This idea proves to be somewhat too simplistic, since several continuous MSV-transformed cell lines have been developed (Berman and Allison, 1969; Aaronson and Rowe, 1970; Bassin *et al.*, 1970; Somers and Kit, 1971; Massicot *et al.*, 1971). Following infection with MSV "normal" BALB/3T3 cells give rise to very small colonies in agar (Aaronson *et al.*, 1970); this phenomenon exhibits single hit kinetics. It must be noted, however, that the continuous cell lines have been derived from tumors or well-established "normal" cell lines instead of primary or

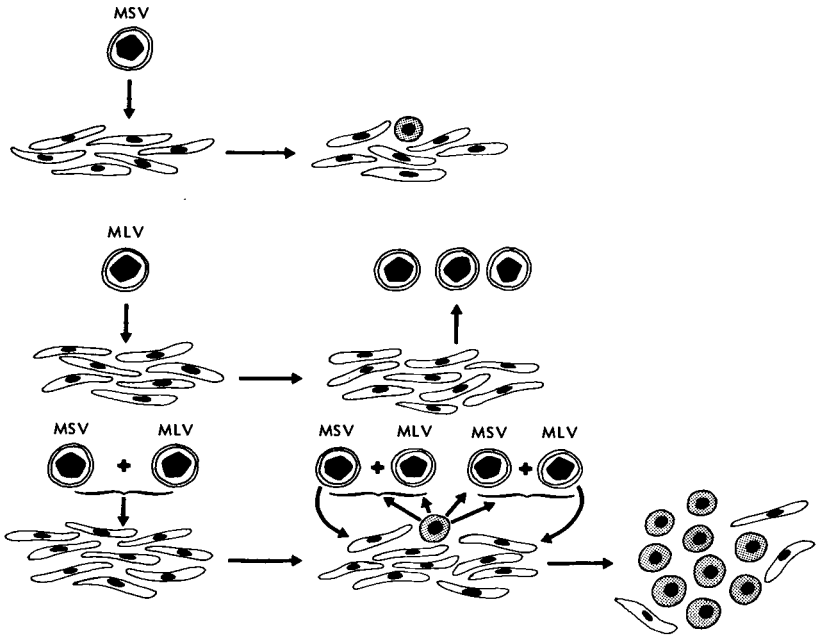

FIG. 1. Focus formation by the MSV–MuLV complex.

secondary embryo cell cultures. Nevertheless, MSV seems to be able to bestow some neoplastic potential on mouse cells.

Nonproducing MSV-transformed cell lines do not synthesize the group-specific internal antigen of the murine sarcoma-leukemia virus group (Aaronson and Rowe, 1970), or at least not all the antigenic components (Fischinger *et al.*, 1972). This would be unlike the avian tumor system where, for instance, RSV-transformed mammalian cells do produce the avian group-specific antigen (Huebner *et al.*, 1964). However, the Gazdar strain of MSV seems to be able to code for this antigen in so-called nonproducing cells (Gazdar *et al.*, 1972b; Sarma *et al.*, 1973).

Peebles *et al.* (1971) studied the rescue kinetics of MSV following MuLV infection on a nonproducing MSV-transformed cell line. There proves to be a 9–12-hour eclipse period followed by simultaneous release of MSV and MuLV. The production of MSV closely paralleled that of MuLV, indicating that replication of MuLV is needed for the release of MSV and that the leukemia virus provides a "late" function, presumably synthesis of a viral envelope, to the defective MSV. Rowe (1971) used an even more elegant method in a study of the same phenomenon. He could identify individual cells for both MSV and MuLV

production and observed that almost every MSV-induced focus coincided with a MuLV-induced plaque in the XC test (Rowe *et al.*, 1970).

In two cloned lines of 3T3 cells transformed by MSV, Bassin *et al.* (1971a) detected the release of C type particles, which are noninfectious, to diverse susceptible cell species because they lack reverse transcriptase.

Stocks of MSV sometimes contain a competent focus-inducing principle (Guillemain *et al.*, 1968; O'Connor and Fischinger, 1968; Fischinger and O'Connor, 1968, 1969a; Hahn *et al.*, 1970). Sedimentation analysis on sucrose gradients and filtration experiments indicated that the "competent" particles represent interviral aggragates of MSV pseudotypes and MuLV (O'Connor and Fischinger, 1969). Such aggregates are formed during ultracentrifugation. When defective MSV is centrifuged together with an excess of another leukemia virus, new pseudotypes may originate that have an altered host range (Fischinger and O'Connor, 1969c).

4. Transspecies Transfer of MSV

The various strains of mouse sarcoma virus can induce tumors in rats (Harvey, 1964; Kirsten and Mayer, 1967; Perk *et al.*, 1968; Soehner and Dmochowski, 1969) and in hamsters (Harvey, 1964; Huebner *et al.*, 1966; Klement *et al.*, 1969b; Soehner and Dmochowski, 1969). *In vitro* transformation has been described for rat (Ting, 1966), hamster (Bernard *et al.*, 1967; Simons *et al.*, 1967a), and bovine cells (Thomas *et al.*, 1968). An interesting feature is the morphological transformation of human fibroblasts by MSV (Boiron *et al.*, 1969; Bernard *et al.*, 1969; Aaronson and Todaro, 1970). Especially cell lines from individuals with neoplasia and genetic or chromosomal abnormalities are generally more susceptible to transformation by MSV than cells from normal individuals or fetuses (Klement *et al.*, 1971).

Often MSV-transformed cells from other species are nonproducing. They may produce some C type particles, which are noninfectious (Somers and Kit, 1971) probably because they lack reverse transcriptase (May *et al.*, 1972; Peebles *et al.*, 1972). With the DNA product made in an endogenous reaction of MuLV, Benveniste and Scolnick (1973) observed the presence of hybridizable RNA in MSV-transformed normal rat kidney cells. This would indicate that the defective MSV genome has some homology with MuLV. Tsuchida *et al.* (1972) made an intriguing observation that in productive MSV-transformed rat and mouse cells 35 S and 20 S RNA subunits are found which hybridize with the MSV product, while in nonproducing hamster cells only hybridizable elements of 35 S are detected. This may imply that the MuLV genome provides a

20 S subunit, which codes for late functions that the MSV genome lacks. Stephenson and Aaronson (1971) hybridized the DNA products of MSV-K and MuLV-K with the RNA of both viruses. They concluded that the defective MSV genome shows less than 50% homology with the RNA of the leukemia virus.

The virus can be rescued from nonproducing xenogeneic cells either by superinfection with MuLV, giving rise to the original MSV (Huebner *et al.*, 1966), or by a leukemia virus from a different species, giving rise to new pseudotypes with a different host range (Sarma *et al.*, 1970). Those pseudotypes would carry the group-specific internal antigen from the superinfecting leukemia virus and not from the murine virus group. However, Gazdar-MSV, rescued by feline leukemia virus, produces both kinds of group-specific antigen (Sarma *et al.*, 1973).

Cell fusion, mediated by inactivated Sendai virus, is the most efficient method of rescue between a nonproducing MSV tumor line and cells, which shed leukemia virus (Kelloff *et al.*, 1971). As early as 2 hours after fusion infectious virus is released, while this takes 9–12 hours following superinfection (Peebles *et al.*, 1971). In the case of superinfection the MuLV must first go through a reproductive cycle (reverse transcription–integration–transcription) before being able to provide late functions, while in the heterokaryon these are immediately available. It takes about 70 minutes from the end of synthesis of viral RNA until budding of complete virions occurs (Bader, 1970).

Clones derived from heterokaryons of a MSV-hamster tumor cell line and MuLV-infected "normal" mouse cells differ in their ability to produce MSV or MuLV (Long *et al.*, 1972). It is assumed that the balance between the chromosomes of both species is of utmost importance for the rate of production of either virus and that the chromosomal constitution differs from one clone to the other.

Stephenson *et al.* (1972a) described morphological revertants of MSV-transformed rat cells, which still contain the viral genome as determined by rescue following MuLV infection. These revertants, which display several physiological attributes of normal rat cells (low glucose uptake and saturation density, etc.), are highly resistant to retransformation by MSV but not by the unrelated SV40 (Stephenson *et al.*, 1973). It seems that a host genetic factor specifically blocks the transforming activity of the MSV genome, but does not induce resistance to transformation.

Sometimes MSV-transformed cell lines of various species release transforming particles with a different host range and serotype than the original mouse virus (Ting, 1967, 1968; Bassin *et al.*, 1968; Klement *et al.*, 1969b; Sarma *et al.*, 1970). The shift in host range observed for

MSV cultured in human cells (Aaronson, 1971a; Bernard *et al.*, 1972) is most interesting. Since Aaronson (1971b) isolated a rat-specific helper virus from a rat-tropic MSV, it seems a probable hypothesis that the MSV, which specifically grows in human cells, has associated with a human helper virus. Bernard *et al.* (1972) observed, however, that human cells supported growth of a virus, which is leukemogenic in mice, but that sarcomatogenic potential for that species was lost. In this case phenotypic mixing between the MSV genome and a human helper virus seems unlikely, since mouse-tropic MSV must then also be produced. The possibility that a stable genetic recombinant has arisen, from MSV with a human-specific "leukemia virus," giving rise to a competent human-specific transforming virus, cannot be excluded. This new virus would not phenotypically mix with MuLV.

5. Immunobiology

Inoculation of newborn mice with MSV leads to the progressive growth of sarcomas, whereas in adult mice the tumor, if it appears at all, will regress sooner or later (Fefer *et al.*, 1967b). The ability of mice to resist progressive tumor growth develops with age in correlation with the maturation of the immune system (Fefer, 1969; Zisblatt *et al.*, 1970). Neonatal thymectomy, which impairs the development of immune competence to a great extent, also causes progressive growth of tumors induced by MSV after inoculation at adult age (Law and Ting, 1965). This effect can be largely abolished by the administration of thymosin, a thymus-derived "hormone" that can restore the immunological abilities of neonatally thymectomized mice to a great extent (Zisblatt *et al.*, 1970).

Treatment of adult mice with anti-lymphocytic serum renders them susceptible to either the induction of tumors by low doses of virus (Law *et al.*, 1968a; Zisblatt and Lilly, 1972) or to progressive growth of tumors, which otherwise would regress (Varet *et al.*, 1968; Hook *et al.*, 1969; Zisblatt and Lilly, 1972). Inoculation with living BCG makes adult mice, depending on the protocol, either partially or completely resistant to MSV sarcomatogenesis (Schwartz *et al.*, 1971). All these findings indicate that an immunological reaction either prevents the induction of tumors by low doses of virus or causes the regression of those appearing after inoculation with a high dose.

Immune sera against MSV when repeatedly injected into neonatally infected mice can prevent the development of tumors (Bubeník *et al.*, 1969). The neutralizing activity of the sera correlated well with the rate of protection against tumorigenesis. Such sera could sometimes also inhibit the growth of transplanted MSV tumors, but enhancement was

also observed. This study suggests that in the initial stages of tumorigenesis, a humoral response may play an important protective role. With Sieglers' (1970a,b) concept in mind, that the initial proliferation is only for repair of tissue injury by the virus and that the lesion proceeds by continuous recruitment of mesenchymal cells by the virus, it will be understandable that interception of the virus by neutralizing antibodies will moderate this proliferation to a large extent.

Serum from mice with regressing tumors contains neutralizing antibodies (Fefer *et al.*, 1967b, 1968; Schlom *et al.*, 1970; McCoy *et al.*, 1972a). This might indicate that these antibodies are instrumental in the destruction of the tumor. However, transfer of spleen cells from mice, in which the tumor regresses, can interfere with the growth of MSV tumors in the recipients (Hellström *et al.*, 1969; Fefer, 1969) even when their immune system has been impaired by cyclophosphamide (Fefer, 1970) or sublethal irradiation (Pollack, 1971). This might imply that cell-mediated immunity is responsible for the regression of tumors in adults.

Hellström and Hellström (1969, 1970) observed that lymph node cells from mice bearing MSV tumors could inhibit the development of colonies of MSV tumor cells *in vitro*. Sera from mice with progressively growing tumors could block this colony inhibition, while sera from mice in which tumors started to regress had no such an effect. Skurzak *et al.* (1972) observed, that, depending on the concentration, sera from tumor-bearing mice could either enhance or reduce the cytotoxic effect of immune lymphocytes on MSV tumor cells. No clear-cut correlation was found with the "progressor or regressor" status in this test.

Leclerc *et al.* (1972) demonstrated anti-tumor cytotoxic activity of lymph node cells from mice with MSV-induced tumors by means of the chromium release test. They observed that cytotoxic cells rapidly disappeared in progressors, while they persisted considerably longer in the regressors. Sera from either kind of mice did not block this cytotoxicity. They favor the hypothesis that in progressors cytotoxic lymphoid cells are blocked *in vivo* by a circulating soluble factor.

Transplantation antigenicity has been well established for MSV-induced tumors by Fefer *et al.* (1967a), Law and Ting (1968), Law *et al.* (1968b), Koldovsky *et al.* (1968), and Chuat *et al.* (1969). Transplantation immunity can be directed against the virus itself (Koldovsky *et al.*, 1968) or against antigens, which are virus-coded but not part of the envelope of the inducing virus (Law, 1972; McCoy *et al.*, 1972b). The latter type of antigens have been detected by immunization with non-virus-producing tumors. The possibility that such tumors would release another virus, unrelated to the inducing virus, must not be excluded, however remote that it may seem. Stephenson and Aaronson (1972a)

TABLE III

Laboratory Strains of Murine Leukemia Virus

Name	Abbreviation	Predominant lesion	Host range	New cellular antigen
Gross leukemia virus	GLV	Lymphosarcoma	N	G
Friend leukemia virus	FLV	Erythroblastosis	N	FMR
Graffi leukemia virus	GiLV	Myeloid leukemia	NB	FMR
Moloney leukemia virus	MoLV	Lymphosarcoma	NB	FMR
Radiation-induced leukemia virus	RadLV	Lymphosarcoma	B	G
Rauscher leukemia virus	RLV	Erythroblastosis	NB	FMR

failed to evoke transplantation immunity with their nonproducing transformed mouse cells. Strouk *et al.* (1972) could not detect serologically distinct cell surface antigens on MSV-transformed cells.

In the chromium release test of Leclerc *et al.* (1972), the lymph node cells seems to detect other antigens as serologically defined cell-surface antigens, which are coded by the murine leukemia viruses (Old *et al.*, 1963a,b, 1964, 1965; Boyse *et al.*, 1964). By means of immuno-electron microscopy Aoki *et al.* (1973) detected in several MSV-transformed cell lines an antigen, which is distinct from the viral envelope of various MuLV strains and MuLV-coded cell surface antigens. The antigen did not occur on spontaneously transformed cells or on those transformed by X irradiation.

D. Murine Leukemia Viruses

Every self-respecting scientist in the mouse leukemia field seems to have felt it necessary to have isolated his own leukemia virus, which resulted in an infinite list of MuLV strains. The most widely used strains are (1) the Gross virus (GLV) isolated from spontaneous lymphosarcomas of AKR mice (Gross, 1951); (2) Friend virus (FLV), an erythroblastosis virus, recovered after inoculation of a cell-free extract of Ehrlich ascites tumor (Friend, 1956, 1957); (3) Graffi virus (GiLV), a myeloid leukemia virus isolated from a transplantable reticulum sarcoma (Graffi, 1957); (4) Moloney virus (MoLV), a lymphatic leukemia virus retrieved from an anaplastic sarcoma (Moloney, 1959, 1960); (5) radiation-induced virus (RadLV), a lymphatic leukemia virus, obtained from a radiation-induced lymphoma (Lieberman and Kaplan, 1959); and (6) Rauscher virus (RLV), an erythroblastosis virus, which fortuitously developed upon repeated passage in BALB/c mice of cell-free material, originally derived from a virus-induced leukemia in adult Swiss mice. An extensive and sometimes touching description of the discovery of these strains of MuLV is given by Gross (1970) (see Table III).

All these strains have been potentiated in their virulence by repeated passage. By mutation and mixing, either phenotypically or genetically with endogenous viruses of their hosts, they may have evolved into mixtures of virus types that differ in pathogenic properties, virulence, and host range. Exemplary in this respect is GiMLV; this virus induced various forms of myeloid and lymphoid leukemia. Depending on the leukemia type, further passage leads to the development of viruses with a less pluriform pathogenic spectrum (Graffi *et al.*, 1966). Following inoculation with the standard GiLV in the preleukemic phase, cell-free extracts are made from the separate organs, a similar pattern of *Aufsplit-*

terung (splitting) is observed (Fey, 1969). The explanation that each organ would provide different histiotropic receptors to the virion does not seem acceptable. More likely, the various target organs support better replication of the corresponding oncogenic virus over that of the different passengers.

For the student of the natural history of leukemia, naturally occurring viruses which induce reticulum cell sarcomas (Fujinaga *et al.*, 1970; Ebbesen *et al.*, 1973) are far more interesting.

1. Classification of MuLV Strains

The MuLV strains can be divided according to their serotype in serum neutralization reactions into a group with GLV as prototype on the one hand (G), and a group with FLV, MoLV and RLV on the other (FMR) (Rowe *et al.*, 1966). Most "naturally occurring" MuLV strains seem to belong to the G group, while various "laboratory artifacts" belong to the FMR group (Hartley *et al.*, 1969). The Gazdar strain of MSV, isolated from a spontaneous tumor of a (NZW × NZB)F_1 mouse, has a helper virus from the FMR subgroup (Gazdar *et al.*, 1972a).

The classification on the basis of neutralization corresponds generally with that of a new cell surface antigen, as detected by cytotoxicity tests (Old *et al.*, 1963a,b, 1964, 1965; for a review, see Pasternak, 1969). According to Levy *et al.* (1968) GiLV induces a new cellular antigen, which cross-reacts with FMR. This observation does not coincide with the pattern of virus neutralization (Steeves and Axelrad, 1967; Levy *et al.*, 1968, 1969).

Heterologous antisera detect a common viral envelope antigen in neutralization tests (Gross, 1965; Igel *et al.*, 1967; McCoy *et al.*, 1968; Levy *et al.*, 1969). In addition, they detect, by means of immunodiffusion, a group-specific soluble internal antigen of the nucleoid (Geering *et al.*, 1966; Fink *et al.*, 1968a; Gregoriades and Old, 1969; Schäfer *et al.*, 1969). We observed that in addition to group-specific determinants this antigen contained type-specific determinants that differ for GLV, RLV, and B/T-LV (Tennant, 1965), respectively (P. Bentvelzen, J. Brinkhof, and S. Offers, unpublished results, 1972).

A completely different classification of the MuLV strains was made on the basis of their host range. Kaplan (1967) observed that RadLV grows better in other mouse strains than does GLV. On the basis of the growth of MuLV in embryo cell cultures of either BALB/c or NIH-Swiss mouse strains, Hartley *et al.* (1970) were able to classify the virus strains in three categories: one which grows well in BALB/c and not in NIH-Swiss (B-tropic), another which exhibits the reverse pattern (N-tropic), and a third group which grows well in both kinds of cells (NB-

tropic). The *in vivo* results of Kaplan (1967) correlate well with these *in vitro* observations.

These differences in tropism do not correspond with serological classification in contrast to the subgroup classification of avian leukosis viruses (Vogt and Ishizaki, 1965). In the latter system resistance proves to be of a more absolute nature, while in the murine system some virus replication still takes place when cells are infected with a virus of adverse tropism.

By means of hybridization of the DNA product of one virus strain with the RNA of another strain, pronounced genomic differences can be found (between GLV, RLV, and MoLV), especially when the melting temperature of the hybrid is taken into account (Haapala and Fischinger, 1973). It is still too early to recognize a subgroup pattern useful for classification.

2. *Pathogenic Spectrum*

The various murine leukemia virus strains can induce a very great variety of neoplastic afflictions of the reticuloendothelial system, ranging from polycythemia to reticulum cell sarcomas or chloromas. The predominant lesion, however, is a diffuse, poorly differentiated lymphosarcoma. This tumor can be found in every mouse strain, usually in a low incidence, except in the C58 and AKR mouse strains. Upon treatment with various carcinogens, such as ionizing radiation or a broad variety of chemicals, lymphosarcomas may appear in a high incidence (for a review, see Kaplan, 1967, 1972).

One virus strain can induce many different lesions, but even in the case of the "Gross Passage A virus" (Gross, 1966), it is doubtful whether this is due to a single viral genotype. Since leukemia viruses can now be plaque-purified in the XC test of Rowe *et al.* (1970), this problem can be settled at the moment.

Host factors are also very important in this respect. Conventional RF mice will develop many myeloid leukemias after X irradiation, while germ-free mice develop lymphosarcomas only (Walburg *et al.*, 1965). The viral etiology of both lesions is well established (Jenkins and Upton, 1969).

3. *Pathogenesis of Various Murine Leukemias*

In most mouse strains the thymus is important for the induction of lymphosarcomas. Thymectomy abrogates their early development, but ectopic transplantation of thymuses restores the suspectibility to this disease (Kaplan *et al.*, 1956; Haran-Ghera *et al.*, 1966). It must be noted that in thymectomized mice lymphosarcomas may appear very

late in life in a moderately high incidence (van Bekkum and Balner, 1973).

In radiation leukemogenesis an important aspect is the injury to the thymus, which is followed by an influx of immature lymphoblastic cells, also seen in the neonate organ. It is believed that this cell type is the target for RadLV (Kaplan, 1972).

Recently, a virus has been separated from MoLV which induces lymphosarcomas that do not involve the thymus (Abelson and Rabstein, 1970). Only on the basis of morphological descriptions, Siegler *et al.* (1972) speculated that this malignancy is not a true neoplasm but a reactive proliferative disease as they had also postulated earlier (Siegler, 1970a,b) for MSV-induced sarcomas. They assume that the virus-infected lymphocytes would not be taken up by the lymph nodes. These organs would then, by normal physiological mechanisms, induce proliferation of stem cells in the bone marrow. Since these cells are infected, they will not differentiate into physiologically mature lymphocytes, which can be taken up by the lymph nodes. There will be therefore a continuous stimulation of the bone marrow, giving it the appearance of a neoplastic disease. This tempting theory must certainly be followed up by extensive experimentation, but is cited here because of its relevance to the work of our laboratory on the pathogenesis of erythroblastosis induction by RLV.

Both RLV and FLV upon inoculation in mice induce a rapidly developing erythroblastosis, which is characterized by splenomegaly and an excess of nucleated red cells in the peripheral blood. Transplantation of leukemic cells readily reproduces the disease, but by means of chromosomal markers it became clear that the resulting leukemia consisted mainly of host cells (Takada *et al.*, 1971; Brommer, 1972; Brommer and Bentvelzen, 1973).

A multi-hit spleen focus formation in several mouse strains was observed by Steeves and Eckner (1970), and Steeves *et al.* (1971) for FLV and by Bentvelzen *et al.* (1972b) for a peculiar RLV preparation. In both cases it could be made acceptable that the focus-forming virus (SFFV) would be defective and needs coinfection with a helper virus. In analogy to the induction of MSV foci *in vitro* (Levy and Rowe, 1971), it was assumed that this defectivity of SFFV would be in its inability to reproduce by itself and that such ability would be necessary for focus formation. This implies that the foci do not develop by proliferation of a single transformed cell but from the spread of SFFV and its helper virus (Bentvelzen *et al.*, 1972b), By passage of RLV through relatively resistant hosts, such as C57BL mice, a lymphatic leukemia virus could be separated from the erythroblastosis agent (Ishimoto and Maeda, 1970).

Thomas and Axelrad (1968) claim to have developed a focus assay for true tumor cells in FLV-induced erythroblastosis. Inoculation of leukemic spleen cells into unirradiated hybrid hosts, which are resistant to focus formation by FLV, would support growth of tumor cells from the susceptible parent. Bentvelzen (1972d) investigated an anologous system for RLV and found indeed that the hybrid (C57BL × CBA)F_1 is resistant to focus formation by cell-free RLV as compared to parental CBA. Leukemic CBA cells produced many colonies in this hybrid, but a high dose of radiation (up to 6000 rad) did not drastically reduce this. It was concluded that the intact cell provides a great protection to the infectivity of RLV as was also found for GLV by Hays (1972). The majority of the colonies did not develop from true neoplastic cells.

With a great effort, transplantable tumors have been developed from FLV-induced leukemias (Buffet and Furth, 1959; Friend and Haddad, 1960; Dawson *et al.*, 1963). Such tumors are classified as reticulum cell sarcomas. They may also be grown in tissue culture (Friend *et al.*, 1966; Patuleia and Friend, 1967). Treatment of such cultures with dimethyl sulfoxide induces the synthesis of hemoglobin, indicating that these cell lines stem from an erythroid precursor cell, in which erythroid maturation has been blocked (Friend *et al.*, 1971).

Some of these tumors do not produce virus. Infection of these tumors with a lymphatic virus leads to the retrieval of SFFV (Fieldsteel *et al.*, 1969). Infection with other MuLV strains has the same effect (Fieldsteel *et al.*, 1971; Dawson and Fieldsteel, 1973), including a lymphatic leukemia virus isolated from FLV (Dawson *et al.*, 1966). The helper viruses determine, to a great extent, the host range of SFFV, but the C57BL mouse strain exhibits a great innate resistance to focus formation, irrespective of the helper virus (Dawson and Fieldsteel, 1973).

As far I know, only one group has been able to develop continuous erythroid cell lines derived from RLV-induced erythroblastosis (Miyoshi *et al.*, 1968, 1969). Numerous attempts in our own laboratory have been unsuccessful. The picture emerges that in FLV, but especially in RLV, induction of erythroblastosis, true tumor cells will appear in a slow rate and that most of the proliferation is reactive, owing to the gradually arising lack of physiologically active erythroid cells. Leukemic mice have an elevated level of erythropoietin in their serum, although not as high as might be anticipated on the basis of the extremely low hematocrits (Ebert *et al.*, 1972; Camiscoli *et al.*, 1972).

Pluznik *et al.* (1966) manipulated the erythroid compartment in mice by inducing hypoxia or polycythemia and observed a pronounced influence on RLV induction of spleen foci. They concluded that a primitive erythroid cell would be the target cell for the virus. Siegel and Morton

(1966a,b) observed an impaired immunological response in RLV-infected mice, and they postulated a competitive effect of the antigens and RLV on the pluripotent hemopoietic stem cell for differentiation into antibody-producing cells or leukemic erythroblasts. Pretreatment of mice with different antigens enhances the leukemic response (Brommer and Bentvelzen, 1973). It has been demonstrated that antigenic stimulation induces proliferation of the hemopoietic stem cell (Boggs *et al.*, 1967; McNeill, 1970). However, a prolonged immunization interferes with the splenomegalic response (Siegel and Morton, 1967, 1969). This phenomenon was attributed to a decrease in available target cells for the virus as a consequence of their commitment to immunological reactivity.

Pretreatment with anti-thrombocyte serum, which does not seem to have an influence on the erythroid compartment, increases the splenomegaly, indicating that a common cell of the erythroid and megakaryocytic compartments is involved in the origin of the disease. The pattern of changes in the number of stem cells in the spleen corresponds with the alterations in average spleen weight or number of spleen foci following inoculation with RLV (Brommer and Bentvelzen, 1973).

Preirradiation of the host initially decreases the susceptibility to RLV, but several days after the irradiation an overshoot reaction will take place (Brommer and Bentvelzen, 1973; Seidel, 1973). This pattern correlates well with the variation in stem cells after irradiation. All of these data seem to suggest that the hemopoietic stem cell is the primary target for erythroblastosis induction by RLV and presumably also by FLV.

The hemopoietic stem cell of RLV-infected mice are able to overcome the bone marrow syndrome of supralethally irradiated mice (Brommer, 1972; Brommer and Bentvelzen, 1973). The restored mice will gradually develop erythroblastosis. It has been hypothesized that upon a differentiation signal into the erythroid direction, an RLV-infected stem cell will become transformed and will not mature along the erythroid pathway (Fig. 2).

With regard to the polycythemia-inducing variant of FLV, sufficient evidence has accumulated that erythropoiesis proceeds in the absence of erythropoietin (Mirand, 1967; Tambourin and Wendling, 1971; Stephenson *et al.*, 1972b). This phenomenon can be regarded as autonomous proliferation, characteristic for neoplasia, although in this case normal maturation occurs.

The reactive nature of RLV-induced erythroblastosis is also evident from the fact that hypertransfusion diminishes the splenomegalic response (Dunn *et al.*, 1966; Brommer, 1972; Seidel, 1972). The transfused mice still display a so-called "hiatus leukaemicus" according to Seidel (1972), in which he interprets that RLV transforms the stem cell

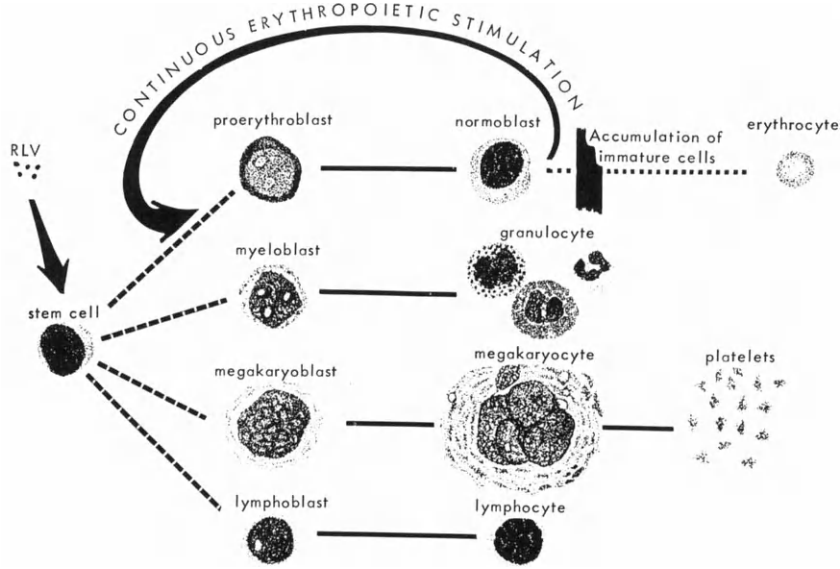

FIG. 2. Schematic representation of the induction of erythroblastosis by RLV.

and when the abnormal erythroid proliferation is inhibited, the aberrant granulopoietic process will continue. The possibility must not be overlooked, that both diseases are induced by separate principles in the RLV mixture.

We investigated the growth pattern *in vitro* of numerous RLV-induced myeloid leukemias. They display a very poor growth rate, but need less "hormone" than normal cells to develop clones in agar. The serum of leukemic mice contains a high level of a colony-stimulating factor, presumably the granulopoietic hormone (Bentvelzen *et al.*, 1974). These findings coincide with a similar model as developed for erythroblastosis (see Fig. 2), but momentarily any indication that the myeloproliferative disease is reactive and that the stem cell is involved is lacking.

4. MuLV in Vitro

Several strains of MuLV can be successfully propagated in tissue culture (Manaker *et al.*, 1960; Ginsberg and Sachs, 1961; Gross, 1961). Transformation has seldom been observed, as was discussed in Section IV. In a series of publications from Huebner's groups, it was indicated that infection of cells with an MuLV makes them much more susceptible to *in vitro* transformation by carcinogenic compounds (Freeman *et al.*,

1970; Price *et al.*, 1971; Rhim *et al.*, 1972a). By preinfection with MuLV the transforming action and presumably also oncogenicity could be established from condensates of city smog (Freeman *et al.*, 1971; Rhim *et al.*, 1972b) and even from a cannabinoid (Price *et al.*, 1972).

The hypothesis has been advanced that the carcinogenic compounds would derepress the transforming oncogenes of MuLV (Huebner and Gilden, 1972). An alternative hypothesis could be that MuLV provides endogenous oncogenes the attributes (for example, by reverse transcriptase) to amplify and to express themselves as envisaged in Temin's (1971) protovirus theory or germinal–somatic provirus hypothesis by Bentvelzen and Daams (1972).

It seems that in one way or the other MuLV makes cells more prone to transformation events. MuLV-infected cells are also far more susceptible to conversion by the oncogenic DNA viruses SV40 (Rhim *et al.*, 1971a) and polyoma (Rhim *et al.*, 1971b) than uninfected ones.

Infection of mouse cells with RLV leads to a rapid attenuation of the virus (Hartley *et al.*, 1965; Barski and Youn, 1965, 1966; Wright and Lasfargues, 1966; Schlom *et al.*, 1971). For the virus passaged *in vivo* the LD_{50} is 10^6 particles, but for the virus passaged a long time *in vitro*, the doses must be 10^{11} particles (Barbieri and Barski, 1973). It is possible that the virus genetically alters in tissue culture or that selective propagation of avirulent types in the RLV mixture takes place. It is interesting to investigate whether such attenuation also take place *in vivo* and whether this would be associated with regression, which is observed following inoculation with low doses of virus.

Rauscher leukemia virus can also grow in human embryonic cells (Wright and Korol, 1969; Chandra *et al.*, 1970; Traul *et al.*, 1972; Ablashi *et al.*, 1972). The virus becomes rapidly attenuated with respect to leukemia induction in mice (Wright and Korol, 1969; Ablashi *et al.*, 1972). After serial passage in human cells, RLV loses its ability to infect mouse cells, but can be propagated in human cells. This human cell passaged RLV still contains the MuLV group-specific antigen, but serum neutralization studies strongly suggest that this viral form has obtained new envelope antigens (Wright and Korol, 1969; Traul *et al.*, 1972; Ablashi *et al.*, 1972). The possibility that RLV has obtained this new coat from a human oncornavirus, either by phenotypic mixing or by true genetic recombination, should be considered.

The human diploid cell line WI38 is restrictive for MoLV. When infected mouse cells are fused with WI38 nonpermissiveness for MoLV prevails (Tennant and Richter, 1972). Loss of many human chromosomes by the cell hybrid leads to the reappearance of the virus, suggesting that human genes exert a repressive control over mouse virus expression.

5. *Immunobiology*

Mice treated with formalin-activated FLV show significant resistance to subsequent challenge with live virus (Friend, 1959). The host therefore seems to be capable of an immune response against the virus. It is also able to produce transplantation immunity to isografts of MuLV-induced lymphomas (Klein *et al.*, 1962; Pasternak *et al.*, 1962; Sachs, 1962).

Immunization of mice with virus-induced leukemic cells leads to formation of cytotoxic antibodies (Old *et al.*, 1963a,b, 1964, 1965). An extensive discussion of the nature of the detected antigens is given by Pasternak (1969). It is not clear at the moment whether the cell surface antigen FMR, induced by FLV, MoLV and RLV, is nonvirion or not (F. Lilly, personal communication to the author).

By means of immunoelectron microscopy Aoki *et al.* (1970) observed that GLV seldomly buds from regions, which contain the so-called G antigen as detected by mouse antisera. This would indicate that this antigen would be nonvirion. Rat antisera to GLV-transformed cells detect two more classes of G cell surface antigens (Stockert *et al.*, 1971), one of which is the viral envelope (Aoki, 1971). Another is a determinant that can also be found on normal thymocytes of some mouse strains. In that case it is an alloantigen, whose expression is controlled by two separate genes (Stockert *et al.*, 1971, 1972; Boyse *et al.*, 1972).

The antigen detected by mouse antisera is a very weak transplantation antigen (Aoki and Johnson, 1972). Its expression can be modulated in immunized hosts, i.e., it will not be formed temporarily under influence of immunosuppression as has also been described for the TL alloantigen (Boyse *et al.*, 1967). A similar finding has been reported by Ioachim *et al.* (1972) for the intracytoplasmic synthesis of the group-specific internal antigen of MuLV in GLV-induced RLV leukemias. The appearance of this antigen is temporarily halted during passage in immunized rats, but can recur in tissue culture.

Adult mice are rather resistant to GLV as compared to RLV and FLV. This might indicate a strong immunological response against GLV. However, mice do not produce many neutralizing antibodies upon immunization with GLV (Wahren, 1964). Passive immunization with mouse antisera to a GLV-induced lymphoma does not retard the growth of transplants despite their *in vitro* cytotoxicity (Aoki and Johnson, 1972), while passive immunization with rat antisera leads to rejection (Old *et al.*, 1967). It seems that neither neutralizing nor cytotoxic antibodies play a great role in the immunologically mediated resistance of adult mice to GLV.

Both FLV (Friend, 1959) and RLV (Fink *et al.*, 1966) can evoke the

production of neutralizing antibodies in mice. The administration of statolon, a double-stranded RNA-containing mycophage, suppress leukemogenesis by FLV for a considerable length of time (Wheelock *et al.*, 1969). During this induced latency phase mice produce many neutralizing antibodies to the virus, but also produce antibodies that are cytotoxic to leukemic cells as tested both *in vivo* and *in vitro*. All antibody levels decline when the mice started to develop leukemia (Wheelock *et al.*, 1972). These data would suggest that in the FLV system humoral immunity would play a great protective role. Rich and Clymer (1971) observed that mice in which the Friend disease has regressed after inoculation with a special strain of FLV do contain many neutralizing antibodies in their serum, but are equally susceptible to reinfection with the standard FLV as normal control mice. This would be in conflict with the idea that humoral immunity would provide protection, but these studies must be repeated on a more quantitative basis.

In the closely related RLV system McCoy *et al.* (1972c) observed that neonatally infected C57BL mice produce antibodies to RLV-producing cells for a long period, while the ability to produce transplantation immunity to RLV tumors does not persist for such a long time and disappears concurrently with the development of primary lymphomas. These studies may suggest that humoral immunity would not play any role in the relative resistance of the C57BL mouse strain to RLV.

McCoy and associates (1972c) studied an antibody that reacts in the indirect membrane immunofluorescence test with an RLV-induced lymphoma. This antibody may be quite different from the ones that neutralize virus or that are cytotoxic *in vivo*.

Glynn *et al.* (1969) could cure a transplantable MoLV-induced lymphoma by the combined therapy of cyclophosphamide and immune spleen cells. Neither treatment alone had any success. This experiment would suggest that cell-mediated immunity can to some extent control MoLV leukemogenesis.

Haran-Ghera (1970a,b, 1971) has collected sufficient evidence that the RadLV is highly antigenic to its natural host, the C57BL mouse strain. Immunosuppression is necessary for lymphoma induction when the virus is inoculated into adult mice. Inoculation of newborn C57BL mice leads to a strong transplantation immunity to RadLV-induced lymphoma cells and to a high titer of neutralizing antibodies to the virus. After about 6 months both immunological responses rapidly decline, concomitant with the development of lymphomas (Haran-Ghera, 1972). The question arises here, too, whether or not humoral immunity is involved in resistance to leukemogenesis.

Inoculation of newborn mice with a MuLV leads to a relative state of immunological inertia in most mouse strains with regard to the virus or virus-associated antigens (Axelrad, 1965; Klein and Klein, 1966; Chieco-Bianchi *et al.*, 1967). The AKR mouse strain, which hereditarily carries GLV and releases this virus early in life (Rowe and Pincus, 1972; Hilgers *et al.* 1972b), also displays an immune deficiency to this agent (Hays, 1973). There is no true tolerance to the virus, however, as deposits of immune complexes of virus-specific antigens can be found in AKR kidneys in association with glomerulonephritis (Oldstone *et al.*, 1972).

Inoculation of a small number of syngeneic leukemia cells after administration of BCG leads to a strong inhibition of spontaneous leukemogenesis in this mouse strain (Lemonde *et al.*, 1971). This inhibition is not achieved by BCG alone. It seems that potentiation of the weak immunity to a cross-reactive antigen can influence the course of spontaneous leukemogenesis. This result is of great importance for cancer prevention.

A chronic infection with the intracellular parasite *Besnoitia jellisoni* leads to a significant delay in the spontaneous development of lymphosarcomas in AKR mice (Lunde and Gelderman, 1971). The authors assume that the activation of macrophages by the protozoan infection leads to a greater resistance to tumor development. It must be noted that severely immunosuppressed mice can be protected against RLV leukemogenesis by pyran copolymer, which exerts its influence through activation of macrophages rather than by potentiation of the cell-mediated immunity (Hirsch *et al.*, 1972).

From this discussion the picture that emerges is that much more research is needed on the question of which hematological compartments are involved in control of viral leukemogenesis in mice. It may be anticipated that pronounced differences will be found between the various MuLV strains, depending upon their biology.

E. Mouse Mammary Tumor Viruses

For more than three decades it has been realized that mammary carcinomas in mice result from the interaction of a virus, hormonal stimulation, and a suitable genetic constitution (Bittner, 1942). The last aspect proves to be a relative one, since genetic resistance can be overcome by increasing the hormonal stimulation (Mühlbock and Boot, 1959, 1960; Heston, 1964; Heston *et al.*, 1972). The problem of whether or not the late appearing tumors, which are induced by such excessive hormonal

stimulation, are due to a virus needs attention. In several cases, the viral etiology could be established, and I believe that every mammary tumor in mice is caused by an MTV or at least a related entity; however the evidence for this opinion is rather circumstantial as yet. All investigators in the mammary tumor field will agree upon one thing: Hormonal stimulation of the mammary gland is an absolute necessity for the induction of these tumors. The main principle in this is the pituitary hormone prolactin, which is released after estrogenic stimulation. The ovarian hormone progesterone can act as a cocarcinogen to prolactin (Mühlbock and Boot, 1967; Boot, 1969).

The standard mammary tumor virus strain MTV is generally known as the "milk factor." It was found that in high mammary cancer strains a maternal influence is responsible for the early development of mammary neoplasia (Staff of the Roscoe B. Jackson Memorial Laboratory, 1933; Korteweg, 1934). This factor proved to be transmitted by the mother's milk to the offspring (Bittner, 1936, 1937). Most research in the MTV field is still being done with this "milk factor," but since then several other MTV strains have been discovered which exhibit another pattern of vertical transmission in their hosts (Bentvelzen, 1968a, 1972b; Mühlbock and Bentvelzen, 1968; Bentvelzen *et al.*, 1970b). In most mouse strains that develop a high incidence of mammary cancer before 1 year of age, the virus is transmitted as a milk factor.

The biology of the various MTV strains has recently been discussed thoroughly by Blair (1971a), Hageman *et al.* (1972), Bentvelzen (1972c), and Nandi and McGrath (1973). A few main points and some new material will be presented in the following survey.

1. Pathogenesis

The development of mammary tumors is usually preceded by the development of many hyperplastic alveolar nodules in the mammary gland (van Rijssel, 1956). These lesions are thought to be preneoplastic precursory stages of mammary carcinoma (DeOme *et al.*, 1959, 1962). One of their abnormal features is that they can grow indefinitely during serial transplantation without hormonal stimulation.

In neonatally infected mice, these lesions can be observed after the first pregnancy, and the number increases with age. They can be induced by artificial hormonal stimulation (Nandi, 1963a,b), and by means of pituitary isografts they can be induced within 6 weeks (Nandi *et al.*, 1971). One must take into account that with this methods several weeks are required for the regression of normal mammary tissue before the hyperplastic nodules may become discernible. This signifies that they have originated within a few weeks after the beginning of the hormonal stimulation. Several months are necessary before a nodule will become a

true carcinoma. Immunological factors will certainly play a role, but probably hyperplastic cells must undergo many changes before a true neoplasm will emerge.

Foulds (1956) described another lesion of the mammary gland which appears in certain mouse strains during pregnancy, attains maximum size by the time of parturition, and regresses soon thereafter. These lesions may reappear during subsequent pregnancies and may evolve to pregnancy-independent carcinomas. These lesions, unfortunately called plaques by Foulds (1956), are regarded by most experts as premalignant, although they may become very large tumors by continued hormonal stimulation. Mainly progesterone and some estrogens are needed for the growth of these lesions (van Nie and Dux, 1971). They can rapidly be induced in GR mice by administration of 17α-ethynyl-19-nortestosterone, which exerts a strong progestative and a weak estrogenic effect (van Nie *et al.*, 1972). The tumors are able to regress to normal mammary ductal elements (Nair, in Nandi and McGrath, 1973). A resemblance comes to mind with the hormone-dependent erythroblastosis induced by RLV, but also with hormone-independent FLV-induced polycythemia, in which normal maturation can occur.

2. MTV Strains

Eight different mammary tumor virus strains have been described thus far (see Table IV). The "milk factor," isolated from American high cancer strains is indicated with MTV-S by Bentvelzen and Daams (1969). It may be expected that the isolates of the various mouse strains will differ in virulence and serology, but this has not yet been studied in great detail. The MTV-S virus (group) is characterized by the rapid induction of hyperplastic nodules, which subsequently are transformed into hormone-independent carcinomas. Their milk transmission, in contrast to other modes of vertical transmission by other MTV strains, is an artificial attribute, which will not be used for classification.

The virus, which is present in the GR, DD, and RIII mouse strains, induces the pregnancy-dependent plaques (also called P tumors by van Nie and Dux, 1971). This virus strain MTV-P is slightly less virulent than MTV-S. It is also serologically distinct (Blair, 1971b; Nowinski and Sarkar, 1972; Hageman *et al.*, 1972; Daams *et al.*, 1973) and displays a somewhat different host range (Mühlbock and Dux, 1971).

In some American sublines of the RIII strain, the resident MTV strain has lost its ability to induce plaques but has gained in virulence. It is antigenically distinct from the MTV-S of C3H mice and displays the same host range as MTV-P from GR. This variant has been named MTV-PS (see Schlom *et al.*, 1973).

When American high mammary cancer strains are liberated from their

TABLE IV

Laboratory Strains of the Murine Mammary Tumor Virus

Strains	Abbreviation	Prototype host strain	Type of tumor	Virulence	Antigenic determinants[a]					
					A	B	C	D	E	F
Standard	S	C3H	Hormone-independent fast-growing carcinoma	+++	+	+	+	+	−	−
Plaque inducing	P	GR	Hormone-dependent P tumors	++	+	+	−	+	−	−
Low oncogenic	L	C3Hf	Hormone-independent slow-growing carcinoma	+	−	+	+	−	+	−
Wild	W	Wild mice	Hormone-independent fast-growing carcinoma	+++	+	−	+	−	−	+
"Overlooked"	O	BALB/c	Hormone-independent fast-growing carcinoma	+++	not tested					
X-ray induced	X	O20	Hormone-independent fast-growing carcinoma	+++	not tested					
	Y	C57BL	?	?	not tested					

[a] According to Blair (1971b).

MTV-S by means of foster nursing of the young on low strain foster mothers, the derived sublines will still carry an avirulent form of MTV, called MTV-L (Bentvelzen and Daams, 1969). This virus strain has also been known as nodule inducing virus (Nandi, 1966), but it is inferior in this respect to MTV-S. Dmochowski *et al.* (1963) claimed to have found oncogenicity for this virus strain. In time-consuming experiments Hageman *et al.* (1972) succeeded in affirming the tumor-inducing activity of this virus after an average of 17 months. MTV-L is antigenically quite distinct from MTV-S with which it occurs in the same mouse strains (Nowinski and Sarkar, 1972; Hageman *et al.*, 1972; Daams *et al.*, 1973).

The BALB/c mouse strain is a low mammary cancer strain, which seems to be free of any MTV. Hageman *et al.* (1972) succeeded in isolating a virulent MTV from mammary glands of old retired breeders, which upon passage in BALB/c mice produces many mammary tumors at an early age. Since the BALB/c strain is so susceptible to infection with any MTV-strain, the virus found in old mice may be due to horizontal transmission. Daams and Hageman (1972) clearly demonstrated that this new virus MTV-O is serologically distinct from any other MTV strain used in their laboratory.

The O20 mouse strain is also a low mammary cancer strain. Irradiation in combination with a treatment with urethan induces early mammary tumors. These tumors contain typical B particles, and cell-free extracts can rapidly induce mammary tumors in BALB/c mice (Timmermans *et al.*, 1969). In view of its derivation from a radiation-induced tumor it has been called MTV-X.

The C57BL mouse strain is one of the most resistant strains to mammary cancer induction. Irradiation, especially when combined with urethane treatment or aging, induces the appearance of MTV-specific antigens in hemopoietic tissues (Bentvelzen, 1972b). Injection of blood cells from irradiated mice induces mammary tumors in otherwise resistant hosts (Boot *et al.*, 1971). Hybridization of cytoplasmic RNA of tissues from aged or carcinogen-treated C57BL mice with the DNA product of MTV occasionally revealed the presence of MTV-specific RNA (Schlom *et al.*, 1973).

No B particle could be found in this strain, not even following carcinogenic treatment. In cultures of C57BL kidney cells, J. Links, O. Tol, F. Buijs, and J. Calafat (unpublished results, 1973) succeeded in inducing the appearance of B particles (MTV virions) after treatment with methylcholanthrene. The oncogenic activity of this particle remains yet to be established. The latent MTV of the C57BL strain has been called MTV-Y by Bentvelzen *et al.* (1970b).

Andervont (1952) found a mammary tumor-inducing milk factor in

wild house mice. Upon passage in BALB/c mice, the agent proved to be a rather virulent one (Andervont and Dunn, 1956). It is possible that this virus strain has been genetically altered in the meantime. Blair (1971b) studied the antigenic determinants that contribute to the immunoprecipitation of intact virions of this virus in Ouchterlony plates. The MTV-W strain proves to have a unique viral coat antigen.

This enumeration of the MTV strains contain some elements of a classification, since all the MTV variants present in the artificially established low mammary cancer mouse strains, such as C3Hf, DBAf, and Af, have been lumped together as MTV-L, while all their virulent counterparts in the parental mouse strains are called MTV-S. A thorough investigation may reveal additional serological differences among members of both groups. The low oncogenic variant in DDf mice also induces the development of so-called plaques (Heston and Vlahakis, 1971). Probably this variant must be regarded as being separate from classical MTV-L. In that case I propose to call it MTV-LP.

Many more studies on serological markers and host range are needed before a good subgroup classification can be made as was achieved for the avian tumor viruses and to some extent for the murine leukemia viruses.

3. Epigenetic Influences on the Expression of Exogenous MTV

Following infection with MTV-S, bioactivity can be retrieved from many different organs (see Nandi and McGrath, 1973). Since infectivity is also found in peripheral blood (Bittner, 1945), the bioactivity of various organs can be due to contamination with blood or blood cells. Consensus has been reached concerning the fact that the virus replicates in hemopoietic tissues (Nandi *et al.*, 1972a; Hageman *et al.*, 1972; Hageman and Calafat, 1972).

The common site for synthesis and budding of complete virions in the mammary gland are the microvilli. B particles are most consistently observed in mammary cells, which are organized for synthesis and secretion of milk proteins (Nakayama, in Nandi and McGrath, 1973). Intracytoplasmic A particles, which are a precursory stage of the MTV virion (Tanaka *et al.*, 1972), are observed in most mammary cells irrespective of their organization for secretion (Nakayama, in Nandi and McGrath, 1973).

B particles (MTV virions) are found in male mice in the epididymis and seminal vesicles (Smith, 1966), which explains the observed infectivity of sperm from high cancer strain mice (Mühlbock, 1950). Virus production by the male accessory sex organs is not associated with malignant transformation.

The state of MTV in the hemopoietic tissues is intriguing. Extensive electron microscopic search did not indicate the presence of B particles (Daams *et al.*, 1970). Incidentally intracytoplasmic A particles can be found in hemopoietic organs of old RIII mice and several lymphomas (Nowinski *et al.*, 1971; Hilgers *et al.*, 1972a; Tanaka *et al.*, 1972; Hilgers and Galesloot, 1973). The internal group-specific antigen of MTV can be observed in the spleen of several mouse strains infected with MTV-S or MTV-L (Hilgers *et al.*, 1972a). An MTV-associated cell surface antigen can be detected on spleen cells by means of immunofluorescence (Daams, 1970; Daams *et al.*, 1970). Immunization with spleen cells from infected animals can evoke transplantation immunity to mammary tumors (Rejthar, 1972; Rejthar and Wotke, 1973).

Preparation of cell-free extracts of peripheral blood cells or hemopoietic tissues drastically reduces the infectivity (Nandi *et al.*, 1972b; Hageman *et al.*, 1972; Hageman and Calafat, 1972). In contrast to Nandi and McGrath (1973), we observed that hemopoietic cells, which have been irradiated with 3000 rads under oxygenated conditions, did not lose their infectivity (P. Bentvelzen and J. Brinkhof, unpublished results, 1973). The viability of the cell does not seem to be a prerequisite for the transfer of the virus. Integrity of the cell surface seems to protect this subviral form of MTV. After lysis the infectivity remains with the ghosts (Hageman and Calafat, 1972). This may eventually be explained by the presence of a few intact cells in the ghost preparation. Titration studies can easily settle this problem. At the moment, I am of the opinion that the subviral form of MTV needs large cell membrane structures for the preservation of its infectivity. It seems that in hemopoietic cells, the virus is not fully expressed. Since transfer of blood cells induces the appearance of B particles in the mammary gland of the recipients (Nandi, 1967), it can be concluded that the whole viral genome is present, but that epigenetic factors prevent its complete expression.

As infectivity appears earlier in the peripheral blood than in the mammary gland (Ritter and Nandi, 1968a,b), a life cycle for MTV has been postulated by Nandi (1967) (see Fig. 3). The virus would first infect the hemopoietic tissues and the blood cell-bound form of the virus would subsequently infect the mammary gland. Ingested B particles would be unable to infect the gland directly. Since the subviral form carried by the peripheral blood cells is not infectious in the cell-free state, it is tempting to assume that infection of the mammary gland takes place by means of intercellular bridges.

By means of a complement-fixation assay for the MTV group-specific antigen, Parks *et al.* (1972b) found that kidneys of MTV-infected mice are consistently positive for MTV. Whether this is due to replication of

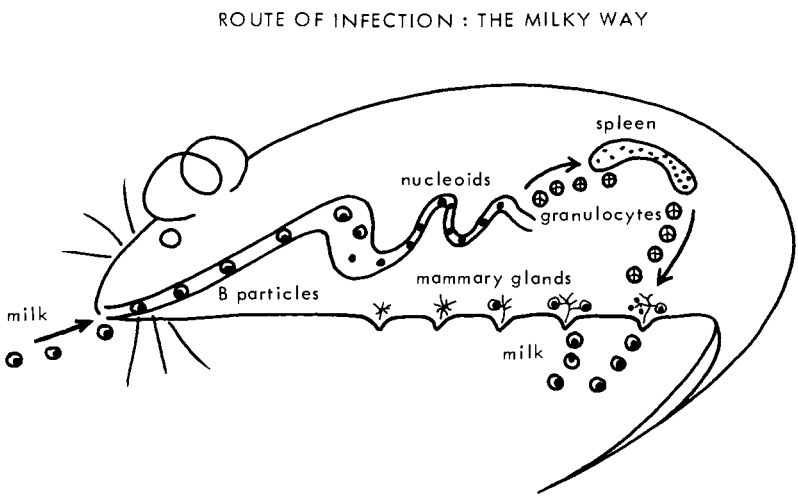

FIG. 3. Life cycle of the mouse mammary tumor virus.

MTV in the kidney or to deposits of immune complexes cannot yet be determined. Links *et al.* (1972) achieved replication of MTV in cultures of baby mouse kidney cells, and we recently observed that kidney cells of BALB/cfC3H mice can transmit MTV (P. Bentvelzen and J. Brinkhof, unpublished results 1973). Schlom and co-workers (1973) detected MTV-specific RNA in kidneys of high mammary cancer strain mice with the DNA product of MTV as a probe. It seems that MTV can indeed replicate in the kidney.

We have never observed the appearance of kidney tumors under the influence of MTV. Felluga *et al.* (1969), however, found a change of a high mammary cancer strain toward a renal tumor strain. These tumors contain intracytoplasmic A particles and produce virions that have some resemblance to B particles. Possibly, the MTV in this strain has mutated into a kidney tumor virus.

MTV-specific antigens and intracytoplasmic A particles have been detected in a Leydig cell tumor (Nowinski *et al.*, 1971). We did find bioactivity of testicular cells (P. Bentvelzen and J. Brinkhof, unpublished results, 1973). MTV-specific RNA has been detected by Schlom *et al.* (1973) in testes of high mammary cancer strain mice. We never observed any testicular tumor to appear under influence of MTV, and therefore, we believe that the tumor studied by Nowinski *et al.* (1971) has arisen independently from the presence of MTV.

From the discussion above, the picture emerges that MTV can infect several tissues, but that the epigenetic state of the cell determines the

rate of expression. In some cell types only some viral antigens will be produced, while in others complete virions are released. In only one organ, the mammary gland, is malignant transformation a regular event that must be triggered by hormonal stimulation.

4. Immunobiology

The antigenicity of MTV to its host species, the mouse, has been well established by the induction of neutralizing antibodies (Blair, 1968; Bentvelzen, 1968a) and antibodies that can precipitate various MTV antigens in the double immunodiffusion test (Blair *et al.*, 1966; Nowinski *et al.*, 1967; Fink *et al.*, 1968b; Bentvelzen *et al.*, 1970a; Hilgers *et al.*, 1971a).

The failure of neonatally infected mice to produce cross-reactive transplantation immunity to MTV-S-induced mammary tumors has been interpreted to mean that such mice would be tolerant to the virus and virus-associated antigens (Morton, 1964, 1969; Weiss *et al.*, 1966). These mice do strongly react against unique transplantation antigens, invalidating the old claim that chemically induced tumors are essentially nonviral because of their non-cross-reactive transplantation antigenicity (Vaage, 1968a,b; Morton *et al.*, 1969a,b).

The humoral response to the virus is still intact as concluded from the ease with which precipitating antibodies against several MTV antigens can be produced (Blair *et al.*, 1966; Bentvelzen *et al.*, 1970a; Hilgers *et al.*, 1971a). Tumor-bearing mice of various strains have a high content of anti-viral precipitating antibodies in their serum (Bentvelzen *et al.*, 1970a; Müller *et al.*, 1971).

By means of the indirect immunofluorescence technique on mammary tumor slices Müller and Zotter (1973) and Zotter *et al.* (1973) detected that neonatally infected mice of both sexes already have antibodies against the viral coat early in life. The titer increases by age, so that antibodies are also detectable by immunoprecipitation. Zotter *et al.* (1973) observed *in utero* transmission of antibodies to the virus. Bentvelzen (1968b) found that infected C57BL mice could passively immunize their offspring to small amounts of MTV-S, whereas the susceptible BALB/c females could not.

By means of the macrophage-inhibition technique, Müller and Zotter (1972) found that this hematological compartment reacts against MTV-associated antigens. Working with another mouse strain and with the colony-inhibition test, Heppner (1969) found only very weak cellular immunity to cross-reactive mammary tumor antigens. Hyperimmunization of mice with MTV leads to an accelerated appearance of "spontaneous" mammary tumors in neonatally (Bentvelzen *et al.*, 1970a) or prena-

tally infected mice (Bentvelzen, 1972e). This earlier appearance of tumors is accompanied by the appearance of precipitating antibodies to the virus. This could be explained either by the fact that these are blocking antibodies, or that cellular immunity is readily exhausted by hyperimmunization on contrast to the humoral response.

Tumors induced by MTV-S are strongly antigenic to syngeneic mice without the virus. The evoked immunity is against a cross-reactive antigen. Zotter *et al.*, (1972) observed by means of immunoelectron microscopy that besides the virion another MTV-specific cell surface antigen exist. Its antigenic strength in transplantation is unknown.

It will be obvious that, as in several other tumor virus systems, continuous extensive research must be done for the elucidation of the role of various immunological systems and their interactions in the control of viral mammary tumorigenesis. The fact that in some instances a significant delay or even inhibition of mammary tumor development can be achieved, by immunological manipulation such as immunization with heated tumor cells (Check *et al.*, 1971), a soluble mammary tumor transplantation antigen (Irie and Irie, 1971), or another MTV-strain (van der Gugten and Bentvelzen, 1969), is encouraging. There is a great danger in multiple immunizations because sometimes tumors will appear even earlier than in the controls (Bentvelzen, 1972e).

VI. Comparative Biology of Avian and Murine Tumor Viruses

In Table V various biological aspects of the oncornaviruses isolated from birds and mice have been summarized. The minus signs may, in

TABLE V

Comparative Biology of the Oncornaviruses

	ASV	ALV	MSV	MuLV	MTV
Multipotential oncogenicity	+	+	+	−	−
Recruitment	+	−	+	+	−
Hormone dependency	−	−	−	+	+
Classification	+	+	−	+	−
Defectiveness	+	+	+	+	−
Interference	−	+	−	+	+
Transspecies transfer	+	+	+	+	−
Immunosuppression	−	+	−	+	+
Horizontal transmission	−	+	−	−	+
Vertical transmission	−	+	−	+	+

several cases, be replaced by a question mark. It often reflects ignorance concerning this aspect of the respective virus group. The plus signs may hold true for only a few members of that group. Most of this table is based upon data presented in the preceding pages. They will either not be discussed at all or only briefly mentioned below. No mention is made of aspects that were presented in Table I, such as *in vitro* transformation. Genetical aspects, such as susceptibility and genetic transmission, are also deleted. They will be discussed in the next section.

A. Recruitment

It is not likely that mammary tumors will grow by means of continuous recruitment of mammary cells by the virus, even not the P type of tumors. In the case of the murine leukemia viruses no recruitment has been observed for thymic-dependent lymphosarcomas, although such a phenomenon cannot be excluded in an early stage. Recruitment has not been reported for avian leukosis viruses, but the defectivity of the myelocytoma-inducing principle in MC29 ALV strain (Ishizaki *et al.*, 1971) suggests that this disease may proceed along this line.

B. Classification

The avian tumor virus subgroups can be neatly classified on the basis of their antigenicity, host range, and interference pattern, especially when the subgroups A and B are involved (Vogt, 1970). The murine leukemia viruses are divided on the basis of their serotypes into two groups (Pasternak, 1969), but this does not seem to have any association with host range (Hartley *et al.*, 1970). Theoretically six subgroups of murine leukemia viruses could be made on the basis of serology and host range, but representatives of two such groups (FMR-B and G-NB) have not yet been found.

C. Defectiveness

Thus far, I have failed to detect a single case of defectiveness in the murine mammary tumor virus system. Hageman *et al.* (1968) observed that high doses of MTV-L could help low doses of MTV-S in the induction of mammary tumors. This phenomenon need not be explained in terms of phenotypic mixing, but on the basis of overcoming the immunological response by a large dose of particles. Terminal dilution of MTV-S has not yet resulted in the isolation of a low oncogenic strain of

MTV, as was reported for FLV by Rowson and Parr (1970). MTV-L, which has been isolated from C3Hf mice, will certainly concur with MTV-S in the milk of C3H mice. The amount of MTV-L in the milk of C3Hf mice is low (Hageman *et al.*, 1968), but it is possible that the production of this virus strain is helped by coinfection with MTV-S. Evidence for this is lacking at the moment.

D. Interference

Both avian and murine leukosis viruses can interfere with the transformation of fibroblasts by the corresponding sarcoma virus, if it has the same envelope as the preinfecting virus (Rubin, 1960a; Sarma *et al.*, 1967). Preinfection of mice with a murine leukemia virus interferes with the induction of splenomegaly (Rowe, 1963) or spleen foci (Bentvelzen *et al.*, 1972b) by subsequent challenge with an erythroblastosis virus.

According to Nandi and DeOme (1965) the low oncogenic mammary tumor virus of C3Hf mice can interfere with subsequent challenge by MTV-S. According to Bentvelzen *et al.* (1970b), who titrated purified MTV-S preparations in several mouse strains, this resistance evoked by MTV-L is only of a moderate degree. DeOme *et al.* (1970) observed that hyperplastic mammary nodular outgrowths of BALB/c mice are resistant to superinfection with MTV-S when they carry MTV-L.

E. Transspecies Transfer

The avian sarcoma viruses can transform many cells of different vertebrate species and are also able to induce tumors in a great array of animals. The avian myeloblastosis virus has been reported to induce leukemia in hamsters (Lacour *et al.*, 1969) and to be reproduced in this malignancy (Delain *et al.*, 1969). The murine sarcoma viruses can only induce tumors in other rodents but can transform many different cell species *in vitro*. The murine leukemia viruses can replicate in cells of unrelated vertebrates, but induce leukemia only in rats. We have attempted to introduce the murine mammary tumor virus in rats and hamsters but the results were negative (P. Bentvelzen and J. H. Daams, unpublished results, 1968).

F. Immunosuppression

The RPL-12 strain of ALV has been reported to cause immunosuppression of the host (Peterson *et al.*, 1966; Purchase *et al.*, 1968). Coinfection with avian leukosis viruses of subgroups A + B leads

to a lesser antibody production to influenza (Meyers and Dougherty, 1971). The immunosuppression caused by murine leukemia viruses is well documented (Peterson *et al.*, 1963; Siegel and Morton, 1966a,b; Salaman and Wedderburn, 1966). This suppression is not only seen at the humoral level but also on the cellular one as measured by the response on phytohemagglutinin stimulation (Häyry *et al.*, 1970). In susceptible strains RLV blocks the synthesis of IgG (Toth *et al.*, 1971). It is not clear whether this is associated with a good replication of the virus in a susceptible host or that the immunosuppression is instrumental in the onset of the disease.

Peled and Haran-Ghera (1971) observed the immunosuppressive action of RadLV. They postulate that the observed immunodeficiency of aged C57BL mice may be due to accumulation of RadLV.

C3H mice, which have been neonatally infected with MTV-S, have a depressed ability to mount a hypersensitive reaction to methylated bovine serum albumin (Griswold *et al.*, 1973). According to Blair *et al.* (1971) mice infected with MTV-S also display an impaired response to sheep erythrocytes and a lower rate of rejection of foreign skin grafts. This immunosuppression at both levels becomes more severe with age. It seems that the residence of MTV in the hemopoietic tissues interferes with the functionability of immunocytes despite incomplete expression.

G. Horizontal Transmission

This pattern of transmission, i.e., from one individual to the other, irrespective of relationship, has been well established for the avian leukosis viruses (Burmester, 1962). Usually such infection does not lead to viremia, vertical transmission by the eggs, and leukosis during the short observation periods poultry breeders watch their flock. It would be interesting to study the effect of immunosuppression on chickens that have been infected in adult life.

Myers *et al.* (1970) found excretion of MuLV in the feces but not in oral swabs or urine. They did not find any evidence for horizontal transmission, however.

No evidence has been found for horizontal transmission of MTV by Mühlbock and Bentvelzen (1968); however, there is a single report that the virus can be transmitted by fleas (Pogossiantz, 1956).

H. Vertical Transmission

Milk-borne transmission has been observed for MTV (Bittner, 1936, 1937) and for MuLV (Law and Moloney, 1961; Gross, 1962; Krischke and Graffi, 1963). The mammary tumor virus can also be transmitted by

the male, although less efficiently than by the milk (for a review, see Mühlbock and Bentvelzen, 1968). I do not know of any report on transmission of MuLV or MTV by the ovum after infection of the mother. Transmission by the gametes in close association with the host genome will be discussed in Section VIII.

VII. Genetics of Susceptibility to Oncornaviruses

> Our understanding of the inheritance of many single-gene diseases is relatively advanced, but the same cannot be said for multiple-gene diseases. Quantitative genetics has long offered us ways of describing multiple-gene traits in terms of their population dynamics. But, for an understanding of the mechanisms whereby a complex genotype influences a biologic character, there seems to be no substitute for the lengthy process of identifying, mapping, and studying one by one the separate genes which act concertedly in each individual (F. Lilly, 1972b).

A. Avian Tumor Viruses

The dissection of polygenic systems that control the chickens susceptibility to virus induction of a malignancy has been considerably facilitated by the use of *in vitro* transformation by RSV. A recent review of such studies by Payne (1972) describes single autosomal loci that control susceptibility to various subgroups of avian tumor viruses. The three genes identified as controlling susceptibility to either subgroup A, B, or C are called *tva*, *tvb*, and *tvc*, respectively (Crittenden *et al.*, 1967). Susceptibility alleles are indicated with, for instance, tva^s, and their resistant allelomorphs with tva^r and so on. The susceptibility alleles prove to be dominant. Probably multiple alleles occur at such loci, leading to different levels of susceptibility as encountered in some inbred chicken lines (Payne and Biggs, 1964). Whereas most genes which confer resistance to specific subgroups are recessive, Dhaliwal (1963) observed a single case of full dominance of resistance. Payne (1972) also found a single rooster in a noninbred Rhode Island red flock which conferred resistance on all his progeny.

The genetics of susceptibility to subgroup D remains to be elucidated. With regard to susceptibility to the endogenous E-type virus, Payne *et al.* (1971) found a locus comparable to the loci controlling susceptibility to the other subgroups. In addition, they found another dominant gene that is epistatic to *tve* and confers resistance to subgroup E despite the presence of tve^s. This gene I^e proves to be at least associated, but probably identical with, the gene that controls the synthesis of the group-specific antigen of the avian tumor viruses in apparently normal cells as

detected by Payne and Chubb (1968; see also Pani and Payne, 1973; Crittenden *et al.*, 1973). Another gene has been described by Crittenden *et al.* (1973) that also causes the release of this viral antigen and is linked to the R_1 erythrocyte antigen. This gene is also coupled with an allele of the *tvb* locus (Crittenden *et al.*, 1970). This gene confers susceptibility to subgroup E, irrespective of the presence of I^e.

B. Murine Leukemia Viruses

A rapid breakthrough in the understanding of the genetic control of susceptibility to murine leukemia viruses has taken place during the last few years (for an excellent review, see Lilly and Pincus, 1973). The tropism of murine leukemia viruses for either Swiss NIH (N) or BALB/c (B) cells proves to be controlled by a single gene (Pincus *et al.*, 1971a). In this study it was found that resistance proves to be dominant, while in the avian system susceptibility usually proves to prevail in hybrids of resistant and susceptible strains. In the avian system there is sufficient evidence that in most cases resistance is associated with a block in the penetration or uncoating of the virus (Piraino, 1967; Crittenden, 1968). In this NB system inhibition of virus replication seems to be an intracellular event as has been elucidated by Fenyö *et al.* (1973) by the use of cell hybridization.

In a study on the susceptibility to spleen focus formation by FLV, Lilly (1970b) detected that two different genes control the response to the virus. One of these genes, *Fv-1*, proved to be identical with the *NB* locus (Pincus *et al.*, 1971b; Axelrad *et al.*, 1972) and suppresses virus growth. The other gene probably controls an intrinsic property of the erythroid compartment to develop erythroblast foci or erythroblastosis irrespective of virus replication (Axelrad *et al.*, 1972; Dawson and Fieldsteel, 1973; Odaka and Hino, 1973; Bennet and Eckner, 1973).

Ware and Axelrad (1972) tested the susceptibility of two congenic mouse strains for susceptibility to N- and B-tropic viruses *in vitro*. One of these mouse lines had been selected for resistance to N-tropic FLV. The differences in the spleen focus assay were reflected *in vitro*. The susceptible line proved to replicate N-tropic virus at a high rate, whereas the derived resistant congenic line is susceptible to B-tropic agents. Ware and Axelrad (1972) made the very interesting observation that when cells are infected by a virus with the proper tropism, single-hit kinetics can be observed, while in the case of adverse tropism, a multi-hit response is found. This can be interpreted that in the latter case more than one particle must penetrate the cell before replication will take place. A similar finding has been reported by Yoshikura (1973b).

The *histocompatibility-2* locus seems to play an important role in the induction of lymphosarcomas by GLV (Lilly *et al.*, 1964; Lilly, 1966, 1970a) or B/T-LV (Tennant and Snell, 1968) and the induction of splenomegaly by FLV (Lilly, 1968). In a congenic line of BALB/c differing only at the *H-2* locus, Lilly (1972a) observed a stronger immunological response to FMR antigens. It is possible that the resistance of this line to the induction of splenomegaly by low doses of virus can be attributed to this immunological response. An alternative hypothesis is that *H-2* controls the appearance of FMR. When under influence of *H-2*, FMR disappears and the tumor eventually escapes from immunological rejection. Persistence of FMR would lead to a strong antibody response. Mutations, which affect the hemopoietic system, also influence the response to MuLV (Steeves *et al.*, 1968; Axelrad, 1968).

C. Mammary Tumor Viruses

Although since the thirties (Korteweg, 1936) extensive genetic studies have been made on susceptibility to mammary cancer in mice, up till now there is little insight into the genetics of this malignancy (for a review, see Bentvelzen, 1972c). If only tumor incidences are taken into account in crosses between high and low cancer strains as presented by Bittner (1958), the data fits a single genic difference in susceptibility to this malignancy. But when also the distribution of tumor ages is considered, multifactorial inheritance becomes evident (Heston *et al.*, 1945).

In a series of backcrosses of the high cancer strain C3H to the low strain C57BL, the standard mammary tumor virus rapidly disappears (Heston *et al.*, 1956). This finding indicates that the number of genes, which affect the propagation of MTV-S, must be small. Mühlbock and Dux (1971) observed that in the C57BL strain substitution of the $H\text{-}2^b$ gene leads to a remarkable increase in susceptibility to MTV-S. Titration of purified MTV-S is necessary to establish subtle differences in susceptibility between these congenic C57BL lines and C3H.

Dux (1972) reported on the development of a congenic C57BL line in which selection had occurred for C3H strain genes that cause a large size in the mammary gland. This line carries the $H\text{-}2^b$ allele, but is quite susceptible to MTV-S. Also in this case titration studies must be done to establish the degree of susceptibility as compared to C3Hf. I have postulated that these two genes control the difference in susceptibility to MTV-S between the C3H and C57BL strains and that they have an additive effect (Bentvelzen, 1972c).

The understanding of the gene physiology of resistance to MTV is far more important than the formal genetics. The role of the *H-2* locus in

resistance to MTV-S suggests an immunological response. The C57BL strain proves to be a good antibody producer to MTV. Bentvelzen, 1968 a,b), but it is indeed doubtful whether this contributes to the resistance of tumorigenesis (Nandi and McGrath, 1973). It would also not explain the unresponsiveness of this locus to MTV-P (Mühlbock And Dux, 1971).

Hybrids of the resistant C57BL and I strains are fully susceptible to MTV-S (Andervont, 1943), indicating that different genes are involved in the determination of resistance. Transplantation of mammary tissues of either strain into this hybrid easily leads to the induction of nodules and tumors by MTV-S, indicating that the target itself is not refractory to the virus (Nandi *et al.*, 1966, 1972b; Nandi, 1967). It must be noted that Dux and Mühlbock (1968) observed in similar experiments that the C57BL mammary gland retains a great deal of resistance. According to Bentvelzen (1968a,b) the resistance of the C57BL strain is caused at different levels by different genetic systems with additive action.

The nodules, which arise in C57BL mammary glands following transplantation into MTV-infected (C57BL × I)F_1 mice, will produce tumors upon transplantation into C57BL mice. Transplantation of I strain nodules, obtained by a similar procedure, into I mice leads to rejection, suggesting a strong immunological mechanism (Nandi *et al.*, 1972b).

Susceptibility of MTV-S is positively correlated with replication of the virus (Hairstone *et al.*, 1964; Bentvelzen, 1968b; Bentvelzen *et al.*, 1970b). We assumed that many viral RNA molecules must be present in the target cell before neoplastic conversion can take place (Bentvelzen, 1968a; Bentvelzen and Daams, 1969; Bentvelzen *et al.*, 1970b). This problem can now be investigated by means of molecular hybridization, using MTV DNA product as a probe.

D. Comparative Aspects

Good humoral antiviral responses in some mouse strains have been reported for MSV, MuLV, and MTV (Fefer *et al.*, 1967c; McCoy *et al.*, 1967; Bentvelzen, 1968a,b). We do not know of similar findings in the avian tumor system. In the cases in which the malignant disease proceeds mainly by continuous recruitment of host cells by the virus, a good humoral response may be an important controlling factor. Since it will take some time before the onset of antibody production and the virus meanwhile will have entered its target cell, humoral responses will have no effect on the development of true neoplasms unless the antibodies are cytotoxic.

Genetic blocking at the cell surface has been reported for the avian

tumor virus system only. It may be that extensive investigations in the murine leukemia system would reveal the existence of a similar mechanism, but the possibility cannot be excluded that the murine viruses can penetrate every cell species. Mammary tumor virus particles cannot penetrate fibroblasts (Lasfargues *et al.*, 1970) and not even mammary cells (Nandi and McGrath, 1973). We found, however, that *in vivo* even the most resistant genotype replicate MTV in their mammary gland to some extent. With the Nandi's (1967) hypothesis of the life cycle of MTV in mind, one could envisage that a block for MTV could occur at the level of the hemopoietic tissues, although this does not seem to be the case.

Intracellular interference with the replication of an oncornavirus is observed with the murine leukemia and mammary tumor viruses but not with the avian leukosis viruses.

Genetic resistance to neoplastic conversion independent of replication of the virus has been detected for lymphoid leukosis induction by RPL 12 virus in chickens (Crittenden *et al.*, 1964). The *Fv-2* locus in mice controls the susceptibility of the erythroid compartment to the induction of erythroblast foci by FLV. The *H-2* locus might play a role in the development of true tumor cells in FLV-induced erythroblastosis by interference with membrane alterations. It may even do so in the development of thymic-dependent lymphosarcomas and mammary tumors.

Genetic factors in the regression of avian tumors have been discussed by Payne (1972). Since these tumors often proliferate by recruitment, it is not clear whether the genetic influence is achieved by a strong cell-mediated immunological response or by resistance to reinfection. The resistance of some mice to progression of MSV-induced sarcomas is most likely due to a good immunological response. The situation is all but clear in the murine leukemia system. In the mammary tumor system, the I strain is very likely to be resistant because of a good cellular immunity.

The five possible ways in which genes can exert their influence on oncogenesis have not been encountered with every tumor virus. This may be accidental, but can also be due to differences in the biology of the virus, such as tumorigenesis by recruitment or an omnipotency with regard to penetration.

VIII. Endogenous Oncornaviruses

> Medical research progresses in waves, and today viruses are riding the crest of the wave of cancer research. Three to four decades ago the genetics of the host was on the crest of the wave and virus was a forbidden word in cancer research . . . the

greater advances in cancer research in the future are going to be made at the union of the host genome and the virus (W. E. Heston, 1972).

A. Avian Leukosis Viruses

Dougherty and DiStefano (1966) observed a flock of leukosis-free chickens in which the embryos occasionally contained the group-specific antigen of the avian tumor viruses. They also observed C type particles in the pancreas and livers of embryos from some flocks (Dougherty *et al.*, 1967). By the use of inbred chicken lines Payne and Chubb (1968) detected that the occurrence of this viral antigen in one line is controlled by a single dominant gene. Recently, a recessive gene was found by Crittenden *et al.* (1973) in another chicken line, which also causes the appearance of this antigen. One of the possible explanations for Payne and Chubb's (1968) findings was genetic integration of an avian tumor virus genome.

A breakthrough in the elucidation of this problem has been the work on the so-called nonproducing RSV-transformed cells. It is a curious note that Temin's (1962, 1964) now famous (somatic) provirus theory found its origin in these nonproducers! These nonproducers sometimes do release transforming virus, but with a different host range than the initial converting agent (Vogt, 1967; Weiss, 1967; Hanafusa and Hanafusa, 1968). The new virus was only made in cells, which synthesized the group-specific antigen (Weiss, 1969a). Genetic analysis demonstrated that the ability to produce the new virus RSV (O) segregates together with the antigen (Weiss and Payne, 1971). It seems that these chicken cells also make, in addition to the antigen, some coat material that can be utilized by the defective RSV genome. T. Hanafusa *et al.* (1970a) independently found the helper effect of seemingly normal chicken cells to defective RSV. Subsequently this led to the discovery of a genetic element in normal chicken cells that could help RSV and which has been called *chf* (chicken helper factor) by H. Hanafusa *et al.* (1970).

The cells, which do not ordinarily produce the group-specific antigen, can upon infection with defective RSV produce C type particles. These virions are noninfectious when tested on a great array of cells (Weiss, 1969b; T. Hanafusa *et al.*, 1970a). The noninfectivity is readily explained by the absence of reverse transcriptase (Hanafusa and Hanafusa, 1971). T. Hanafusa *et al.* (1970b) succeeded in retrieving a new Rous-associated virus from cells with *chf* following infection with another leukosis virus. They assumed that recombination between *chf* and the superinfecting virus genome would give rise to a new ALV

(RAV-60) with a new host range. Vogt and Friis (1971) observed the spontaneous release of an ALV in some embryos, which they named RAV-O. Some cells that lack the antigen can nevertheless provide the helper function to defective RSV (Weiss, 1972; Hanafusa, 1972; Hanafusa *et al.*, 1972). This may be due to the relatively insensitive techniques used for the detection of the antigen, but it seems that most, if not all, embryo cells contain the genetic information for helping defective RSV and that the expression of this information is controlled by a regulatory gene (Hanafusa *et al.*, 1972).

By means of carcinogenic chemicals or ionizing radiation Weiss *et al.* (1971) succeeded in retrieving C type particles from normal chicken cells, whether they expressed *chf* and the antigen or not. This indicates that every normal chicken cell carries the information for an oncornavirus, but that its expression is controlled by regulatory elements.

Molecular hybridization studies by Rosenthal *et al.* (1971) using the DNA product of RAV-60 as a probe, indicated that both chf^+ and chf^- chicken cells contain the same number of DNA copies of RAV-60 RNA. A similar finding has been reported by Varmus *et al.* (1972a), who utilized the DNA product of RAV-O. Baluda (1972) and Baluda and Drohan (1972), who used the DNA product of avian myeloblastosis virus (AMV), detected 2–4 genome equivalents in the cells of normal chickens, while after infection with AMV this number increased up to 4–13. Increase in the number of DNA copies after exogenous infection was also reported by Rosenthal *et al.* (1971). These results would support both the notion that normal cells contain genetic information for an oncornavirus (germinal provirus) and that upon infection with an exogenous virus a DNA copy (somatic provirus) of the viral genome will be made and integrated into the host genome.

Hayward and Hanafusa (1973) using a viral DNA probe observed that in chf^+ cells only 3–40 viral RNA molecules would be present, while in RAV-2-infected cells this number would be several thousand. In the case of the normal cells the virus-specific RNA showed less than 50% hybridization. These findings may indicate that only partial transcription of the germinal provirus would take place and then at a low rate.

B. Murine Leukemia Viruses

The AKR mouse strain is a high leukemia strain that cannot be converted into a disease-free strain by transfer of freshly fertilized eggs into low leukemia strains (Fekete and Otis, 1954). In 1951, Gross discov-

ered a leukemogenic agent in this strain which led to the emphasis on the viral etiology of the disease. Introduction of GLV in other mouse strains does not lead to transmission by the gametes but only by the milk (Gross, 1962), which led Law (1966) to the conviction that in the AKR strain the virus is transmitted as a genetic factor of the host.

In primary cultures of AKR embryos only a few cells release infectious MuLV (Rowe *et al.*, 1971). Upon further passage the number of positive cells increases considerably, although it has been possible to isolate several clones that do not spontaneously release any virus. The virus can be retrieved from these lines by either irradiation (X rays and UV) or transformation by SV40. It takes several passages before virus appears after the exposure to these carcinogens.

Halogenated pyrimidines proved to be very efficient inducers of MuLV in these nonproducing AKR cell lines (Lowy *et al.*, 1971). A great variety of carcinogenic and/or mutagenic chemicals have been tested by Teich *et al.* (1973) in this system, but they were ineffective or considerably less efficient than the halogenated pyrimidines.

In analogy to the model we had developed for the genetic transmission of murine mammary tumor viruses, we postulated that several mouse strains would carry a germinal provirus for MuLV that ordinarily would be repressed except in the AKR strain, which would carry a mutation in the regulator gene (Bentvelzen *et al.*, 1968). This model proved to be too simplistic in several respects. It seems that most cells will not release the virus spontaneously. The chance for a few cells to produce virus will nevertheless be considerably higher in the AKR strain than in the C57BL or other low leukemia strains. The regulatory system of the AKR therefore seems to be much weaker, although it can keep the virus unexpressed in most cells.

The fact that mass cultures of AKR embryonic cells become prolific virus producers after several passages cannot be explained by the mere summation of cells that at one time or the other will start to produce virus spontaneously. The virus released by some cells will infect other cells in the culture, which then become secondary producers. The spread of virus seems to be an important factor *in vitro*, and probably also *in vivo*, because soon before birth and thereafter virus can be detected in appreciable amounts in the AKR strain (Rowe and Pincus, 1972).

In crosses between AKR and C57L mice (a low leukemic strain), Taylor *et al.* (1971) obtained evidence that two "duplicate" genes would independently cause the appearance of the group-specific antigen of MuLV in the spleen as detected by complement fixation. Rowe (1972), who tested in similar crosses for the presence of infectious virus in the

tip of the tail, found also two genes that independently from each other can cause virus release. When embryos of such matings are tested *in vitro* for their inducibility of a MuLV, similar Mendelian segregation data were found as were found *in vivo*.

All these crosses mentioned above have been done with mouse strains, which support to some extent the growth *in vitro* of N-tropic MuLV. Introduction of the dominant *Fv-1*^{b} gene, which causes resistance to N-tropic MuLV, also interferes with the expression of endogenous N-tropic MuLV to some extent. In hybrids between AKR and strains with the *Fv-1*^{b} gene, the virus of AKR appears several weeks later than in hybrids with *Fv-1*^{n} (Rowe and Hartley, 1972). In the various backcrosses and test crosses of this study, incidences did not fit a clear-cut two-gene pattern as found in crosses with *Fv-1*^{n} strains. Hilgers *et al.* (1972b) studied, by means of the immunofluorescence-absorption test, the presence of the group-specific antigen of MuLV in spleens of mice from segregating populations of crosses between the AKR and C57BL (*Fv-1*^{b}) strains. The initial impression was that a single AKR gene controls the presence of this antigen. Hilgers *et al.* (1972b) observed the same for the high leukemia strain C58 (*Fv-1*^{n}) as compared to C57BL. Hilgers and Galesloot (1973) completed the studies on AKR versus C57BL with an additional backcross to the C57BL strain and then found a nice fit with a three-gene hypothesis. Two independently dominant genes from AKR would cause release of MuLV, but their expression can be inhibited by the *Fv-1*^{b} gene when homozygous.

Rowe and Hartley (1972) did not observe *in vitro* an influence of the *Fv-1* locus on the activation of a MuLV by 5-iododeoxyuridine. It was concluded that this gene would only interfere with the spread of virus. A more quantitative study is needed with various doses of IUdR and congenic lines differing only at the *Fv-1* locus as have been developed by Axelrad (1968), Odaka (1969) and Lilly (1970b). In addition to tests for infectious virus with the XC tests (Rowe *et al.*, 1970), it might be worthwhile to look for the group-specific antigen by means of immunofluorescence as was done by Grollé *et al.* (1973) in studies on the induction of an oncornavirus by BUdR in rat cells. I would not be surprised if it was found that *Fv-1* exerts some influence on the induction of endogenous MuLV.

Halogenated pyrimidines can also activate the release of MuLV in BALB/3T3 cells (Aaronson *et al.*, 1971b). In crosses between BALB/c and noninducible NIH-Swiss mice, one single gene of the BALB/c causes inducibility of MuLV (Stephenson and Aaronson, 1972b).

In the other high leukemia strain C58, more than two genes can cause the release of MuLV after exposure to IUdR (Stephenson and

Aaronson, 1973). These authors compared the biological activity of the induced virus from the C58 and BALB/c as measured by the number of plaques in the XC tests, the size of the plaques, and the quantity of reverse transcriptase in the medium. The BALB/c strain virus proved to be inferior in all three respects. The three properties segregated together in crosses of both strains with noninducible NIH-Swiss. Stephenson and Aaronson (1973) interpret their findings to be that the observed genes represent viral structural information. It would be better to explain their data to be that the detected genes are weak regulatory elements, which are closely associated with structural viral information (proviruses). The latter entities can also be seen segregating in these crosses because of the nature of their corresponding regulatory genes.

Another line of research, which has led to the conviction that mouse cells contain genetic information for MuLV, has been the study of spontaneously transformed cells *in vitro* (Aaronson *et al.*, 1969). Mass cell cultures of murine embryonic fibroblasts rapidly transform, whereas cultures that are passaged with low cell numbers (the so-called 3T3 regimen) retain their normal appearance (Aaronson and Todaro, 1968). Several transformed cell lines proved to produce C type particles (Aaronson *et al.*, 1969).

According to Todaro (1972) and Lieber and Todaro (1973) clones of transformed cells, which do not shed virus, can easily be induced by IUdR to produce a C type virus. In normal cells virus production after treatment with the halogenated pyrimidine is a transient phenomenon, indicating resistance of the normal cells to superinfection with their own endogenous virus. This "repression" is not so apparent in the transformed cells, since the virus continues to replicate (Todaro, 1972).

It is not clear whether the released virus is causally associated with the transformed state of the cell. Grady and Campbell (1973) demonstrated that in transformed cells more transcription of nonrepetitive DNA takes place than in exponentially growing normal cells. It is possible that release of endogenous virus is due to a specific general state of derepression in the transformed cell.

The murine leukemia virus proves to be ubiquitous and can even be found in low leukemia mouse strains (Kajima and Pollard, 1968; Nowinski *et al.*, 1968; Hartley *et al.*, 1969; Abelev and Elgort, 1970; Hilgers and Galesloot, 1973). The expression or release of MuLV in low leukemia mouse strains is often associated with aging (Hartley *et al.*, 1969; Hilgers and Galesloot, 1973). Frequently, the expression of MuLV is incomplete (Huebner *et al.*, 1970; Huebner and Gilden, 1972; Taylor *et al.*, 1973), as was described for the chicken helper factor system by Weiss (1972) and Hanafusa (1972).

In young mice from low leukemic mouse strains the appearance of

virus, or at least viral antigens, can be rapidly induced by treatment with chemical antigens or ionizing irradiation (Gross and Feldman, 1968; Igel *et al.*, 1969; Ball and McCarter, 1971; Gardner *et al.*, 1971; Fowler *et al.*, 1972). Often the expression of MuLV is incomplete after carcinogenic treatment (Pasternak *et al.*, 1972). In molecular hybridization studies, using the DNA product of MSV as a probe, Tsuchida *et al.* (1972) obtained indications that irradiated NIH-Swiss cells contain virus-specific RNA that does not represent the whole viral genome.

The etiological role of the induced viruses in chemical or radiation oncogenesis is demonstrated by the inhibition of tumor development by active (Whitmire and Huebner, 1972) or passive immunization (Balner, 1971; Haran-Ghera and Peled, 1972). The activation of MuLV (or at least its antigens) is quite remarkable by lymphocytic choriomeningitis virus (Oldstone *et al.*, 1971), graft versus host reactions (Hirsch *et al.*, 1970; Armstrong *et al.*, 1972, 1973; Cornelius, 1972), and host versus graft reactions (Hirsch *et al.*, 1973.

Varet *et al.* (1973) observed that graft versus host (GvH) also promotes leukemogenesis by infection with GiLV. Hays (1972) is of the opinion, however, that virus appearance and subsequent leukemia development in animals with GvH is due to transfer of unrepressed virus in the spleen cell inoculum. With regard to the host versus graft situation, Reid *et al.* (1972) observed that foreign skin grafts inhibited leukemogenesis by either exogenous or endogenous MuLV.

Hormonal activation of MuLV, or at least its group-specific internal antigen, has been reported by Chen *et al.* (1972) and Fowler *et al.* (1972). Dwarf mice, which have a genetic anomaly of the pituitary, have no MuLV group-specific antigen in their spleen in contrast to their normal littermates (Chen *et al.*, 1972). Administration of prolactin, which restores various functions in dwarf mice, also induces the synthesis of this antigen. In the normal littermates the hormone induces the precocious appearance of the viral antigen. With regard to the dwarf mice, the DNA-dependent RNA synthesis is very depressed. This can be restored by the administration of prolactin. The induction of the viral antigen is therefore probably a nonspecific event, associated with a general increase of protein synthesis. An interesting observation in this study of Chen *et al.* (1972) is that the liver, even after hormonal stimulation, does not produce the viral component.

In uterine extracts of ovariectomized NIH-Swiss mice no MuLV can be detected by complement-fixation or reverse transcriptase assay. Estrogenic treatment can induce the appearance of both viral elements (Fowler *et al.*, 1972). It is quite remarkable that in AKR mice uterine tissues are one of the richest sources of MuLV, although this is not as-

sociated with a high incidence of uterine tumors (Rowe and Pincus, 1972).

In most studies on the activation of MuLV, there has been the implicit thought that only one type of virus would be induced by various treatments. It seems, however, that BALB/c mice "latently" carries several C type viruses with different host range and serotype (Hartley *et al.*, 1970; Rowe *et al.*, 1972; Peters *et al.*. 1972; Aoki and Todaro, 1973).

C. Mammary Tumor Viruses

The notion that murine mammary tumor viruses may be genetically transmitted can be first found in a paper by Moore (1963) in which he tries to explain the erratic behavior of the B particle in mice, which have been liberated from the virulent MTV-S by foster nursing.

More serious background for the hypothesis of genetic transmission of MTV was the observation by Mühlbock (1965) that in the GR mouse strain a virulent MTV is transmitted by the male to its offspring. In the other high mammary cancer strains, male transmission is a rare event (Mühlbock and Bentvelzen, 1968). When the GR strain virus (MTV-P) is introduced into other mouse strains, no transmission by the male takes place. There is a close association between the GR genome and MTV-P with regard to male transmission. Genetic analysis of this characteristic proves it to be controlled by a single dominant gene (Bentvelzen, 1968a, 1972b; Mühlbock and Bentvelzen, 1968; Bentvelzen and Daams, 1969). The virus proves also to be transmitted by the ovum (Zeilmaker, 1969), a phenomenon that is controlled by the same gene as male transmission (Bentvelzen, 1968a, 1972b). Cell-free extracts of GR liver and brain can transfer MTV-P, but extracts of the same tissues from BALB/c mice carrying either MTV-P or MTV-S are negative (Bentvelzen and Daams, 1970).

The low oncogenic virus (MTV-L) harbored by C3Hf mice is also transmitted by either parent at conception (Pitelka *et al.*, 1964). We observed that in crosses of C3Hf to the low mammary cancer strains O20 and C57BL the expression of MTV-L is repressed which is caused by a single dominant gene (Bentvelzen and Daams, 1969, 1973). The BALB/c strain, which does not have an overt expression of an MTV at a young age, also does not inhibit the release of MTV-L. The BALB/c genome even seems to enhance the oncogenic activity of MTV-L. In contrast to the less susceptible C3Hf mouse strain, BALB/c mice cannot transmit MTV-L by the sex cells. This indicates that the C3Hf strain has a genetic system solely concerned with this mode of vertical

transmission, and the most economical hypothesis was that MTV-L is transmitted as a genetic factor of the host. Nandi (1972) came up with a sophisticated hypothesis that the C3Hf embryos would be extremely susceptible to MTV-L, whereas the newborns would be less susceptible. He postulates a similar phenomenon for the case of MTV-P. Although this hypothesis cannot be completely ruled out, it need not to be taken all too seriously. It fails, among other things, to explain the infectivity observed in extracts of GR livers and brains (Bentvelzen and Daams, 1970).

Results of crosses between C3Hf and BALB/c proved that the transmission and release of MTV-L are controlled by a single gene. This gene is located in the same linkage group as the gene for transmission of MTV-P in the GR strain (Bentvelzen and Daams, 1969, 1973). In the test cross BALB/c × (C3Hf × GR)F_1 a few virus-negative mice were noted. The incidence was too low to be explained by location of the wild-type alleles of the respective transmitter genes on different chromosomes. It was postulated that these virus-free mice resulted from crossing-over between the two respective transmitter genes, giving rise to a chromosome with the two corresponding wild-type alleles that do not allow virus release (Bentvelzen and Daams, 1973). In this cross 55% of the animals proved to carry MTV-P and 41% to carry MTV-L.

These results strongly suggest Mendelian segregation of the two virus types in this cross. It could signify that mouse chromosomes can carry genetic information for viral properties. Definite proof for the existence of murine proviral genes can be obtained when recombination is observed between the transmitter (regulator) genes and structural genes coding for viral properties.

The spontaneous release of MTV was regarded as an abnormal situation, and it was assumed that in the "normal" low mammary cancer strains a proviral form of MTV would be present, whose release was repressed (Bentvelzen, 1968a). Treatment of the resistant strain O20 with X irradiation and urethane led to the activation of a virulent MTV (Timmermans *et al.*, 1969). This procedure was not as successful in the C57BL strain, where only MTV-specific antigens were found in the hemopoietic tissues of carcinogen-treated C57BL mice (Bentvelzen, 1972b). Inoculation of "virus-free" mice with blood from irradiated C57BL mice led to a significant acceleration of mammary tumor development, suggesting that at least a blood cell-bound form of MTV has been activated (Boot *et al.*, 1971). Recently, J. Links, O. Tol, F. Buijs, and J. Calafat (unpublished results, 1973) observed that kidney cultures of baby C57BL mice release B particles after treatment with methylcholanthrene.

Varmus *et al.* (1972b) found in DNA–DNA hybridization studies using MTV DNA product as a reagent that cellular DNA of both GR and C57BL tissues contain the same number (90) of MTV DNA copies per cell.

Since the O20 and C57BL strains are rather resistant to superinfection with MTV-S (Bentvelzen and Daams, 1969; Bentvelzen *et al.*, 1970b), it was assumed that a classical repressor interferes with both the release of endogenous virus (transcription of a germinal provirus) and with the replication of administered virus. Especially with regard to exogenous virus, the inhibition is far from absolute, but the replication is considerably less than in susceptible mouse strains (Bentvelzen, 1968b).

An interesting observation is that very old C57BL mice (2–3 years old) also release MTV-specific antigens in their hemopoietic tissues (Bentvelzen, 1972b). Using MTV DNA product as a probe Schlom *et al.* (1973) occasionally detected MTV-specific RNA in several tissues of carcinogen-treated or old C57BL mice. They did not observe detectable amounts of virus-specific RNA in similar tissues of control mice. Bishop *et al.*, (1973) claims to have found, with a somewhat similar methodology, appreciable amounts of MTV-RNA in the mammary glands of C57BL, although considerably less than, for instance, in the GR mouse strain. The difference in quantity of MTV RNA seems to be controlled by a single gene.

The differences between both groups may be due to a less sensitive method used by Schlom *et al.* (1973) or to a different DNA probe. During reverse transcription *in vitro* the whole genome would not be copied. It is possible that the two groups have worked with copies of different parts of the genome. If so, than in normal mammary glands only a part of the C57BL germinal provirus would be transcribed, and after carcinogen treatment or aging, the whole genome. In that case the C57BL strain would be comparable to chf^+ in chickens.

The BALB/c strain has been widely used in MTV research because the strain was claimed to be virus-free but extremely susceptible to superinfection with any kind of MTV. Hageman *et al.* (1972) succeeded in isolating a virulent MTV from mammary glands of old retired breeders of this strain. It induces a high incidence of early arising tumors upon inoculation into young BALB/c mice. Spleen cells of old BALB/c mice of either sex can also transfer this virus, which we have called MTV-O (Bentvelzen *et al.*, 1970b). Since this virus can also be detected in germ-free mice, it can be excluded that the virus in BALB/c mice is due to a latent infection after horizontal transmission. Furthermore, according to Daams and Hageman (1972), MTV-O has a unique antigen not found in any other MTV strain with which they have worked.

The genetic factor(s) that inhibits the release of endogenous virus in young BALB/c mice does not repress the release of MTV-L in hybrids with the C3Hf strain (Bentvelzen and Daams, 1969). The hybrids of C3Hf with other low cancer strains, such as O20 and C57BL, show a considerable inhibition of MTV-L expression (Bentvelzen and Daams, 1969; Hilgers and Galesloot, 1973). On the other hand, the C3Hf strain gene(s), which allows the release of endogenous MTV-L, does not evoke the spontaneous production of MTV-O by the BALB/c genome in the hybrid (C3Hf × BALB/c)F_1.

Hilgers and Galesloot (1973) are of the opinion that the classical work by Rowe and Hartley (1972) on the influence of the *Fv-1* locus on the expression of endogenous MuLV can be used as a model for the problem mentioned above. I am arrogant enough to believe that the reverse holds true. The situation in the BALB/c strain is a beautiful example of position effect. The inhibiting factor of BALB/c acts only upon that provirus with which it is in a cis position. It does not affect transcription of the MTV-L provirus with which it is in a trans position. It also does not affect the transcription of the somatic provirus established after infection with endogenous MTV-O.

The C57BL and O20 strains contain an overall repressor, which affects not only the release of their own MTV but also expression of that provirus with which the regulatory element of the "wild-type" strains occurs in trans position. It also influences expression of somatic proviruses. In our past publications we assumed that the C57BL and O20 strain repressor would inhibit transcription of either provirus. It is very well possible that this overall repressor acts on the level of translation (see Fig. 4).

The gene of the GR for release of MTV-P is not inhibited by genes from ten different mouse strains (Bentvelzen, 1968a, 1972b, unpublished results, 1972). One possibility is that the GR strain is mutated at the proviral operator gene. In the case of a translational control by the overall repressor, this mutation must then also be expressed in the released RNA because otherwise virus expression could be inhibited at this level. This may be true, since the GR strain virus (MTV-P) replicates considerably better in C57BL mice than MTV-S. An alternative possibility is that the site-specific regulator gene of the GR is mutated, giving rise to transcription of the provirus and that in addition the provirus carries such a mutation that it is not recognized by the overall repressor of various mouse strains. We assumed that the GR strain would synthesize the overall repressor, since it proved to be resistant to superinfection with MTV-S (Bentvelzen and Daams, 1969). The observed resistance can also be explained by interference of resident MTV-P with

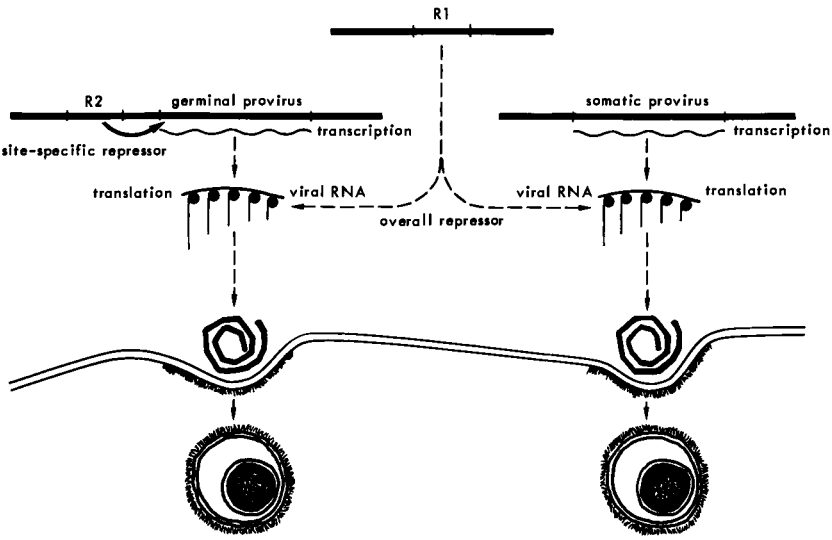

FIG. 4. Schematic representation of repression of germinal and somatic proviruses of MTV.

superinfecting MTV-S. Molecular biological tools are available at present to investigate the new model, which has been presented in Fig. 4.

In a study of six quite genetically different mouse strains, a good correlation was observed in susceptibility to spontaneous urethane and viral carcinogenesis of the mammary gland (Bentvelzen *et al.*, 1970b; Bentvelzen, 1972b). This signifies that the same genes control susceptibility to all three modes of tumorigenesis. It supports the notion of the all-viral etiology of this malignancy and the ubiquity of the germinal provirus of MTV.

IX. General Discussion

> Thus, it appears probable that within a few years the existence of a provirus of DNA and of RNA oncogenic viruses will be demonstrated. The problem of the inhibition and induction of viral functions of course remains. This is to my mind one of the key problems of cancer research (A. Lwoff, 1972).

During the last three years, ample evidence has been collected for the one-time controversial theory by Temin (1964) that for transformation and also replication of an oncornavirus a DNA provirus will be made,

which will be integrated into the host genome. Highlights in this respect have been the discovery of reverse transcriptase (Temin and Mizutani, 1970; Baltimore, 1970) and infectious DNA (Hill and Hillova, 1972). The finding of infectious MSV DNA by Karpas and Milstein (1973) also proves the existence for a somatic provirus for MSV.

Although no experiments on infectious DNA have been reported as yet for any of the other three oncornavirus groups, the ubiquity of reverse transcriptase infers that a provirus would be made for each of them. Since DNA synthesis is needed for the establishment of infection with avian leukosis viruses (Duesberg and Vogt, 1969; Vigier, 1970) and the murine leukemia viruses (Bentvelzen *et al.*, 1974), it is also very likely that for these groups of viruses integration of a DNA provirus is needed for replication.

It will be obvious that the establishment of a somatic provirus is not the sole answer to the problem of neoplastic conversion of cells by oncornaviruses. Probably sarcoma viruses transform every cell species they are able to infect, although the rate of establishment of a virogenic relationship is considerably higher than that of transformation (Kotler, 1971). Leukemia viruses replicate well in fibroblasts, but will rarely convert this kind of cell, except for the notorious cell line of Hackett and Sylvester (1972). This irregularity may be explained by the fact that the cell line is already in such a high "excitation state" with regard to transformation that it needs little stimulation to be pushed over.

In his still controversial protovirus theory, Temin (1972) postulates that rare transformation of fibroblasts by leukemia viruses may be explained by integration of the (somatic) provirus in a usually forbidden site or by association of the viruses with cellular episomal elements, called protoviruses, which gives rise to the establishment of genes for neoplastic transformation. An association such as this may have occurred when passage of an avian sarcoma virus in rat cells gave rise to an altered virus with a high efficiency for transformation of rat cells (Altaner and Temin, 1970).

Several leukemia viruses can rapidly transform hemopoietic cells *in vivo* and in some instances also *in vitro*. One may hypothesize that in such cells integration sites are available for the leukemia proviruses, which are usually not accessible to them in fibroblasts. This assumption will be very unlikely, since from RLV-infected hemopoietic stem cells either transformed erythroblasts or untransformed cells of the other hemopoietic series (Brommer, 1972) may be derived. In the case of the forbidden-site hypothesis, this would mean that upon differentiation into the erythroid series the somatic provirus may switch to the forbidden site. For other differentiation signals the provirus would stay at its place.

This hypothesis is highly untenable, and I therefore assume that epigenetic factors strongly influence the transforming activity of the somatic provirus of the leukemia viruses.

The epigenetic control is presumably at the translational level, since transcription of the whole somatic provirus takes place. An alternative possibility is that in cells, which are not permissive for the transforming activity of a given oncornavirus, complete translation of the transcript takes place but that the "oncogene products" must interact with cellular genes, which are expressed following, for instance, hormonal stimulation.

The mouse mammary tumor system is the most beautiful example of epigenetic control of the expression of somatic proviruses. In the hemopoietic tissues only a subviral form of MTV can be found. It will be of utmost importance to investigate whether transcription of the whole provirus takes place. Molecular hybridization studies, using the DNA product of MTV as a probe, will certainly give an answer to this problem and will have a bearing on the epigenetic control of the transforming action of murine leukemia viruses.

In the section on endogenous oncornaviruses, ample evidence has been presented that chickens have genetic information for an ALV and mice for MuLV and MTV. It is not likely that reverse transcriptase plays a role in the genetic transmission of oncornaviruses as has been suggested by various authors. We, as well as other workers, have never observed that infection with another oncornavirus would lead to its genetic transmission instead of the endogenous virus. Mendelian segregation ratios that have been observed in various studies cannot be expected in the aforementioned case, since the enzyme might then cause 100% transmission. Integration of the germinal provirus into the sex cells may have taken place several million years ago (Gilden, 1972), but since then, the germinal provirus has been a stable genetic element that has coevolved with the host. This germinal provirus seems to be neutral to several mutations, as becomes obvious from the great variation in several traits among the endogenous mammary tumor viruses that I have been studying. There will only be selection against the spontaneous release of a virulent mammary tumor virus.

Factors, which control the incomplete expression of germinal proviruses as in the chf situation, are poorly understood. We have hypothesized that for the continuous release of infectious virus the reverse transcriptase may play a great role (Bentvelzen and Daams, 1972). After temporary release of viral RNA following carcinogenic treatment, the enzyme could make a new somatic provirus, which can be inserted at a different site out of reach of the specific repressor. It is also possible that

such a system exists for the incomplete expression of the germinal provirus. The enzyme will certainly play a great role in the horizontal spread of virus.

In our initial theory on the genetic transmission of mammary tumor viruses, we assumed that low mammary cancer strains have a repressor, which inhibited the transcription of their own germinal provirus and also that of other strains in various hybrids. This repressor would also cause resistance to superinfection by interfering with the replication of exogenous virus. Studies on the BALB/c strain have led to a new insight that there are site-specific repressors and overall repressors. The first category inhibits only the transcription of that provirus, with which it is in a cis position. The overall repressor has a qualitative effect on the expression of every kind of provirus. It remains to be established whether it exerts this on the level of transcription or translation. I assume that the two AKR strain genes, which control the inducibility of MuLV, are site-specific regulatory genes. Their dominance is explained by the fact that the alleles from the low leukemia strains cannot repress proviruses in trans position. Yoshikura (1973a) postulated that the *Fv-1* locus would produce a repressor, which would affect either N- or B-tropic viruses with regard to their replication. In that case it would be comparable with the overall repressor of the mammary tumor virus system. An objection to this hypothesis may be that *Fv-1* does not seem to influence the inducibility of MuLV by halogenated pyrimidines *in vitro* (Rowe and Hartley, 1972). Possibly, the treatment in their studies has been so harsh that both types of regulatory elements have been inactivated by the compound. Dose–response studies may reveal another additional relationship in compatible *Fv-1* types than in incompatible ones.

In chickens, a dominant mendelian factor has been found, which causes the release of the group-specific antigen of the avian tumor viruses and some other attributes. This factor probably reflects a mutation in a site-specific regulatory gene of the germinal provirus of RAV-O. Since the overall regulatory gene is not mutated, no excessive susceptibility to superinfection may be anticipated. Release of viral coat material may even cause resistance by interference at the level of penetration (Payne *et al.*, 1971).

Crittenden *et al.* (1973) detected a recessive gene that causes release of the viral antigen and also susceptibility to superinfection. This factor may be a mutation in the overall regulatory gene.

Tumor virological research has gained impetus by the oncogene hypothesis by Huebner and Todaro (1969). One of the stimulating aspects of this hypothesis was the realization that no complete expression of the virogene (germinal provirus) is necessary for the development of a tumor. One of the serious shortcomings of this theory is that it assumes

every tumor to be caused by one and the same oncogene. Indeed, the avian and murine transforming viruses (sarcoma viruses) can also cause many different types of tumors, but in reverse not every tumor needs necessarily to be caused by one and the same virus. Mühlbock (in Bentvelzen, 1972b) observed a great variation in susceptibility to spontaneous tumors between the many inbred mouse strains he had in his colony. Some strains display a great susceptibility to hepatomas, others to pulmonary tumors, and so on. It is very likely that different oncogenes with their associated regulatory elements are responsible for these strain differences. The finding by Aoki and Todaro (1973) that different C type viruses can be induced in BALB/c cells plead for this conviction.

X. Epilogue

Some tumor virological research is being done for agricultural reasons, but most investigations on oncogenic viruses are financed by medical institutions. The question that is warranted, is what relevance the animal models contribute to the solution of human cancer problem. From this chapter, it will be obvious that many different neoplasms in two very different groups of vertebrates are caused by an oncornavirus. In several other species oncornaviruses have been detected, and these have not been reviewed in this chapter. In my opinion it would be extremely arrogant not to believe in the causation of various human neoplasms by oncornaviruses. This does not imply that oncogenic DNA viruses would not play an important role in human cancer.

The present review has outlined numerous pitfalls in the work with oncornaviruses, such as defectivity; passengers; incomplete expression due to epigenetic factors; phenotypic mixing; genetic recombination, especially following transspecies transfer; interference; host range limitations; and most importantly, noninfectivity in cell-free form.

There is, with few exceptions, no evidence for the infectious nature of cancer in man, although epidemiological investigations in this respect will be hampered by the anticipated protracted latency period of viral carcinogenesis. The emerging theory that every chicken or mouse carries genetic information for oncornaviruses will be extremely helpful in understanding the natural history of human cancer. The realization that such genetically transmitted viruses are often incompletely expressed is of extreme importance. The insight that animals and probably also man carry their own tumor viruses in their hereditary material may cause the pessimistic view that nothing can be done in terms of prevention, since these viruses cannot be eradicated. The animal studies show,

however, that no true tolerance exists to the inborn viruses, and that in some noteworthy cases immunological manipulations either delay or sometimes even inhibit the development of spontaneous tumors in high cancer strains.

It is certainly necessary to investigate further the relationships between virus and host genome in the animal models, but the most important is the study of the immunobiology of naturally occurring oncornaviruses.

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CHAPTER 13

The Cell Surface, Virus Modification, and Virus Transformation

ROSE SHEININ

I. Introduction*

Role of the Cell Surface

The surface plays a crucial role in the biology of a single cell, and also in the interplay of cell with cell, contributing to and molding the organismic whole. Of particular interest in the present context are those phe-

* Abbreviations used: $GlcNH_2$, glucosamine; GlcNAc, *N*-acetylglucosamine; RSV, Rous sarcoma virus; ts, temperature-sensitive; UV, ultraviolet light.

TABLE I

Cell Functions Mediated by the Surface

Function	Modified by neoplastic transformation	Selected references
Enzyme activity		
Various	+	Emmelot and Benedetti, 1967
Adenyl cyclase	+	d'Armiento *et al.*, 1972; Anderson *et al.*, 1973
Proteases	+	cf. Burger, 1973
Glycosyl transferases	+	Roth and White, 1972
Transport systems	+	cf. Holley, 1972; Kalckar *et al.*, 1973
Interaction with viruses	+	cf. Allison, 1971; Križanová *et al.*, 1971; Taylor *et al.*, 1971; Drzeniek, 1972; Sturman and Takemoto, 1972; Weiss, 1973
Interaction with agglutinins		
Lectins	+	Inbar *et al.*, 1972; cf. Burger, 1973
Polymers of basic amino acids	+	cf. Inbar *et al.*, 1972
Antigenicity		
Blood group substances		Pann and Kuhns, 1972; Dimmock *et al.*, 1972; Hakomori *et al.*, 1972
H-2 antigens	+	Klein, 1971, 1972; Lilly, 1971
HL-A antigens	+	Reisfeld *et al.*, 1971; Osoba and Falk, 1974
Tissue-specific antigens	+	Boyse and Old, 1969; Dickinson *et al.*, 1972; Iyje *et al.*, 1972; cf. Tillack, 1972; Snell *et al.*, 1973
Fetal antigens	+	Alexander, 1972
Forssman antigen	+	Hakomori and Kijimoto, 1972
Immunoglobulins	+	Aisenberg and Bloch, 1972
Interaction with immune system		
Immunogenicity	+	cf. Haughton and Nash, 1969; cf. Klein, 1971
Reaction with immune lymphocytes		Wekerle *et al.*, 1972
Reaction with macrophages	+	Hibbs, 1973
Reaction with cytotoxic sera	+	cf. Haughton and Nash, 1969; cf. Klein, 1971; Kurth and Bauer, 1972
Reaction with complement		Müller-Eberhard, 1972

TABLE I (*continued*)

Function	Modified by neoplastic transformation	Selected references
Growth, contact inhibition		
Of movement	+	Abercrombie, 1967
Of mitosis	+	cf. Martz and Steinberg, 1973
Of progression through cell cycle	+	cf. Dulbecco, 1971
Morphogenesis		
Cell recognition	+	cf. Lilien, 1969; Roth *et al.*, 1971; Pessac and Defendi, 1972
Adhesion	+	cf. Weiss, 1973
Intercellular junction Formation	+	cf. Goodenough and Gilula, 1972
Interaction with regulatory molecules		
Hormones	+	Krug *et al.*, 1972; Amir *et al.*, 1973; Lesniak *et al.*, 1973
Acetylcholine		Patrick *et al.*, 1972; Sytkowski *et al.*, 1973

nomena that are greatly modified during neoplastic conversion by oncogenic viruses. The illustrative information set out in Table I indicates the wide range of phenomena involved. It will be the assumption of this discussion that specific molecules at the surface of cells carry out the various functions here tabulated. In some cases, this postulate rests on very few, but intriguing, observations; in others the data have converted hypothesis to established fact.

The working model for the cell surface derives from that proposed by Singer and Nicolson (1972). The plasmalemma is considered to comprise a fluid lipid phase into which are inserted structural and functional proteins and glycoproteins, the distribution of which is subject to continual quantiative and qualitative modification during growth and development. A central problem in the present consideration is the mechanism whereby the cell surface is formed from its constituent building blocks. There is considerable information on the general patterns of synthesis of neutral lipids (Spector, 1972), phospholipids (McMurray and Magee, 1972), sterols (Rothblat, 1972), glycolipids (cf. Roseman, 1970), proteins, and glycoproteins (cf. Schachter and Roden, 1973) by animal tissues. But little is known about the reactions by which individual cells generate these components and mold them into a functioning plasmalemma.

Of specific importance will be the synthesis and incorporation into the cell surface of glycolipids and glycoproteins. It will be assumed that *de novo* synthesis of these molecules by the various fibroblast types discussed proceeds by procedures already established. In the case of the glycolipids, the sugars are added stepwise to a preformed ceramide base through the action of specific glycosyl transferases (cf. Roseman, 1970). The protein of glycoproteins is synthesized on polyribosomes and there, upon completion, accepts the first sugar residue. Once again carbohydrate residues are added sequentially by specific sugar-transferring enzymes, as the glycoprotein is transported through the Golgi apparatus to its site at the cell periphery (cf. Schachter and Roden, 1973). Our own studies on the synthesis of surface glycoprotein by mouse fibroblasts are in accord with the general aspects of the latter model, since treatment of cells with puromycin or cycloheximide brings rapid inhibition of formation of peptide and oligosaccharide moieties (Sheinin and Onodera, 1970).

This chapter will address itself to the following questions: (i) What are the modifications to the metabolism of surface molecules, which are inflicted upon cells by their infecting viruses? (ii) Under what conditions, can these modified pathways become stablized, thereby producing a permanent alteration of the cell surface? (iii) In what way do the oncogenic viruses resemble, or differ from, their nononcogenic counterparts in their effects on cell surface metabolism?

II. Interactions of Viruses with the Cell Surface

A. Introduction

The interactions of viruses with the surface of cells may perhaps be classified under three headings: (i) reactions by which an infecting virus is permitted entry into the cell; (ii) reactions by which newly replicated virus is permitted exit from the cell; (iii) reactions of biogenesis of the integral plasma membrane (Singer and Nicolson, 1972) and its peripheral components which are directly or indirectly modified as a result of virus infection.

This chapter will concern itself primarily with class (iii) phenomena, for these appear to offer the most suitable target for oncogenic conversion. The many processes embraced under classes (i) and (ii) are discussed elsewhere (Allison, 1971; Poste, 1970; Krizănová *et al.*, 1971; Dales, 1973a,b; Medzon, 1973). Of these, the action of viral neuramini-

dases (cf. Drzeniek, 1972) may be relevant, and will be commented upon below.

As noted in Tables II and III, the genome of many viruses carries information that directly, or indirectly, results in modification of the metabolism of molecules at the surface of the host cell. The ingenuity of many investigators has been employed in isolating virus strains, a major expression of which is seen in this function, normal or mutant (Ghendon, 1972; Vogt, 1972; Roizman *et al.*, 1973). Such variants are known to occur in nature (Poste, 1970; Ichihashi and Dales, 1971; Ichihashi *et al.*, 1971; Nagata *et al.*, 1972; Higashi, 1973; Roizman *et al.*, 1973; Dales, 1973b). Many more undoubtedly remain to be discovered, and perhaps even to be produced.

B. Action of Enveloped Viruses

The most profound direct effects on the synthesis and turnover of the plasmalemma is to be observed in vertebrate cells infected by enveloped viruses. These microorganisms contain a core of nucleoprotein surrounded by the capsid proteins, which in turn are encased in a membranous envelope. The genome of enveloped viruses codes directly for the synthesis of proteins and glycoproteins of the viral envelope membrane, which resembles in many ways the membranes of host cells (Guidotti, 1972). So close is the structural analogy that the biogenesis of viral membrane has been taken as a most effective model for the generation of cellular membrane (cf. Dales and Mosbach, 1968; Ben-Porat and Kaplan, 1972; David, 1973).

The same general picture emerges from the study of myxoviruses, paramyxoviruses, rhabdoviruses, herpesviruses, poxviruses, and oncornaviruses (Eiserling and Dickson, 1972; see also references cited in Tables II and III):

(i) The virus genome codes directly for the formation of specific proteins of the viral membrane, while perhaps only indirectly contributing to the composition of the lipid moiety.

(ii) Viral envelope polypeptides and glycoproteins are synthesized within the cytoplasm of the cell and are then transported from the machinery of synthesis to the site of maturation (be it nuclear or cytoplasmic).

(iii) Viral envelope proteins become associated with membrane lipid either before, or in the process of, becoming a part of the membrane site of maturation.

TABLE II

Vertebrate DNA Viruses Affecting Cell Surface

Class	Species (selected examples)	Genome size ($\times 10^6$ daltons)	Natural host		Cells transformed *in vitro*	Envelope	Cell surface modification		Selected references
			Replication	Tumor formation			During replication	In transformed cells	
Poxvirus	Vaccinia	100–200	Cattle	Unknown (U)	U	+	+	U	Dales, 1973; Dales and Mosbach, 1968
	Rabbit fibroma		Rabbit	Rabbit (cottontail, domestic)	U	+	+	+	Shope, 1966
	Yaba		Monkey	Monkey	Primate, human	+	+	+	Tsuchiya and Rouhandeh, 1972
Herpesvirus	Herpes simplex, type 1	100	Human	U	Hamster[a]	+	+	+[a]	Heine *et al.*, 1972; Roizman *et al.*, 1973; Nahmias *et al.*, 1972; Tevethia *et al.*, 1972; Rapp, 1973
	Herpes simplex, type 2		Human	U	Hamster[a]	+	+	+[a]	Heine *et al.*, 1972, Roizman *et al.*, 1973, Nahmias *et al.*, 1972, Rapp, 1973
	Epstein-Barr		Human	Human	Human, Marmoset	+	+	+	Klein, 1972; zur Hausen, 1972
	Lucké		Frog	Frog	U	+		+	Klein, 1972
	Marek's disease		Chickens, turkeys	Chickens	U	+	+	+	Klein, 1972; Nazerian, 1973

Adenovirus	Human	20–25	Human	U	Hamster, human	−	+	+	Schlesinger, 1969; Vasconcelos-Costa *et al.*, 1973
	Canine		Dogs	U	U	−	U		
	Swine		Swine	U	U	−	U		
Papillomavirus	Shope	5	Rabbit	Rabbit	U	−	U	+	Shope, 1966
	Human		Human	Human (benign)	U	−	U	+	Butel, 1972
	Equine		Horse	Horse (benign)	U	−	U	+	Fulton *et al.*, 1970
Papovavirus	Polyoma	2–3	Mouse	U	Rodent	−	+	+	Habel, 1965; Klein, 1971
	SV40		Monkey	U	Rodent, Human	−	+	+	Häyry and Defendi, 1970; cf. Sambrook, 1972
Parvovirus	Minute virus of mice adeno-associated virus	2	Mouse	U	Mouse[b]	−	U	U	cf. Crawford, 1969

[a] Observed with UV-irradiated virus (Rapp, 1973).

[b] One unconfirmed observation.

TABLE III

Enveloped Vertebrate RNA Viruses Affecting Cell Surface

Class	Species (selected examples)	Genome size ($\times 10^6$ daltons)	Natural host		Cells transformed (*in vitro*)	Envelope	Cell surface modified		Selected references
			Replication	Tumor formation			During replication	In transformed cells	
Myxovirus	Influenza	2–5	Primates, birds	Unknown (U)	U	+	+	U	Eiserling and Dickson, 1972; Rott *et al.*, 1972; Compans, 1973
	Fowl plague		Birds	U	U	+	+	U	
Paramyxovirus	Simian virus 5	6–8	Monkeys	U	U	+	+	U	Kingsbury, 1972
	Sendai		Mouse, pigs	U	U	+	+	U	Bachi *et al.*, 1973
	Sindbis		Birds	U	U	+	+	U	Bose and Brundige, 1972
	Measles		Human	U	U	+	+	U	Knight *et al.*, 1972
	Mumps		Human	U	U	+	+	U	Kingsbury, 1972
	Newcastle disease		Birds	U	U	+	+	U	Kingsbury, 1972
Rhabdovirus	Vesicular stomatitis	3–4	Swine, cattle, Rodents, dogs	U	U	+	+	U	Howatson, 1970; Kingsbury, 1972
	Rabies		Rodents, dogs	U	U	+	+	U	Howatson, 1970; Higachi, 1973
Oncornavirus	Mouse mammary	10–12	Mouse	Mouse	U	+	+	+	Bentvelzen, 1972; Dalton, 1972

B type C type	Murine leukemia-sarcoma	10–12	Mouse	Mouse	Rodent, human	+	+	+	Aoki *et al.*, 1973; Mann *et al.*, 1973; Salzber *et al.*, 1973
	Feline leukemia-sarcoma		Canine	Cat	Feline, canine, human	+	+	+	Boone *et al.*, 1973
	Avian myeloblastosis		Avian	Bird	Avian	+	+	+	
	Rous sarcoma		Avian	Chicken	Avian, rodent, human	+	+	+	Rao *et al.*, 1966; Quigley *et al.*, 1972; Robinson and Robinson, 1972
Coronavirus	Human		Human	U	U	+	+	U	Bradburne and Tyrrell, 1971
	Murine		Murine	U	U	+	+	U	Sturman and Takemoto, 1972
Togavirus	Rubella		Human	U	U	+	+	U	Higashi, 1973
	Japanese encephalitis		Avian	U	U	+	+	U	Shapiro *et al.*, 1972

(iv) Viral envelope proteins can be detected (by biochemical and immunological methods) as an integral component of the plasmalemma of infected cells before virion maturation begins.

(v) The surface biology and biochemistry of host cells is profoundly altered when viral envelope proteins are inserted into the plasma membrane. This is reflected in an altered immunopathology and in the reaction of infected cells with lectins.

Thus enveloped viruses can modify the reactivity of cells by coding for the synthesis of specific proteins and glycoproteins which become an integral part of the cell surface. The myxoviruses, and some of the paramyxoviruses, are potentially effective in such functions by virtue of the fact that they carry a neuraminidase as a virion component (cf. Drzeniek, 1972). These enzymes have the ability to remove sialic acid from complex carbohydrate residues at the cell surface, thereby altering the immunological, biochemical, and biophysical properties of cells, their capacity to react with neighboring cells, and their progression through metabolic events of the cell cycle (cf. Weiss, 1973).

C. Action of Nonenveloped Viruses

Unlike the enveloped viruses, those without a lipoprotein–glycoprotein coat appear not to have a direct effect on the synthesis of molecules that become a part of the plasma membrane or other membranous cell constituents. They may, however, indirectly bring about the turnover of membrane components, particularly in association with infection and release of virus from cells (Allison, 1971; Poste, 1970; Dales, 1973a,b; Medzon, 1973) or with transmission of virus from cell to cell.

As noted above, these phenomena will not be discussed further because they do not appear to provide a mechanism for the stable alteration of the surface of surviving cells.

D. General Considerations

When one considers the biological consequences of cell surface modulation by viruses, it is clear that most instances of productive infection are of little direct relevance to the phenomenon of viral oncogenesis because the affected cell dies. There are, however, at least three types of virus–cell interaction in which host cells would survive, but with virus-determined biochemical alteration of the cell surface. These include (i) cells which have established a carrier state and continue to bud off

complete or defective enveloped virions from the cell surface (e.g., Bradburne and Tyrrell, 1971; Howatson, 1970; Lunger and Clark, 1972; Matsumoto, 1972; Higashi, 1973); (ii) abortively infected cells in which are expressed those functions determining synthesis and movement to the surface of viral envelope proteins (cf. Poste, 1970); and (iii) cells infected with virus that is a mutant in some terminal function of the replication cycle, but which does express those functions that modify the cell surface (e.g., Nagata *et al.*, 1972).

These observations are especially important in assessing the potential for oncogenic conversion by viruses. They lead to an appreciation of a major difference between oncogenic and nononcogenic viruses, even within the same class. Tumor viruses can function as donors of genetic information (perhaps even as transducing agents) (cf. Luria, 1959). Cells that survive infection by the oncogenic viruses and undergo stable transformation are known to carry virus DNA as an integral part of the chromosomal DNA (Winocour, 1971; Sambrook, 1972; Temin, 1972; Todaro and Huebner, 1972; zur Hausen, 1972, 1973). This would provide a fourth, and perhaps most efficient, method whereby surface metabolism of host cells could be stably modified.

III. Oncogenic Viruses

A. Introduction

We now know that virus transformation is a two-step process analagous to that illustrated in Fig. 1. Genetic transformation, which results from incorporation of virus genetic material into the chromosomal DNA, is necessary but not sufficient to produce a neoplastically transformed cell. It requires in addition phenotypic expression of a virus function which ultimately modifies the surface of the affected cell. It is the cell surface that embodies a key lesion of neoplastic transformation, for it mediates those phenomena of cell–cell interaction that underlie both normal and cancerous growth and development (see Table I).

To understand the mechanism of oncogenesis by viruses, it is of importance to establish which, if any, virus genes participate in neoplastic conversion and how these interact with genes for cell surface biogenesis. At the biochemical level the problem becomes one of defining the chemical basis of the physiological surface modification to permit identification of the metabolic processes involved.

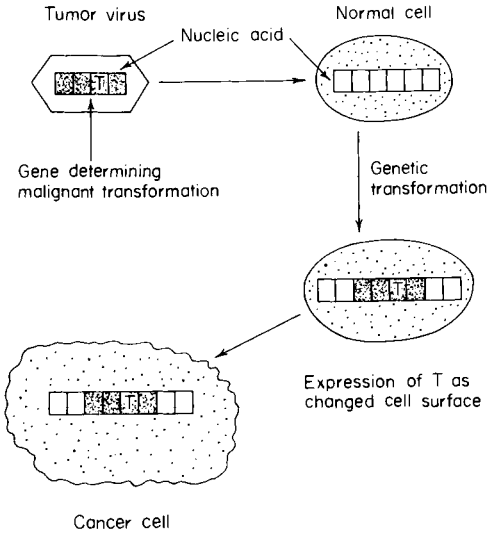

FIG. 1. Model for neoplastic transformation by oncogenic viruses.

B. Genetic Considerations

There seems little doubt that genetic information for modulation of cell surface macromolecules is carried by the genome of oncogenic viruses. Such a conclusion derives from the following observations: (i) Infectious nucleic acid, isolated from highly purified virus, brings about transformation both *in vivo* and *in vitro* (Ito, 1962; cf. Crawford, 1969; cf. Sambrook, 1972; Graham and van der Eb, 1973). (ii) Infection and transformation by these viruses is sensitive to high energy irradiation and mutagenic chemicals (cf. Sachs, 1967; Eddy, 1969; Sambrook, 1972; Vogt, 1972). Because the capacity for viral replication is more sensitive to these damaging agents than is the ability to transform cells, it seems likely that information for the latter function resides only in a segment of viral genome. (iii) Recently a correlation has been established between the nucleic acid composition of various isolates of RSV and their capacity to replicate and/or transform host cells (cf. Duesberg and Vogt, 1973). (iv) Some of the surface alterations characteristic of transformed cells are expressed early in cells infected productively or abortively, by oncogenic virus (Ben-Bassat *et al.*, 1970; Häyry and Defendi, 1970; Girardi and Defendi, 1970; Hakomori *et al.*, 1971, 1972; cf. Eckhart, 1972; cf. Benjamin, 1972). (v) Mutants of these viruses have been isolated [polyoma (cf. Eckhart, 1972; Benjamin,

1972; Sambrook, 1972), Rous sarcoma virus (cf. Vogt, 1972), and murine leukemic-sarcoma virus (cf. Vogt, 1972; McCarter *et al.*, 1974)] which cannot transform cells under nonpermissive conditions and which elicit specific, virus-determined cell surface changes only under permissive conditions.

Clearly, there is ample data implicating the virus genome as the repository of information for neoplastic transformation. The problem to be posed concerns the nature of this information. Does it code for specific molecules that are continually elaborated and incorporated into the plasma membrane? Or does it simply provide for alteration of the pattern of regulation of cellular metabolic pathways?

Somatic cell genetics, not yet at a stage that is amenable to sophisticated analysis, has permitted the isolation of cell variants that do shed light on the present problem. Many revertants of virus-transformed cells have been isolated and studied at the genetic, physiological, and biochemical levels (cf. Macpherson, 1971; Wyke, 1971; Nomura *et al.*, 1972; Sambrook, 1972; Stephenson *et al.*, 1973; Yamamato *et al.*, 1973). Some were free of detectable virus genome, indicating the essential contribution of virus. However, other revertants did still carry virus genetic information but were phenotypically normal. These observations suggested a delicate interplay of virus and cellular genome in maintaining the transformed state.

Temperature-sensitive variants of virus-transformed cells have been obtained that do not express the transformed cell phenotype at the nonpermissive temperature (Noonan *et al.*, 1973; Renger and Basilico, 1972). As far as can be ascertained, the virus genome remained associated with these cells and appeared not to be temperature sensitive.

These studies suggested that the virus genome coding for transformation does so through interaction with cell genetic information that determines structure and function of surface molecules. With the early demonstration that virus genome acts in this way, one turned with some confidence to seek out its biochemical expression. As will be noted below, the experimental harvest was bountiful in the extreme, proving something of an embarrassment. It became increasingly difficult to account for the very large number of changes observed in association with virus transformation with the available genetic repository.

This is particularly so in the case of the smaller papilloma and papovaviruses [and even adenoviruses (see Tables II and III)], with genome molecular weights of 2 to 5×10^6 daltons. This relatively small amount of DNA, which must code for some seven (or more) virion and nonvirion proteins (cf. Sambrook, 1972), is unable to accommodate each component change shown in Tables I and IV, if indeed each depends

upon the expression of an unique virus gene sequence. The available genetic analyses suggest that two gene products at most could serve this function (cf. Sachs, 1967; Eckhart, 1972; Benjamin, 1972).

C. Biochemical Studies

The results obtained in studies designed to understand the biochemical basis of the altered cell surface physiology associated with viral oncogenesis, are briefly summarized in Table IV. They embrace a large number of individual biochemical changes, from those involving terminal sialic acid residues at the extreme cell periphery inward to the internal plasma membrane components.

Our own studies compare the surface structure of control 3T3 mouse fibroblasts, and those transformed by polyoma virus and SV40, at the level of the glycoprotein at the extreme periphery of the cell and the proteins, glycoproteins, and glycolipids of the underlying plasma membrane. These two plasmalemma domains are most easily defined in terms of the model shown in Fig. 2. As suggested by Singer and Nicolson (1972) the plasma membrane is considered to be comprised of a lipid bilayer into which are inserted globular proteins and glycoproteins. The extreme periphery, or surface component, of the cells can be defined as that portion of the plasmalemma which is sensitive to treatment with enzymes, under conditions that leave the cells with the underlying plasma membrane functionally intact with respect to the maintenance of cellular integrity and the transport of nutrients for survival.

In our studies, trypsin has been used to define these two plasmalemma domains. Thus, treatment of cells with this enzyme (see Fig. 2) was shown to release surface glycoprotein from the extreme periphery of cells. This left the underlying plasma membrane intact, as indicated by the fact that the plating efficiency of treated cells was unaltered, as was their uptake of nonvital stains (cf. Onodera and Sheinin, 1970; Sheinin and Onodera, 1970).

Figure 3 illustrates the operational definition employed for surface glycoprotein. It relies on the fact that 3T3 mouse fibroblasts (as well as most other animal cells), when grown to confluence, are in a pseudo-G-1 phase of growth. If subcultured with trypsin, and plated at subconfluent concentrations, they will grow synchronously and immediately regenerate their surface glycoprotein. If such cultures are incubated with radioactive $GlcNH_2$ at 12–13 hours post-plating, this precursor is preferentially incorporated into surface glycoprotein. Such specifically labeled surface glycoprotein can be isolated, purified, and characterized (cf.

TABLE IV

Biochemical Changes Observed in Surface of Virus-Transformed Cells

Component affected	Virus	Selected references
Glycoproteins at the periphery of the plasma membrane	Polyoma, SV40	Wu *et al.*, 1969; Meezan *et al.*, 1969; Onodera and Sheinin, 1970; Greenberg and Glick, 1972; cf. Warren *et al.*, 1973
	RSV	cf. Warren *et al.*, 1973; Wickus and Robbins, 1973; Stone *et al.*, 1974; Wickus *et al.*, 1974
Glycoproteins within the plasma membrane	Py, SV40	Sheinin *et al.*, 1971; Sakiyama and Burge, 1972; Sheinin, 1972; Sheinin and Onodera, 1972
	RSV	Wickus and Robbins, 1973; Stone *et al.*, 1974
	EBV	cf. zur Hausen, 1972
Glycolipids	Py, SV40	cf. Hakomori *et al.*, 1972; cf. Sheinin, 1972; Yogeeswaran *et al.*, 1972; cf. Brady *et al.*, 1973; Hammarström and Bjursell, 1973; Schengrund *et al.*, 1973; Murray *et al.*, 1973
	RSV	cf. Hakomori *et al.*, 1971, 1972; Warren *et al.*, 1972; cf. Sakiyama and Robbins, 1973
Enzymes	Polyoma, SV40	cf. Burger *et al.*, 1972; cf. Johnson and Pastan, 1972
	RSV	cf. d'Armiento *et al.*, 1972; Anderson *et al.*, 1973
Sialic acid residues	Polyoma, SV40	cf. Kraemer, 1971; Schengrund *et al.*, 1973; cf. Weiss, 1973

Onodera and Sheinin, 1970; Sheinin and Onodera, 1970).

Preparations of underlying plasma membrane, greatly depleted of surface glycoprotein, were obtained using cells removed from their solid growing surface by gentle enzyme treatment (cf. Sheinin *et al.*, 1971; Sheinin and Onodera, 1972). Recently we have applied a procedure developed by Mr. Svein Carlsen (Ontario Cancer Institute) to produce plasma membrane preparations carrying almost all of the surface glycoprotein (Sheinin *et al.*, 1973).

We have now studied in detail the surface glycoprotein fraction derived from 3T3 mouse fibroblasts, and derivative cells transformed by

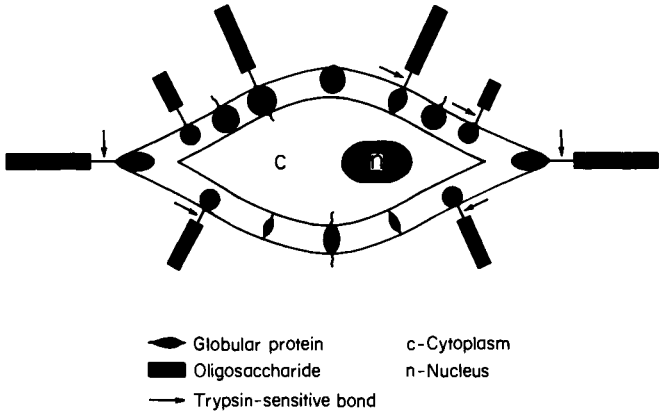

FIG. 2. Model for the structure of the cell and its surface.

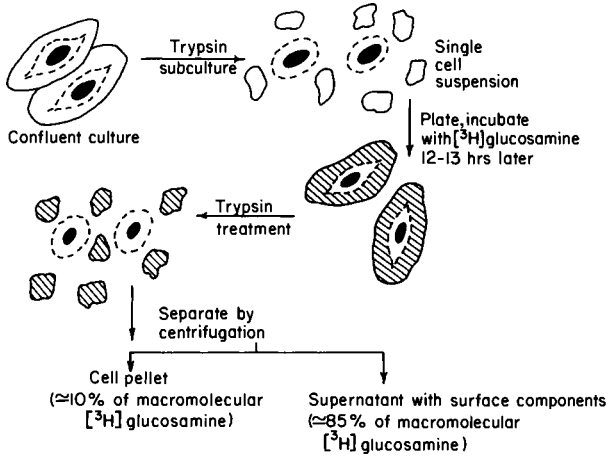

FIG. 3. Operational definition for surface glycoprotein. When confluent cultures of normal and virus-transformed 3T3 mouse fibroblasts are subcultured at $<10^4$ cells/cm^2 of growing surface area, they grow synchronously and proceed to regenerate the surface components released by trypsin treatment. If incubated with [^{3}H]GlcNH_2 at 12–13 hours postplating, this precursor is very largely incorporated into surface macromolecules shown to be glycoprotein. (Onodera and Sheinin, 1970; Sheinin and Onodera, 1970; Sheinin *et al.*, 1973).

polyoma and SV40 viruses. The physiological properties of these cells (3T3-Py6, 3T3-SV479, 3T3-SVCE56, 3T3-SVA26) have been described elsewhere (cf. Yogeeswaran *et al.*, 1972). The surface glycoprotein, as released from cells by sonic vibration and purified, was recovered as a soluble component, which remains in the supernatant after sedimentation at 105,000 *g* for 3 hours. This fraction exhibited heterogeneity when examined by polyacrylamide gel electrophoresis (cf. Sheinin *et al.*, 1973), with a major fraction moving very slowly through the gels in the region of material having molecular weights in excess of 10^5 daltons. When subjected to sedimentation in neutral sucrose density gradients [5–20% (w/w), 16 hours at 23,000 rpm at 2°C) the greatest proportion of the surface glycoprotein exhibited an apparent molecular weight of about 62,000 daltons (Sheinin and Onodera, 1970). The surface glycoprotein, excluded from beads of Sephadex G-200, was barely included after treatment with 8 *M* urea (Sheinin *et al.*, 1973).

The biochemical properties of the surface glycoprotein fraction of control and virus-transformed cells, uncovered to date, are summarized in Table V. Of particular interest is the finding that all of the surface glycoprotein, as defined herein, is homogeneous in the nature of the linkage between peptide and carbohydrate moieties. Its resistance to alkali, and the enrichment for asparagine (cf. Frohlich, 1972) in the protein suggest that this linkage is between an *N*-aspartamido residue and a GlcNAc residue. Such a linkage is characteristic of immunoglobulins and other glycoproteins found at the surface of animal cells (cf. Schachter and Roden, 1973).

Comparative analysis of surface glycoprotein derived from 3T3 cells and from 3T3 cells transformed by polyoma and SV40 viruses, revealed that these were not the same (Onodera and Sheinin, 1970). Preliminary amino acid analyses have uncovered no gross qualitative or quantitative differences; however some quantitative variations in carbohydrate content have been detected (Frohlich, 1972).

The latter findings are in general agreement with those of others (listed in Table IV) in which differences have been observed between glycoproteins at the surface of normal and virus-transformed cells. Although definitive evidence is still to be obtained, it has been suggested that the chain length of the carbohydrate residues may be shorter in the latter situation (Grimes, 1970; Greenberg and Glick, 1972; Sakiyama and Burge, 1972; Warren *et al.*, 1973).

Four kinds of experiments were performed to compare the compositions of surface glycoprotein-depleted plasma membrane preparations obtained from 3T3 cells and virus-transformed 3T3 cells. In the first purified plasma membrane preparations were fully solublized using SDS,

TABLE V

Properties of a Purified Surface Glycoprotein Fraction from Control or Virus-Transformed 3T3 Mouse Fibroblasts

1. Contains peptide and carbohydrate covalently-linked[a]
2. Linkage is alkali-resistant[b]
3. Free of lipid, RNA, DNA[a]
4. Constitutes no more than 0.2% of total cellular protein[a]
5. Carbohydrate makes up approximately 26% of the weight percent (about 14% neutral sugar, 5% amino sugar, and 7% sialic acid)[b]
6. Carbohydrate contains $GlcNH_2$, galactose, mannose, fucose[b], and sialic acid[a], but not glucuronic acid[b]
7. A full complement of amino acids is present with enrichment of asparagine and aspartic acid[b]
8. Insensitive to hyaluronidase,[a] sensitive to neuraminidase[a,b]

[a] Sheinin and Onodera, 1970.
[b] Frohlich, 1972.

mercaptoethanol, and heating. The solublized material was subjected to electrophoresis in polyacrylamide gels to resolve some 30-odd peptides, varying in molecular size from about 15,000–200,000 daltons, (Sheinin *et al.*, 1971; Sheinin, 1972; Sheinin and Onodera, 1972). The summary data shown in Fig. 4 indicated that although the major peptide pattern was similar for various plasma membrane preparations, nevertheless significant differences could be observed. In the second group of studies, plasma membrane harvested from cells generally labeled with radioactive $GlcNH_2$, to tag glycolipids and glycoproteins, were solublized and analyzed as noted above. Quite profound differences were observed between the normal and virus-transformed preparations (Sheinin, 1972; Sheinin and Onodera, 1972). It seems likely that the components here analyzed by their radioactivity were primarily plasma membrane glycoproteins, although the presence of protein-associated glycolipid was not excluded.

Protein of purified plasma membranes was obtained, solubilized, and analyzed by electrophoresis in polyacrylamide gels. Once again the peptide patterns of the normal and virus-transformed cells were generally similar (Sheinin *et al.*, 1971; Sheinin, 1972; Sheinin and Onodera, 1972). However significant differences were observed. It was especially interesting to note (Fig. 5) that the peptide patterns for the plasma membrane preparations derived from the three SV40-transformed cells were remarkably similar, perhaps suggesting a primary expression of virus genome.

A closer examination of the electrophoretic patterns shown in Figs. 3 and 4 reveals that the large molecular weight components (running at the

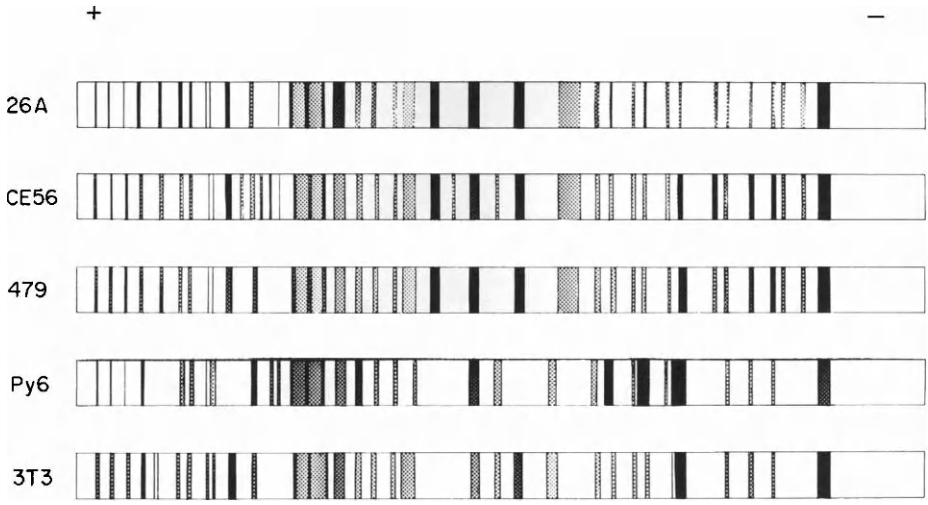

FIG. 4. Electrophoretograms of plasma membrane preparations of various 3T3 mouse cell types, analyzed in sodium dodecyl sulfate-containing polyacrylamide gels. Plasma membrane preparations (depleted of surface glycoprotein) were obtained from 3T3-SV A26, 3T3-SV CE56, 3T3-SV 479, 3T3-Py6, and control 3T3 mouse fibroblasts. These were dissolved in sodium dodecyl sulfate plus mercaptoethanol and analyzed in polyacrylamide gels as described elsewhere (Sheinin and Onodera, 1972). Material in 10 μl volumes containing, respectively, 368, 432, 443, 327 and 250 μg protein was subjected to electrophoresis at 8 mA/gel for 5–6 hours. Intensity of staining with Coomassie blue, is designated as follows: most intense, solid black, decreasing density of stain by decreasing degree of stipling. Reprinted, with permission, from Sheinin and Onodera (1972).

positive end of the gels with apparent molecular weight of $> 10^5$ daltons) are present in virus-transformed cells in lesser amount than in control 3T3 fibroblasts. In addition, the low molecular weight protein components, running at the negative end of the gels, are increased.

These observations obtained with cells transformed by the DNA viruses, polyoma and SV40, are in general agreement with those recently seen in chick embryo cells relatively early after infection with various isolates of RSV (Stone *et al.*, 1974), and under permissive conditions with a ts mutant (Wickus *et al.*, 1974). In addition, the latter infection results in a decrease in the amount of another protein of much lower molecular weight [45,000 daltons (Wickus and Robbins, 1973)].

It seemed likely from our studies with $GlcNH_2$-labeled plasma membrane preparations (Sheinin and Onodera, 1972) that the high molecular weight peptides affected in the course of transformation by polyoma and SV40 viruses are glycoproteins. This conclusion has received strong support from our recent studies (Sheinin *et al.*, 1973), in which we have

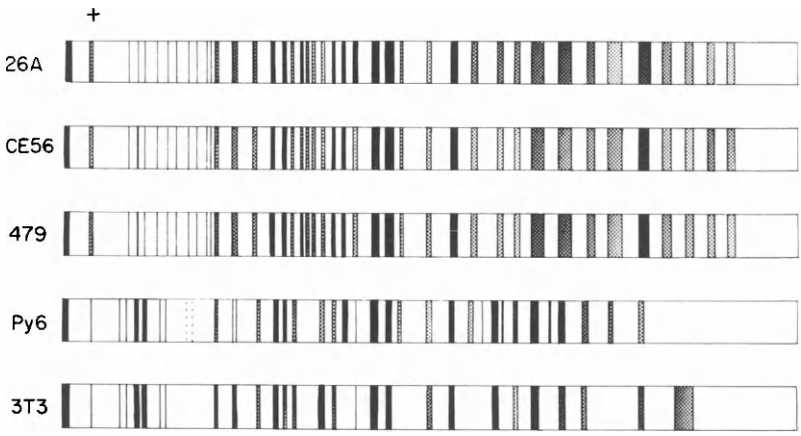

FIG. 5. Electrophoretograms of lipid-extracted plasma membrane preparations of various 3T3 mouse fibroblasts. Plasma membrane preparations (depleted of surface glycoprotein) were freed of lipid, dissolved in phenol–acetic acid and urea, and analyzed on polyacrylamide gels containing acetic acid and urea, as described elsewhere (Sheinin and Onodera, 1972). Preparations derived from 3T3-SV A26, 3T3-SV CE56, 3T3-SV 479, 3T3-Py6, and 3T3 cells contained, respectively, 40.0, 57.6, 56.8, 41.2 and 62.8 μg protein. Reprinted, with permission, from Sheinin and Onodera (1972).

been able to isolate presumably intact plasma membrane by direct processing of cells on the growing surface, without trypsin treatment. Plasma membrane so obtained from cells specifically labeled with $GlcNH_2$ in their surface glycoprotein gave profiles of electrophoresis in which the majority of the label was associated with one or two of the large molecular weight peptides.

These observations add weight to a conclusion drawn earlier (Onodera and Sheinin, 1970), that transformation by polyoma and SV40 viruses results in a change in glycoprotein at the extreme periphery of the plasma membrane. In this context it is of interest to note that the analogous large molecular weight peptides of plasma membrane from chick embryo cells, which decreased as a result of transformation by RSV, also appear to be at the external surface of the cells, as indicated by the fact that they can be labeled using iodination with lactoperoxidase (Podluso *et al.*, 1972; Stone *et al.*, 1974; Wickus *et al.*, 1974).

The final series of experiments in our comparison of the plasma membranes of normal and virus-transformed 3T3 cells were concerned with the glycolipids, which appear to be concentrated primarily in the plasma membrane of animal cells (Sheinin *et al.*, 1971; Sheinin, 1972; Yogeeswaran *et al.*, 1972, 1973). Assays of cell extracts, and extracts

of plasma membrane, again revealed great differences between normal cells and those transformed by polyoma and SV40 viruses. The data obtained with one polyoma- and one SV40-transformed cell line were generally in accord with those reported by others (see Table IV) in that marked simplification of the ganglioside pattern had occurred. However, two other SV40-transformed cell lines exhibited a complex pattern similar to that of control cells, but with a quantitative difference.

We can as yet say little with precision about the chemical basis underlying the differences observed between the glycoproteins and proteins of the plasma membranes of normal and virus-transformed cells. Analyses of glycolipids does indicate that at least in some virus-transformed cells the gangliosides have shorter and less complex carbohydrate chains, carrying fewer sialic acid residues. These observations and those of others are in accord with the hypothesis that formation of the more complex, highly sialyl-substituted gangliosides is modified in virus-transformed cells (cf. Hakomori *et al.*, 1972, 1974). Similar conclusions, but with less direct evidence, have been drawn with respect to the glycoproteins of such cells.

IV. Virus Transformation: Its Relationship to Cellular Metabolism of Surface Molecules

The extensiveness of the surface changes that accompany neoplastic transformation by viruses is evident from the data just described, as well as those summarized in Tables I and IV. An accommodation of these myriad biochemical changes, with virus genome available for coding for cell surface modulation with transformation, may come from a consideration of the postulate that integrated virus genome interferes with cellular regulatory processes that normally control the formation and turnover of surface molecules (Wallach, 1969). Such a model, shown in several forms in Fig. 6, would readily embrace the following observations.

(i) Virus transformation is associated with the appearance of species-, tissue-, or even cell-specific surface alterations, which seem not to be mediated by virus genome (cf. Haughton and Nash, 1969; cf. Alexander, 1972; cf. Tillack, 1972).

(ii) Cells that undergo virus transformation suffer a derepression of certain pathways of surface molecule synthesis. These, normally operative in cells during embryonic life, are repressed during subsequent differentiation (cf. Sachs, 1967; Boyse and Old, 1969; Hakomori and

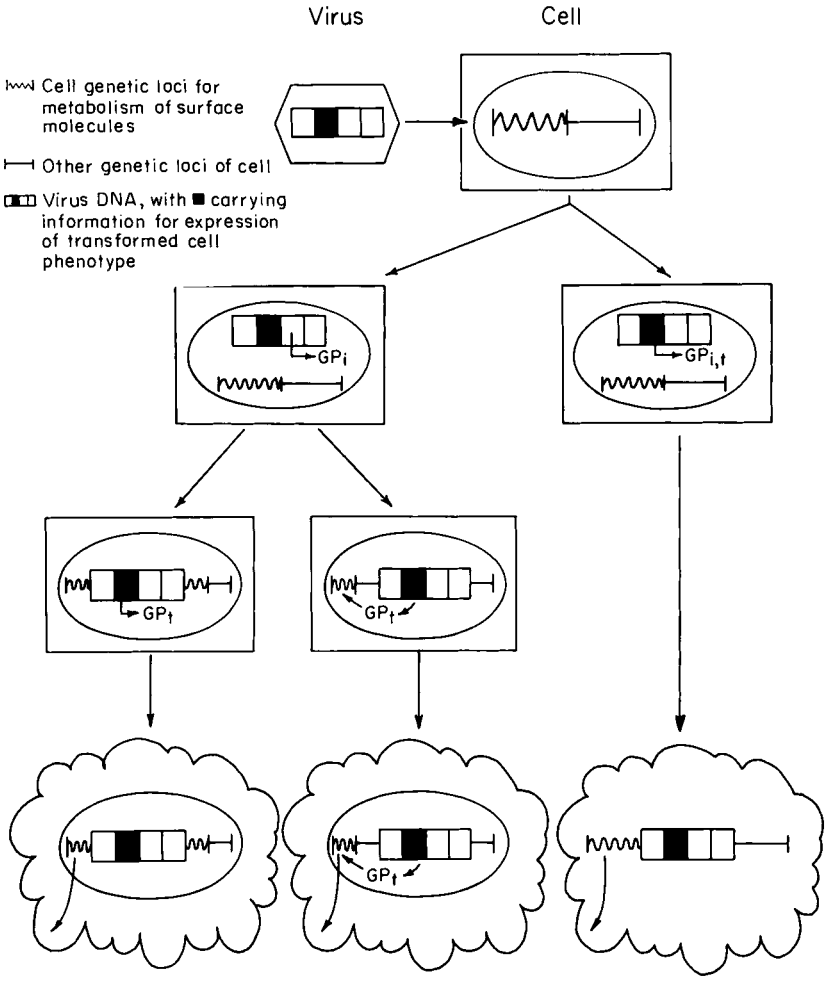

FIG. 6. Models for modulation of metabolism of surface molecules by oncogenic viruses. GP, gene product; i, for integration; t, for transformation.

Kijimoto, 1972). The end products of such metabolic sequences, present on both embryonic and transformed cells, are embraced within the general class of oncofetal antigens (cf. Alexander, 1972).

(iii) During the *in vitro* growth of normal cells, the formation of plasma membrane constituents is carefully regulated such that the formation of membrane phospholipids (cf. McMurray and Magee, 1972), neutral lipids (Bosmann and Winton, 1970; cf. Spector, 1972), glycolipids (cf. Bosmann and Winton, 1970; Hakomori *et al.*, 1972; Sakiyama

and Robbins, 1973), and glycoproteins (Bosmann and Winton, 1970; Onodera and Sheinin, 1970; cf. Kraemer, 1971; Nowakowski *et al.*, 1972; cf. Warren, 1972) assumes different patterns throughout the growth cycle. Of importance is the finding that such a shift in metabolic activity is not exhibited by virus-transformed cells.

(iv) Regulation of cell surface formation may be seen in the fact that the core plasma membrane is synthesized primarily at one stage of the cell cycle (Sheinin and Onodera, 1973; Pasternak *et al*, 1974, whereas specific glycolipid (cf. Hakomori *et al.*, 1972; Warren *et al.*, 1972; Sakiyama and Robbins, 1973), glycoprotein (Sheinin and Onodera, 1973), and protein (Kiehn and Holland, 1970; Sheinin and Onodera, 1970, 1973; cf. Warren, 1972; Pasternak *et al*, 1974) reactive groups appear to be inserted or mobilized at other, but equally specific, periods. Some properties of virus-transformed cells mimic those of cells which have not completed their full cycle of duplication (Shoham and Sachs, 1972; cf. Burger, 1973).

(v) Normal cells exhibit many of the properties of virus-transformed cells, if they are first subjected to very gentle treatment with proteolytic enzymes (cf. Burger, 1973). Recently evidence has been obtained suggesting that in virus-transformed cells, plasma membrane-associated proteases may be more active than in normal cells (cf. Burger, 1973; Schnebli, 1974).

(vi) A number of agents are known which, in normal cells, evoke many of the pleiotypic surface alterations exhibited by virus-transformed cells (cf. Kram *et al.*, 1973). In addition, virus-transformed cells selected for reversion with respect to one altered surface function can be at least partially reverted with respect to others (Wollman and Sachs, 1972; Wright, 1973).

V. Conclusions and Comments

The model that invokes modification of cellular reactions of formation of cell surface, as a primary mechanism of phenotypic expression of integrated genome of oncogenic viruses, is an agreeable one. It permits one to consolidate what has often appeared to be an enormous amount of unrelated data showing great changes in surface physiology and biochemistry as a consequence of virus transformation. It is no longer necessary to try to force agreement from different cell types (at various stages of differentiation) transformed by the same, or even different, viruses. Clearly, if the virus genes of transformation code for a product that interferes at the level of regulation of cellular pathways, then one

might expect to observe great variability depending upon the cell under study. Since major structural and functional cell surface molecules are glycolipids and glycoproteins (cf. Winzler, 1970), it is not surprising to find that the metabolism of these molecules is severely affected by transformation.

However, the key problems still remain unresolved. What is the virus gene product that so severely affects the metabolism of surface molecules in transformed cells? And what is its primary biochemical target? The second question may prove to be the more difficult to answer, since the target may vary from cell to cell. Already a number of postulates have been proposed, none of which has proved entirely satisfactory. The metabolism of cAMP, with its extremely far-reaching consequences for growth and development, has received a great deal of attention (Burger *et al.*, 1972; Johnson and Pastan, 1972; Otten *et al.*, 1972), as have transport of nutrients and their regulation of metabolic events (cf. Holley, 1972). Recently the focus has shifted to regulation of function of surface-associated enzymes, the normal action of which may be to modulate surface structure and function (Roseman, 1970; Roth *et al.*, 1971; Roth and White, 1972; Burger, 1973).

Perhaps the evidence may yet enforce the still very attractive suggestion (cf. Hakomori *et al.*, 1972) that the synthesis of the complex heteroglycopolymers (glycolipids and glycoproteins) is curtailed or modified by the virus gene product for transformation. This hypothesis rests directly on chemical evidence described above. In addition it leans on the physiological data concerning altered lectin-binding sites, receptors, antigen reactivity, cell–cell interactions of contact inhibition, adhesion and junction formation, many of which are known to be mediated by glycoproteins and glycolipids of the cell surface (cf. Winzler, 1970; and references cited in Table I). In apparent support of this hypothesis have come studies on specific glycopeptidyl and glycolipid glycosyltransferases (Grimes, 1970; Den *et al.*, 1971; Kijimoto and Hakomori, 1971; cf. Brady *et al.*, 1973; Warren *et al.*, 1972; Schengrund *et al.*, 1973) which show that the level of activity of these enzymes is lower (to the extent of 2- to 11-fold) in virus-transformed cells as compared with normal cells. However whether the decreased enzyme activity is a primary effect of virus transformation, or results from feedback control or repression, is not at all clear. The variations in enzyme activity observed are relatively small, considering the fact that the activity of glycopeptidyl-GlcNAc and sialyl transferases have been observed to vary by as much as 20-fold in 3T3 cells and 3T3-SV479 cells as they move through the cell cycle (R. Sheinin and H. Schachter, unpublished, 1972).

Perhaps there is a common target in all cell types for the action of the genes of transformation of the oncogenic viruses. It is to be hoped that this will emerge from studies with normal and virus-transformed cell variants in which surface expression can be regulated by moving from permissive to nonpermissive conditions. Similar expectations lie with revertants and re-revertants of virus-transformed cells (cf. Macpherson, 1971; McNutt *et al.*, 1973; Yamamoto *et al.*, 1973).

Turning to the question of the nature of the gene product for transformation, here too the possibilities may be several, depending upon the virus in question. For example, the simplest explanation for the action of genome of enveloped viruses is that a portion of the information for viral envelope is transmitted continuously in transformed cells, giving rise to the biogenesis of virion envelope as an integral part of the plasmalemma. Although this possibility cannot be ruled out as an essential component of the mechanism for cell surface modulation by the particular microorganisms involved, it is made less likely by the following observations.

(i) In the case of every tumor virus, neoplastic cells have been derived in which no trace of virus gene product can be detected.

(ii) Virus mutants have been isolated [from RSV (Martin, 1970; Kawai and Hanafusa, 1971) and from polyoma virus (cf. Eckhart, 1972; Benjamin, 1972)] in which the mutated gene does not define a virion peptide, but does determine a protein that is required for maintenance of the transformed cell phenotype.

Our knowledge of the reactions of plasma membrane biogenesis are still rudimentary. New avenues of biochemical and biophysical analysis of the cell surface are now opening up. These, coupled with the powerful tools of virus mutants and somatic cell variants, should permit the development of a clear picture of the synthesis, structural relationships, and structural-functional relationships of surface molecules. From such studies will undoubtedly issue a sharper comprehension of the mechanism of viral oncogenesis as it is expressed in modulation of the surface metabolism of host cells.

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CHAPTER 14

Comparative Morphology, Immunology, and Biochemistry of Viruses Associated with Neoplasia of Animals and Man

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I. Introduction

It is an interesting fact that one of the first animal viruses to be discovered was Rous sarcoma virus, an oncogenic virus (Rous, 1911). The scientific basis for oncogenicity of at least some animal viruses has therefore existed for well over half a century. In spite of this, however, a very long interval of time elapsed between the remarkable discovery of Rous

and the demonstration of an association between viruses and any mammalian tumor. Bittner, in 1936, recognized that milk of mice of certain strains contained a transmissible "factor" etiologically related to the origin of mammary cancer. However, that this factor was in fact a virus was not clearly established until the report of Dmochowski in 1953. The pioneering work of Gross (1951) that demonstrated the viral origin of leukemia in mice, while fulfilling the expectations of many workers, was received with considerable skepticism and viewed with doubt for several years. However, the two decades, since the classic report of Gross appeared, have seen the definitive establishment of viruses as etiological agents of a variety of neoplastic diseases in animals of a number of species, including amphibians, reptiles, birds, and mammals, from mice to gibbon apes (see references in Dutcher, 1970).

Viruses of a number of groups have been shown to produce tumors and/or cell transformation under laboratory conditions. The herpesvirus group (Biggs *et al.*, 1972) and the group of RNA viruses termed "oncornaviruses" by Nowinski *et al.* (1970) appear to include the agents of most virus-induced tumors of animals thus far studied. This discussion will emphasize the RNA tumor viruses and their role in the origin of neoplasia in animals and man.

Dmochowski and his associates (1956, 1969; Dmochowski and Grey, 1958) first demonstrated the presence of virus particles in the tissues of some patients with leukemia, lymphoma, and lymphosarcoma. These virus particles were morphologically similar to the virus particles responsible for murine leukemia. This observation raised the possibility of a viral etiology of human leukemia and lymphoma. The accumulated evidence from research of the past several years has strongly implicated viruses in the origin of at least some types of tumors in man, including leukemia, lymphoma, and a variety of solid tumors. Isolation of oncogenic viruses from human neoplastic tissues has, unfortunately, proved difficult and frustrating. For this reason, it has been necessary to apply knowledge gained from a variety of studies on the comparative properties of known animal tumor viruses to the problem of human neoplasia. It will be the aim of this chapter to describe some of the results of an integrated team research effort in a broad and multifaceted investigation of the relationship of viruses, particularly RNA viruses, to the etiology of selected human neoplastic diseases. The studies to be described are based on the simultaneous application of ultrastructural, biological, immunological, and biochemical methods to an investigation of viral etiology of human tumors and comparison with appropriate animal model systems. The goal of these studies has been to help close the gap between our understanding of the viruses of animal tumors and

the inferential data on human tumor cells in which virus involvement is suspected.

II. Morphological Studies

Since the initial observation of viruslike particles in the tissues of leukemic mice and of patients with leukemia (Dmochowski *et al.*, 1956; Dmochowski and Grey, 1958), many laboratories have engaged in an intensive electron microscopic search for virus particles in a variety of human and animal neoplasms. In the case of animal studies, comparison of morphological and biological findings have established the etiological role of type C virus particles (Dalton *et al.*, 1966) in leukemia, lymphoma, and lymphosarcoma of rodents, cats, and gibbons. Type C virus particles have also been demonstrated to be the causative agents of a variety of sarcomas, including soft tissue sarcomas, fibrosarcomas, and osteosarcomas in animals of a number of different species, including nonhuman primates (Dutcher, 1970; Dmochowski and Bowen, 1973). Type B virus particles (Dalton *et al.*, 1966; Dmochowski *et al.*, 1967) have now been established as the etiological agents of mouse mammary tumors.

Figure 1 (1–41) shows a composite electron micrograph of type C virus particles in normal and neoplastic tissues of animals of different species, including mice, rats, hamsters, and cats. The examples, selected from hundreds of observations of type C particles of different animals, illustrate the variation in the appearance of the type C virus particles in different tissue specimens and in different stages of maturation (Dmochowski, 1970a). A similar composite electron micrograph of type B virus particles is shown in Fig. 2 (1–41) illustrating the structural variation of type B virus particles.

Figures 1 and 2 also illustrate among the viruses of a particular morphological type, the essential similarity in the ultrastructural features of the virions and of their sites and modes of replication, no matter in what species of animals the virus particles are observed. Dalton has pointed out (1972a,b,c), as confirmed by us, that special fixation permits observation of distinct and taxonomically significant differences in the intermediate membranes of budding murine, feline, and ESP-1 type C virus particles (Priori *et al.*, 1971a) which would not be apparent in most preparations.

What then, is the situation in human tumor cells? Figure 3 illustrates some examples of the viruslike particles observed in human osteosarcoma, rhabdomyosarcoma, leukemia and lymphoma. These viruslike

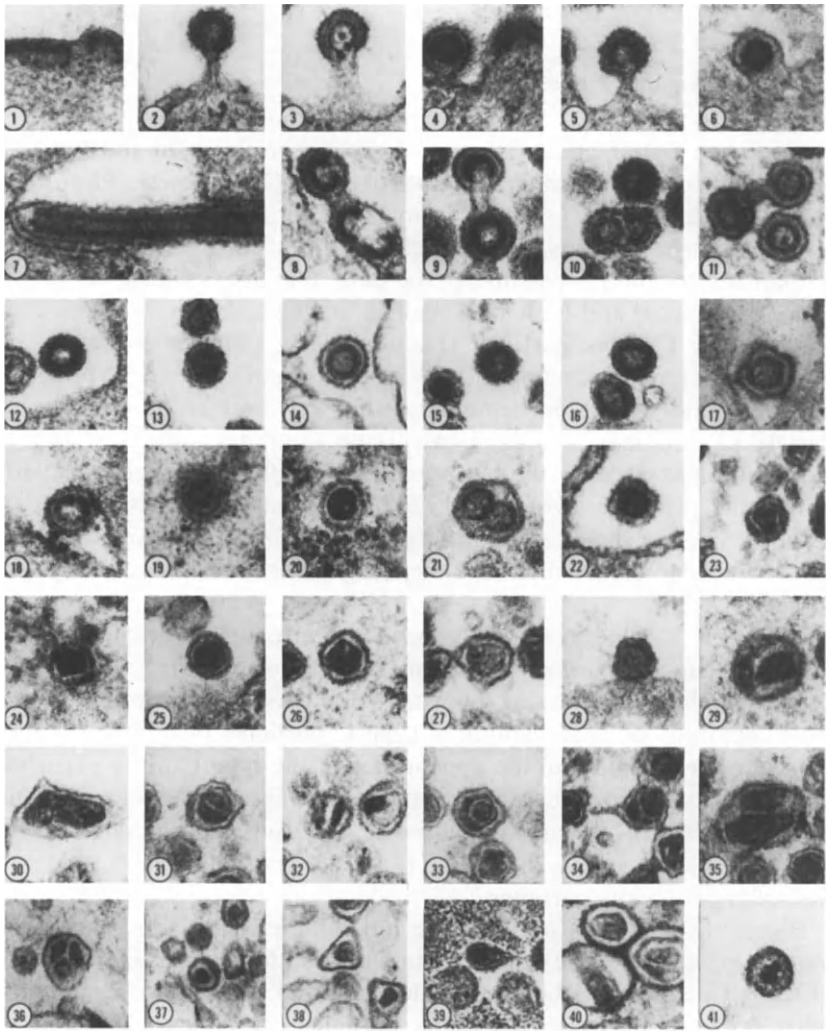


FIG. 1. Examples of type C virus particles from different species of animals, illustrating appearance of virions in various stages of maturation and some morphological variations. All virus particles are magnified ×100,000. Type C virus particles from *mice* are shown in insets 3, 4, 5, 7, 8, 9, 10, 11, 12, 15, 16, 17, 19, 20, 21, 22, 24, 25, 26, 27, 28, 31, 33, 34, 35, 37, 38, and 39. Type C virus particles from *rats* are shown in insets 1, 13, 14, 23, 30, 36, and 40. Type C virus particles from *hamsters* are shown in insets 6, 18, and 32. *Feline* type C virus particles are illustrated in insets 2 and 28. The appearance of a mature type C virion in *chicken* tissue is shown in inset 41. Tissue specimens were prefixed in buffered 3% glutaraldehyde, rinsed in phosphate buffer, then post-fixed in buffered osmic acid.

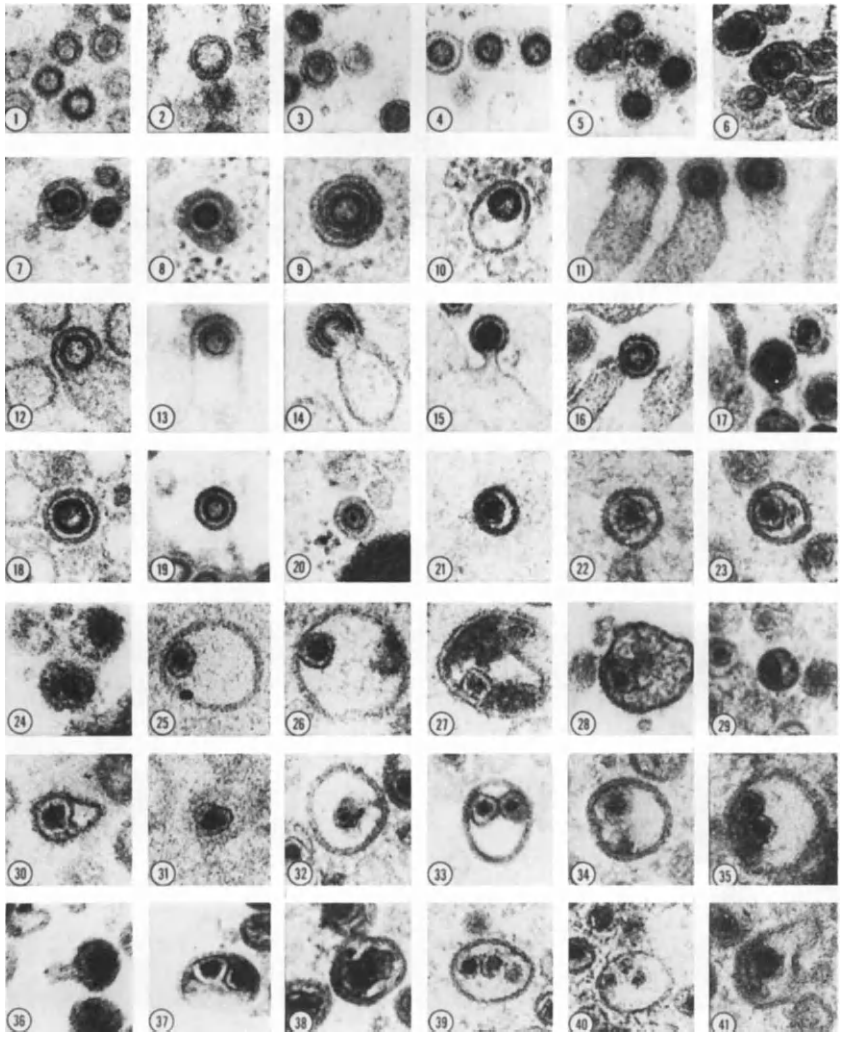
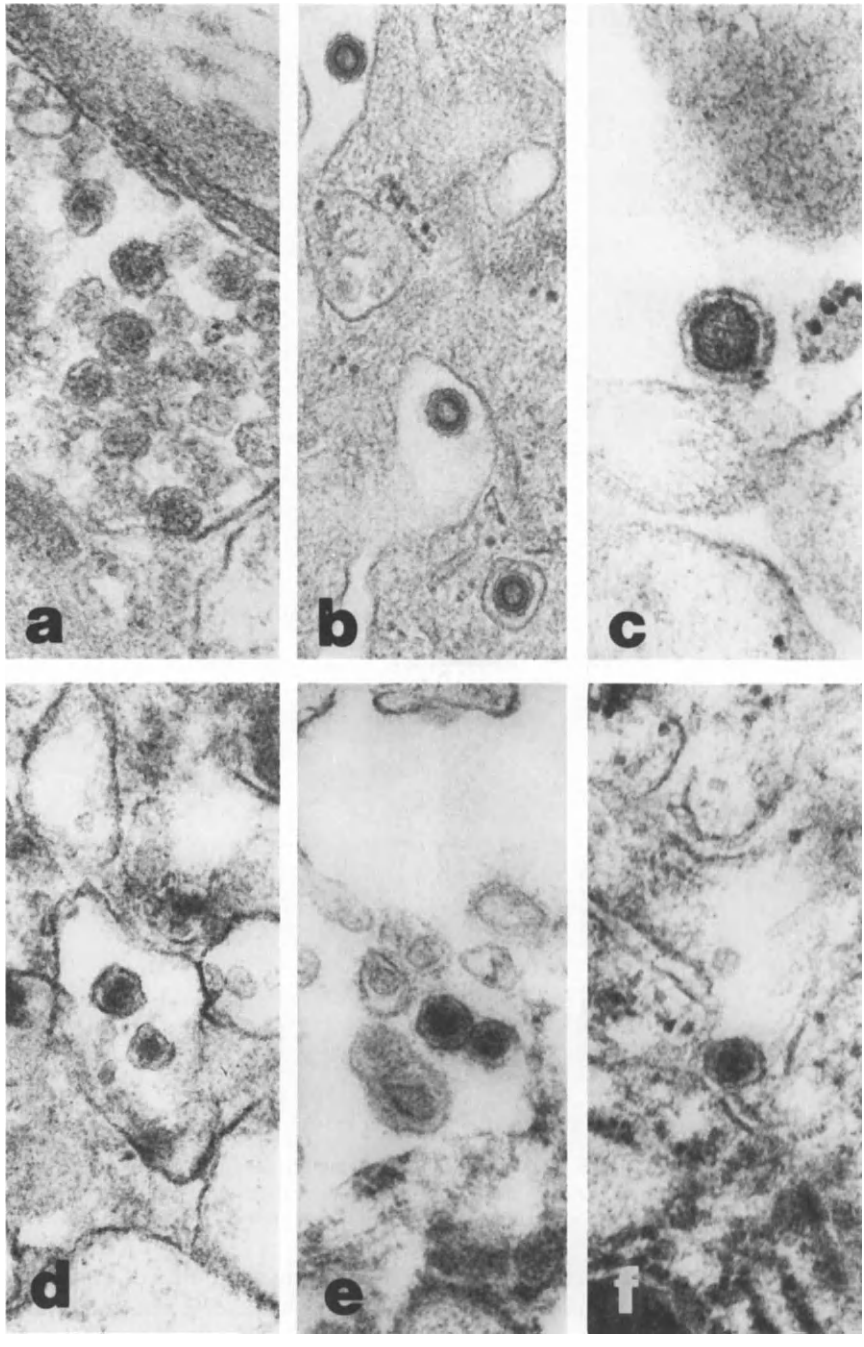


FIG. 2. Examples of type B virus particles from mammary tumor tissue, milk, or normal tissue of mice of various strains, illustrating morphological features and variations. All virus particles are magnified ×100,000. Specimens fixed as in Fig. 1.

particles show some variation, but are well within the limits of variability seen among type C virus particles in animal tissues. As a general conclusion, it can be stated that these "human type C viruslike particles" are morphologically indistinguishable from the type C virus particles in animals of various species. This statement also applied to the viruslike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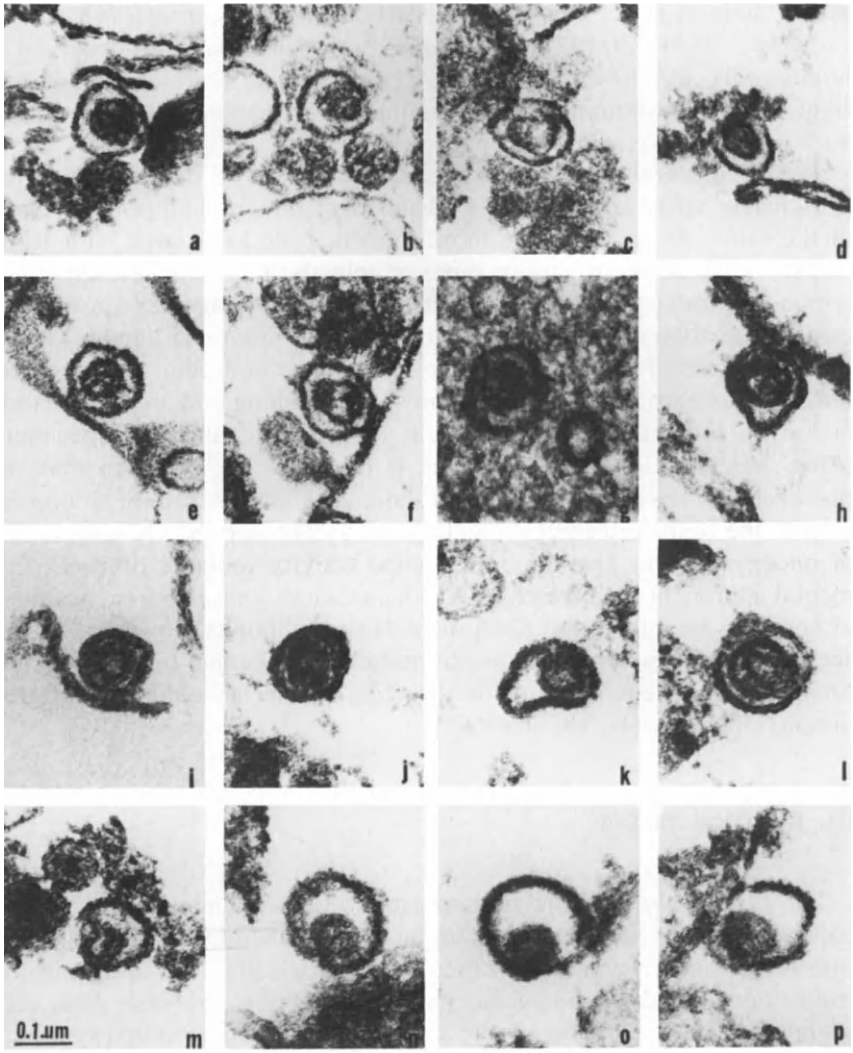


FIG. 4. Examples of type C and type B viruslike particles in different samples of human milk. ×70,000. Specimens fixed as in Fig. 1.

FIG. 3. Examples of type C viruslike particles in neoplastic tissues of a patient with leukemia (a), with lymphoma (b), with rhabdomyosarcoma (c), and of three different patients with osteosarcoma (d,e, and f). ×60,000. Specimens fixed as in Fig. 1.

particles observed in the tumor tissues of women with breast cancer and in the milk of some normal women (Feller and Chopra, 1968; Dmochowski *et al.*, 1969, 1972b). Examples of viruslike particles observed in human milk are shown in Fig. 4. A characteristic feature in studies of tissues and milk of humans is the finding of viruslike particles resembling both type B and type C virus particles.

Thus, the results of morphological studies have demonstrated the presence of viruslike particles in tissues from human neoplastic diseases of the same diverse types as those known to be associated with RNA viruses in neoplasia of various types of animals. These include leukemia, lymphoma, and a variety of solid tumors. These findings have provided a basis for further studies on the relationship of viruses to human cancer, but two points of caution should be raised. The first point is that detection of virus particles in human tissues requires long and arduous search, and even then it is unsuccessful in a high percentage of specimens (Dmochowski, 1970a). In addition, it must not be forgotten that the presence of virus particles in human neoplastic tissues cannot be considered as the basis for establishing their oncogenic activity. Demonstration of oncogenicity or any other biological activity requires other experimental approaches. In the case of human cancer, these approaches must of necessity be inferential. Regardless of its limitations, however, the observation of characteristic viruslike particles in a human tumor specimen provides an excellent basis for directing further investigation of that tumor (Dmochowski, 1973b,c).

III. Biological Studies

The type C virus particles associated with leukemias and with sarcomas of animals bear an important biological relationship to each other. Indistinguishable from each other morphologically and antigenically, leukemogenic and sarcomagenic viruses may infect the same host cell, leading to transformation of the cells and productive infection which give rise to both types of virus progeny. The sarcoma virus has been shown to be defective (Huebner *et al.*, 1966) and produces cell transformation without productive infection in the absence of a helper leukemia virus. Cells transformed by sarcomagenic viruses or derived from virus-induced sarcomas do not spontaneously release sarcoma viruses, but can be induced to do so by superinfection with appropriate leukemia viruses or by cocultivation of the transformed cells with cells producing leukemia viruses.

The observation of foci of morphologically altered cells in a monolayer culture infected with a sarcoma virus provides a biological parameter for the detection and quantitation of sarcoma virus activity in tissues of animals of different species.

The characteristic appearance of Soehner-Dmochowski virus-induced transformation of mouse cells is shown in Fig. 5. Figure 5a illustrates the appearance of an uninfected control culture, and Fig. 5b shows the appearance of an infected culture with focal areas of transformation.

Using the animal leukemia-sarcoma system as a model, studies were carried out in an attempt to induce or activate sarcomagenic activity in cells derived from selected human solid tumors. Cells derived from human osteosarcomas, fibrosarcomas, and giant cell tumors of bone were taken at biopsy, dispersed with trypsin, and cultured. Growing cultures of these cells were inoculated with fresh bone marrow aspirates from patients with different types of leukemia. The cocultures were maintained, subcultured when necessary, and observed for evidence of morphological changes. In a number of these mixed cultures, foci of morphologically altered cells appeared (Fig. 6). The changes appeared several weeks and at least five subcultures after cocultivation was initiated. Cells in the foci were picked and transferred, resulting in apparently pure cultures of rapidly growing epithelial cells. Cells of untreated cultures grew slowly, retained their fibroblastic appearance, and never showed morphologic transformation (Fig. 7), except for one culture of giant cell tumor that spontaneously transformed some 50 subcultures later.

The morphologically transformed cells of these cultures exhibited many of the properties of malignant cells, including growth in semisolid agar and production of tumors in immunosuppressed mice (Maruyama and Dmochowski, 1973; Maruyama *et al.*, 1973; Dmochowski *et al.*, 1973). Intensive electron microscopy of these transformed cells and their parent lines failed to reveal the presence of virus particles. Other studies, however, which will be presented elsewhere in this chapter, revealed the appearance of newly acquired biochemical properties that suggested partial activation of an RNA viral genome.

Repeated attempts at inducing virus production in the transformed cells, the parental sarcoma cell lines, or in cells of cultures derived from breast tumors have been unsuccessful. These attempts included various types of treatment of the cultures such as 5-iododeoxyuridine, 5-bromodeoxyuridine, and other compounds shown to induce type C virus particle replication in cells of animal (Lowy *et al.*, 1971) and some human (Stewart *et al.*, 1972a,b) cultures.

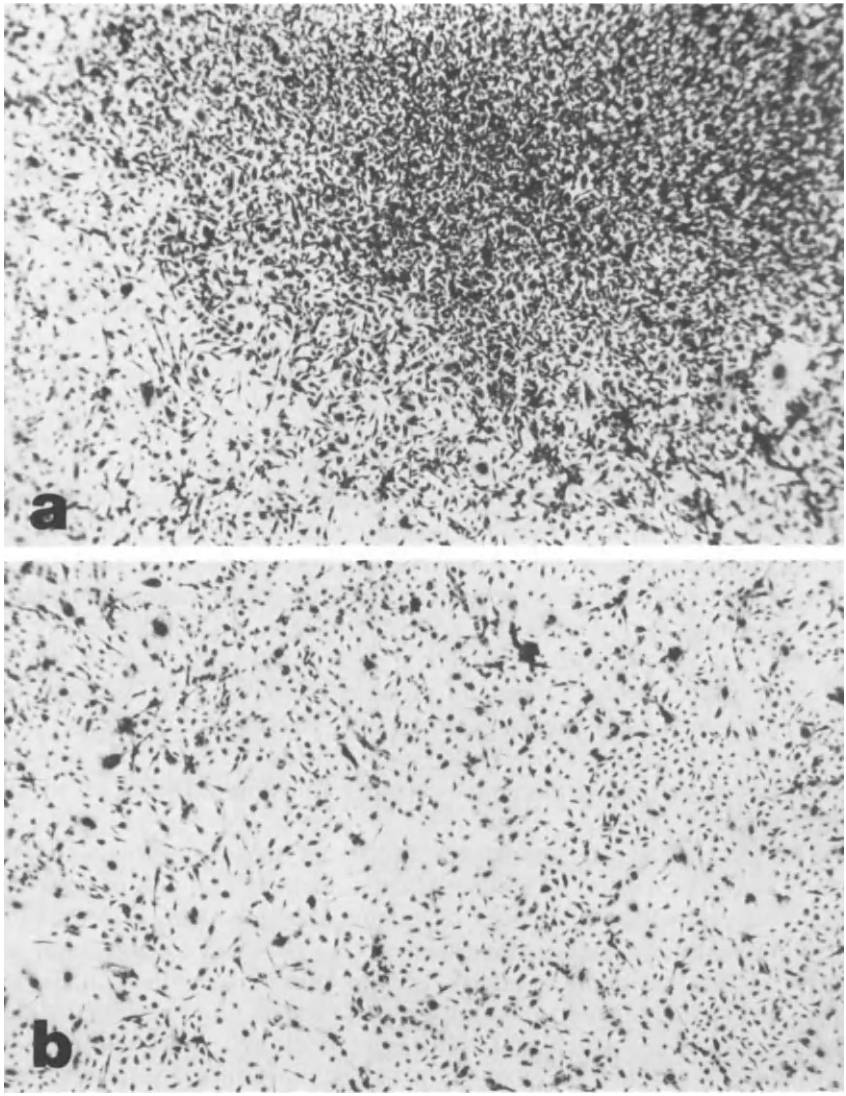
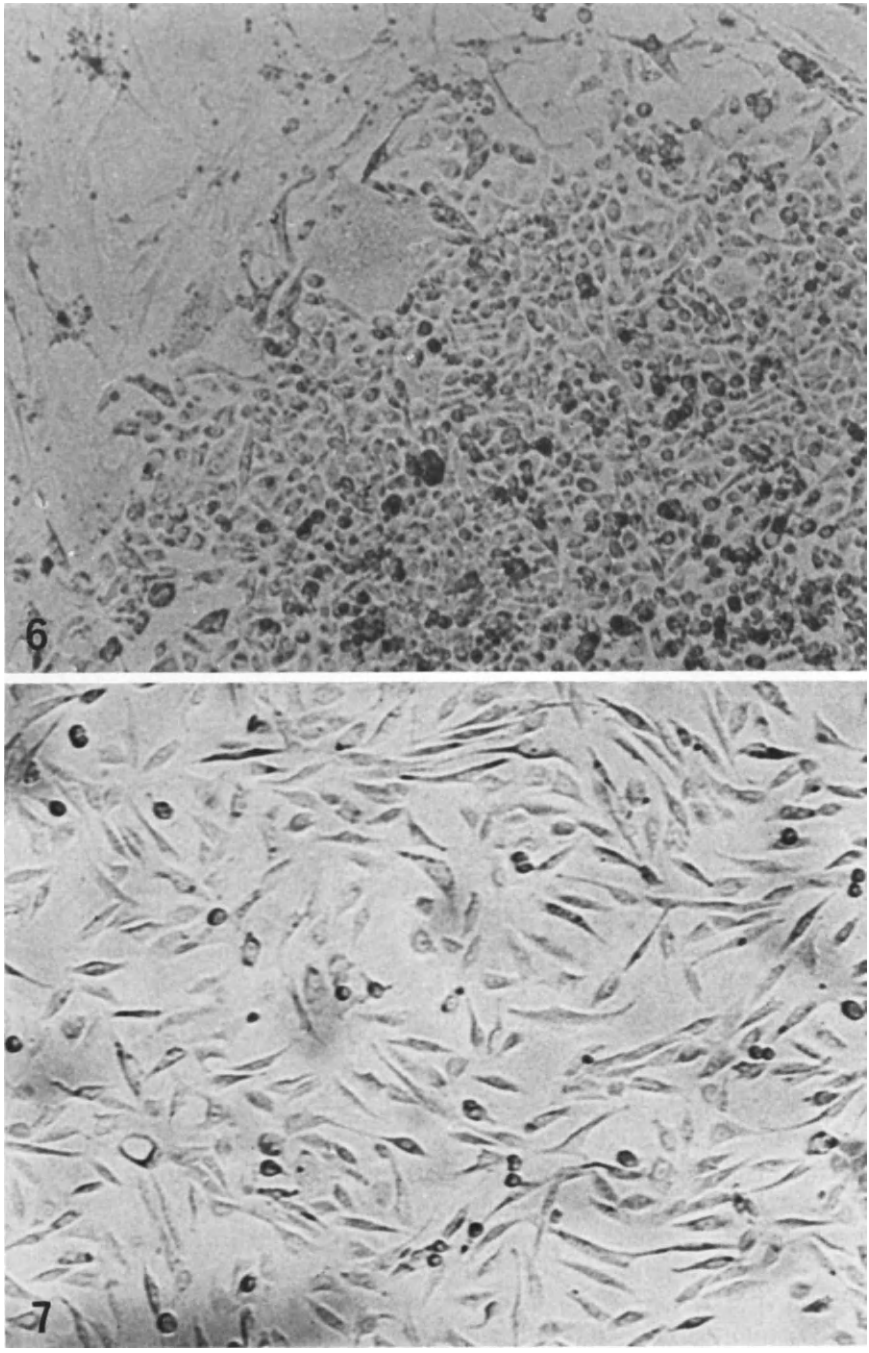


FIG. 5. A focal area (a) of 3T3 mouse fibroblasts transformed by Soehner-Dmochowski murine sarcoma virus. An uninfected control is shown in (b). ×100.

FIG. 6. Cells of a culture derived from a human giant cell tumor of bone which transformed following inoculation with bone marrow aspirate from a patient with acute lymphatic leukemia. Part of a focal area of transformed cells is shown. ×150.

FIG. 7. Cells of the uninoculated parental culture of a human giant cell tumor of bone, which showed no morphological transformation. ×150.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IV. Immunological Studies

The morphological similarity of RNA tumor virus particles in neoplastic tissues of animals of different species raises the question of their possible antigenic relationships. RNA tumor virus particles appear to contain antigens of three different classes: type-specific antigens, group-specific antigens shared by viruses of a single species (or within a species), and group-specific antigens shared by viruses of different species (Old *et al.*, 1964; Geering *et al.*, 1968). These classes overlap, and there is good evidence (Schäfer *et al.*, 1969, 1972; Gilden *et al.*, 1971) that certain virion components may carry antigenic sites of more than one class.

Immunological studies on virus-induced animal tumors have shown that tumors induced by the same oncogenic virus in animals of different species share common antigens. These antigens may be of several different types, including viral, tumor, embryonic, and heterophile, or Forssman antigens (Dmochowski, 1973; Maruyama and Dmochowski, 1974). Animals bearing virus-induced tumors may produce antibodies against some or all of these antigens (Dmochowski *et al.*, 1972b; Hoshino *et al.*, 1973). Cells of some virus-induced tumors may show the presence of viral antigens, even in the absence of demonstrable production of virus particles. These antigens may provide specific means for detecting the interaction of the oncogenic virus with the host cell leading to malignant transformation.

Extensive immunological studies have shown that sera of patients with different types of neoplasia contain antibodies to antigens of their own and homologous tumors. Further, sera of some patients with a tumor of one histologic type cross-react with antigens in cells of tumors of different histological types (Priori *et al.*, 1972). Such antibodies have been detected in the sera of patients with leukemia, with osteosarcoma (Morton and Malmgren, 1968; Priori *et al.*, 1971b), with soft tissue sarcomas, and with breast cancer (Priori *et al.*, 1972; Dmochowski *et al.*, 1972b). Extensive cross-reactions have been observed between osteosarcoma and breast cancer serum-cell combinations. The results of absorption of positive sera with appropriate material have demonstrated that the majority of these reactions are not due to heterophile, allotypic, or other tissue antigens. Further, these results indicate that the reactions may reflect the presence of viral and/or tumor antigens in the cells of the human tumors examined. These antigens have not been positively identified as viral, however, and their true nature remains to be elucidated. It is interesting to note that some sera from patients with tumors of dif-

ferent types give positive cytoplasmic fluorescence with type C virus-producing animal cell lines. These reactions have not as yet been demonstrated to be directly associated with virus particles.

The results of recent studies have clarified the nature of the immunofluorescent reaction in the case of breast cancer. They have added further support for the idea that human breast cancer is viral in origin and have indicated that the "human breast cancer virus" is antigenically related to the mouse mammary tumor virus. In an attempt to analyze the nature of the antibodies in sera of breast cancer patients giving positive reactions with cytoplasmic antigen in breast cancer cells, an investigation was carried out in an attempt to find out whether these sera react with antigens in virus-producing cells derived from mouse mammary tumors. In initial studies, the indirect immunofluorescence test was used (Priori *et al.*, 1972). The results of these tests indicated the presence of antibodies in sera of some patients with breast cancer, of some of their relatives, and of some normal individuals which react with cells derived from mouse mammary tumors and which produce type B virus particles, type C virus particles, or both types of virus particles. A typical immunofluorescent reaction between a serum of a patient with breast cancer and a type B particle-producing mouse mammary tumor cell line is illustrated in Fig. 8a. For comparison, the reaction of this serum with cells derived from a human breast tumor is shown in Fig. 8b.

The results of an extensive survey of these sera against various mammary tumor cell lines are shown in Table I. Sera of 50% of the patients, 40% of relatives of some of the patients, and 15% of the donors reacted with cells of mouse mammary tumor cell lines producing type B and type C virus particles. Absorption of the positive sera with preparations of type B virus particles removed the immunofluorescent reaction, while tissues of normal mice, heterophile antigens, or human embryonic tissues failed to absorb out the activity. These results strongly indicated that the positive immunofluorescence reactions between human sera and mouse mammary tumor cells were due, at least in part, to viral antigens. In an attempt to answer this question, the indirect immunoperoxidase technique was employed to determine whether the antigens reacting with antibodies in the sera of patients with breast cancer were associated with type B virions. A number of the human sera found positive with virus-producing cells of mouse mammary tumors gave strong immunoperoxidase reactions with mature and budding type B virus particles of these cells. These observations strongly support the conclusion that human breast cancer is associated etiologically with a virus that is antigenically related to the virus of mouse mammary cancer. The extent and nature of this antigenic relationship is currently under intensive investigation.

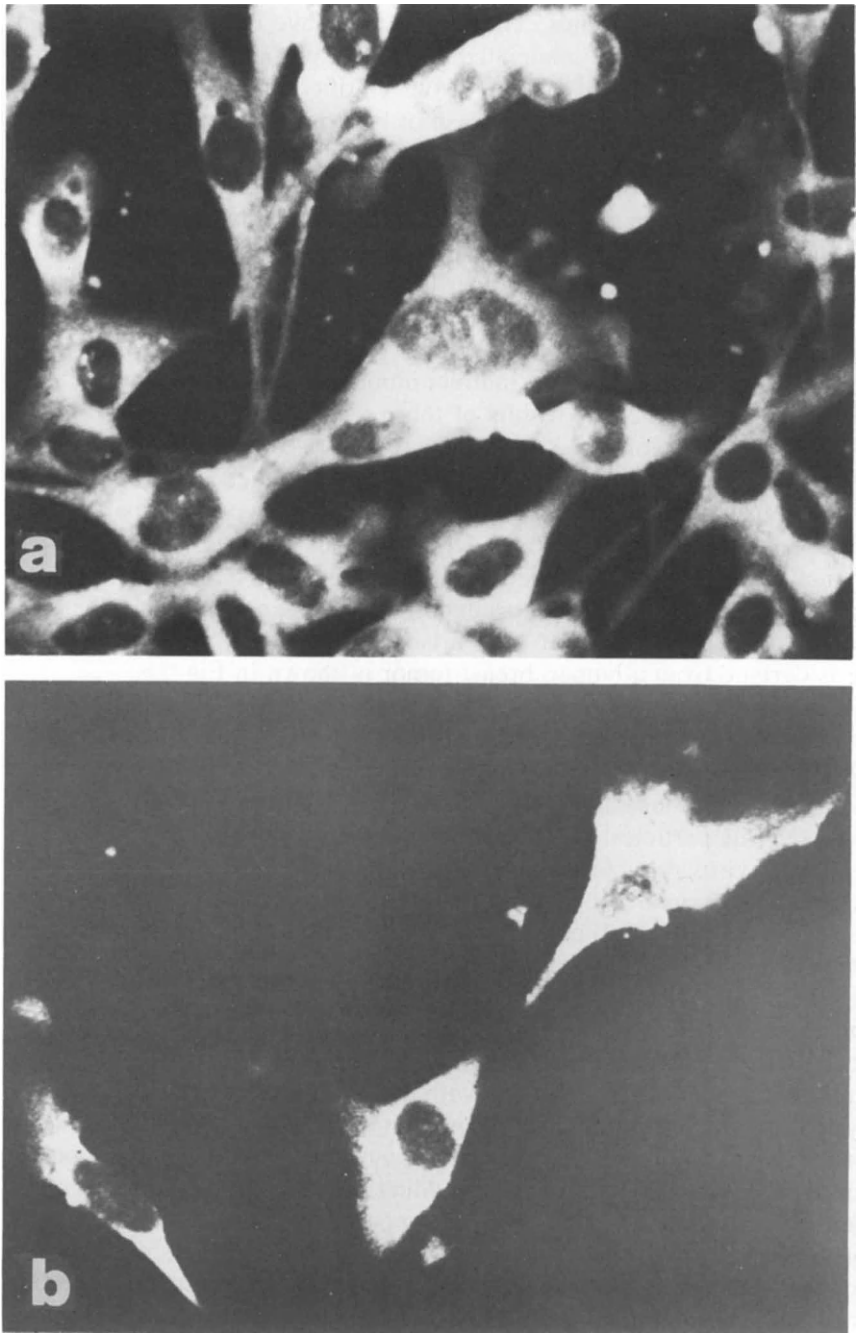
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TABLE I

Summary of the Results of Immunofluorescence Tests of Sera of Patients with Breast Cancer and of Some of Their Relatives with Virus-Producing Cells of Various Mouse Mammary Tumors[a]

		Mouse mammary tumor tissue cultures					
		B+C+		B−C+		B+C−	B−C−
Patients	No.	RIII	C3H/Z	BALB/c	C3H/f	C57 × A_{F1}	C3H/Z
Early	5	0	2	3	2	4	0
Middle	7	5	5	3	2	3	1
Late	11	9	8	6	3	7	1
Totals	23	14	15	12	7	14	2
Relatives							
Early	1	1	0	1	0	0	0
Middle	13	5	7[b]	2	4	8[b]	0
Late	9	7	2[b]	6	7	5[c]	0
Totals	23	13	9	9	11	13	0
Donors	20	NT	NT	NT	8[d]	3	4
Totals					8	3	4

[a] See text for details.
[b] One of these + sera was from one male relative.
[c] Two of these + sera were from male relatives.
[d] Cell line from DBA mouse mammary tumor was tested.

V. Biochemical Studies

A number of significant new avenues in studies of the role of viruses in human neoplasia have opened as a result of recent advances in the development and application of molecular probes. Particularly apparent among the newer biochemical approaches in viral oncology have been the characterization of the complex, high molecular weight RNA

FIG. 8. Fluorescence photomicrographs of type B virus particle-producing mouse mammary tumor cells (a) and of cells derived from a human breast cancer (b) fixed in acetone and treated with the serum of a patient with breast cancer. The reaction was developed with fluorescein-conjugated goat anti-human IgG. Note the strong cytoplasmic immunofluorescence given by this serum with both types of cells. The results of subsequent immunoperoxidase studies revealed that the reaction between the human serum and the mouse mammary tumor cells was due to labeling of type B virus particles. (a) ×1200, (b) ×750.

genome of oncornaviruses (Duesberg, 1968; Dmochowski, 1973a;); the discovery and characterization of viral RNA-dependent DNA polymerase complexes (reverse transcriptase, Baltimore, 1970; Temin and Mizutani, 1970; Spiegelman *et al.*, 1970; Gallo, 1971); and the application of molecular hybridization techniques to elucidation of genetic relatedness between viruses of different animal species (Spiegelman *et al.*, 1970; Haapala and Fischinger, 1973).

Our studies have combined these biochemical approaches with other experimental methods in an attempt to obtain meaningful correlative data on viral genetic information in animal and human tumor cells. Extensive use has been made of the Soehner-Dmochowski rat bone tumor-inducing virus (MSV-SD) that was derived from Moloney sarcoma virus (Soehner and Dmochowski, 1969; Dmochowski *et al.*, 1972a). MSV-SD has the biological, biophysical, and biochemical properties characteristic of the murine leukemia-sarcoma complex. The results of biophysical characterization of MSV-SD virions and of their genomic RNA are illustrated in Fig. 9. The left-hand profile in Fig. 9 shows the characteristic buoyant density of MSV-SD virions banded in an isopycnic sucrose gradient. The peak fraction of radiolabeled virus particles is at a density of 1.164 gm/ml. RNA extracted from these particles and analyzed by rate zonal ultracentrifugation in sucrose gradients exhibits a mean sedimentation value of about 55 S as shown in the right-hand profile of Fig. 9. The RNA of MSV-SD, like that of other oncornaviruses, is unique in that the 55 S–60 S molecule is an aggregate consisting of subunit components held together by hydrogen bonding (Duesberg, 1968; East *et al.*, 1973). During maturation of MSV-SD virions, the genomic RNA undergoes a structural rearrangement (East *et al.*, 1973) that is characterized by a shift in buoyant density from 50 S to the heavier molecule. Studies now in progress suggest that different groups of RNA viruses may fall into at least three categories, depending on the mode of formation of the final aggregate genomic RNA molecule. This feature may be of future use in an attempt at subgrouping of oncornaviruses.

As an initial step in the biochemical characterization of RNA from human tumor tissues or from transformed human cultures, the RNA of known animal tumor viruses was studied. For determination of the sedimentation characteristics of RNA from animal RNA tumor viruses, virus-producing animal cell cultures were exposed to tritiated uridine, the released virus particles were purified, and the RNA was extracted and analyzed in rate zonal sucrose gradients (East *et al.*, 1973). Examples of typical sedimentation profiles of the RNA of two different animal viruses, MSV-SD (murine) and Crandell (feline) are shown in

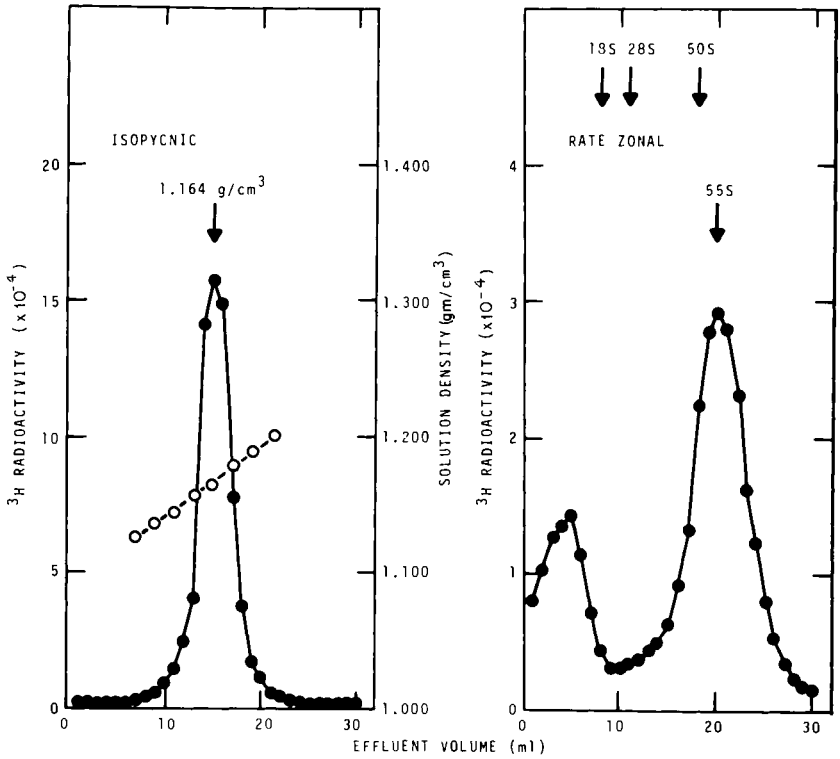

FIG. 9. Biophysical characterization of Soehner-Dmochowski murine sarcoma virus (MSV-SD) and its genomic RNA. The left-hand panel shows MSV-SD virions concentrated from culture medium from a virus-producing rat bone tumor tissue culture and analyzed on an isopycnic sucrose gradient. RNA was extracted from these particles with sodium dodecyl sulfate and phenol and analyzed by rate zonal centrifugation on linear sucrose gradients. The sedimentation coefficient of 55 S was determined by comparison with three internal markers – 18 S and 28 S ribosomal RNA from HeLa cells and 50 S genomic RNA of Newcastle disease virus.

Fig. 10A and B. These values are characteristic for these viruses and are reproducible over a very narrow range from experiment to experiment.

The unique properties and relative ease of demonstration of fast-sedimenting RNA has led to the use of the presence of particles containing such RNA as an important criterion to indicate the presence of RNA tumor viruses or their genetic expression in cells derived from human neoplasms. The human cell cultures were exposed to tritiated uridine for extended periods (up to 5 days). The tissue culture fluid was then concentrated and the RNA extracted and analyzed by rate zonal centrifugation on sucrose gradients in the same manner as that used for

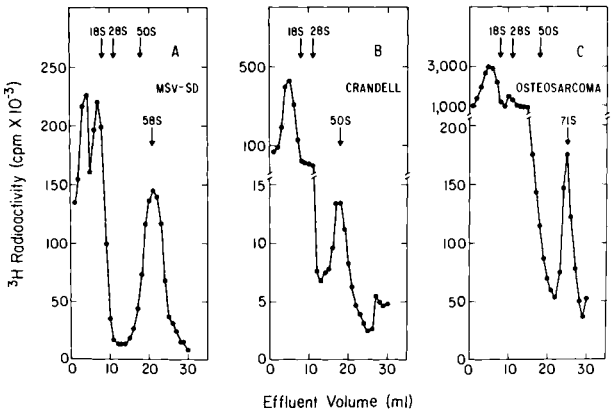

FIG. 10. Sedimentation profiles of RNA extracted from Soehner-Dmochowski murine sarcoma virus (A), from Crandell feline type C virus (B), and from particles released by cells of a culture of human osteosarcoma (C). The RNA was extracted and analyzed as in Fig. 9.

the animal viruses. Figure 10C shows, for comparison with the animal viruses, the sedimentation profile of RNA extracted from a particulate fraction of tissue culture medium from a culture of human osteosarcoma. The typical profile of oncornaviruslike fast-sedimenting RNA is apparent in these results.

The characteristic sedimentation values obtained for several animal viruses are illustrated in the left-hand columns of Table II. The sedimentation values include those for both leukemia and sarcoma viruses of murine, feline, and simian origin as well as for mammary tumor virus particles and for type C particles of unknown oncogenicity. A rather wide distribution of sedimentation values of genomic RNA's was observed, varying from 58 S for several of the viruses to 71 S for the type C virus particles from cells of a BALB/c/Dm mouse mammary gland culture. The sedimentation coefficients of fast-sedimenting RNA's from two viruses originating in human cells, RD-114 and ESP-1, and from medium of selected cultures of cells derived from human neoplasms are shown in the right-hand columns of Table II. The genomic RNA of RD-114 virus had a sedimentation constant of 50 S, whereas ESP-1 viral RNA gave a value of 64 S.

Fast-sedimenting RNA's were also detected in the medium from a number of human neoplastic cell cultures, which suggests the production of oncornaviruslike nucleic acids by cells of these cultures. The total number of counts in the region of indicated fast-sedimenting RNA's of course varies widely, depending upon the amount of virus or RNA

TABLE II

Sedimentation Coefficients of RNA's from Selected RNA Tumor Viruses and from Human Tumor Cells in Tissue Culture[a]

Animal system	cpm per culture	S value	Human system	cpm per culture	S value
Murine viruses			Candidate cancer viruses		
MuLV-Friend (C)	48,045	58	RD-114	160,260	50
MuLV-Rauscher (C)	497,940	62	ESP-1	11,620	64
MSV-SD (C)	1,309,240	58			
MSV-Kirsten (C)	326,562	58			
MSV-Moloney (C)	493,035	62			
MMT-DW (C + B)	191,055	58			
MMT-C_3H (B)	25,795	58	Human tumor cells		
MMT-S (B)	1,875	68	Giant cell tumor of	662	55
MMG-BALB/C (C)	8,997	71	bone (L)	812	68
			Fibrosarcoma (B)	634	68
			Osteosarcoma	198,923	71
Feline viruses			Osteosarcoma	93,090	71
FeLV-Rickard (C)	70,675	58	Leukemia	4,415	64
FeLV-Thielen (C)	19,620	64			
FeSV-Rickard (C)	145,007	62			
Feline-Crandell (C)	27,301	50	Cocultures		
			Giant cell tumor of	1,583	55
Simian viruses			bone + leukemia (LG)	3,553	68
SiSV-Marmoset (C)	50,642	64	Fibrosarcoma +		
SiMTV-MP (B2)	9,390	63	leukemia (BT)	14,820	68

[a] Sedimentation values were obtained with RNA preparations extracted and analyzed by rate zonal centrifugation in sucrose gradients as described in the text.

produced by the different test cultures. It is of particular interest to compare the RNA's obtained from the culture of a human giant cell tumor of bone (L) and from the culture of a human fibrosarcoma (B), with the RNA's obtained from cultures transformed after treatment with bone marrow aspirates from patients with acute lymphatic leukemia (LG and BT). Both parental cultures released particles containing fast-sedimenting RNA, but at very low levels. The parental culture derived from the giant cell tumor of bone consistently showed two species of fast-sedimenting RNA with sedimentation coefficients of 55 S and 68 S. The parental culture derived from the fibrosarcoma produced a single species of 68 S. After transformation, these cultures produced RNA species of the same sedimentation values, but at three- to twentyfold higher levels, suggesting that genetic information contained in the parental tumor cells was activated during transformation resulting from treatment with leukemic bone marrow cells.

The observation of RNA's with sedimentation coefficients similar to those of RNA tumor viruses is merely suggestive of the presence of viral genomes in these cells. The RNA's must be further characterized before their identities can be firmly established. Molecular hybridization experiments are now in progress to determine whether the fast-sedimenting RNA species released by human neoplastic cells have nucleotide sequences in common with the RNA genomes of mammalian RNA tumor viruses.

These findings are particularly encouraging in view of the findings of Spiegelman and his associates of genetic information in human leukemia, lymphoma, and sarcoma tissues homologous to nucleotide sequences in Rauscher leukemia virus (Axel *et al.*, 1972; Hehlman, *et al.*, 1972; Kufe *et al.*, 1972). Similarly, Spiegelman *et al.* (1972) and Das *et al.* (1972) have demonstrated common nucleotide sequences between mouse mammary tumor virus genome and DNA from human breast cancer tissue. Schlom *et al.* (1971, 1972) have further shown that particles in human milk which morphologically resemble type B virus particles of mice also contain reverse transcriptase and high molecular weight RNA. This provided further evidence of viral origin of human breast cancer. The simultaneous detection of reverse transcriptase and high molecular weight RNA has been accepted as another major criterion for the presence of RNA tumor viruses or their genetic expression in animal and human tumors. Spiegelman and his associates (Axel *et al.*, 1972; Hehlman *et al.*, 1972; Kufe *et al.*, 1972) Gallo and his associates (1970, 1971, 1972; Todaro and Gallo, 1973) and investigators in our group (Allen *et al.*, 1973; Bowen *et al.*, 1973) have shown the presence of reverse transcriptase and/or high molecular weight RNA in tissues from patients with leukemia, lymphoma, sarcomas of various types, and certain other tumors, including giant cell tumors of bone. We have applied the simultaneous detection assay described in Schlom and Spiegelman (1971) to concentrated fluid from a number of human tumor tissue cultures. Unfortunately, the results have been variable and the DNA counts in the 60 S to 70 S region of the sucrose gradients have been low, as presented in Fig. 11A–C. The data in Fig. 11 illustrate three different experiments on material from a culture established from the bone marrow of a patient with acute lymphatic leukemia. A standard simultaneous detection assay was performed on concentrated tissue culture medium from the culture. In each case a small peak of fast-sedimenting DNA was observed, raising the possibility that the culture is releasing particles with some of the properties of RNA tumor viruses. Thus, the application of molecular probes to the search for viruses in human neoplastic tissue has led to further evidence that viruses, with

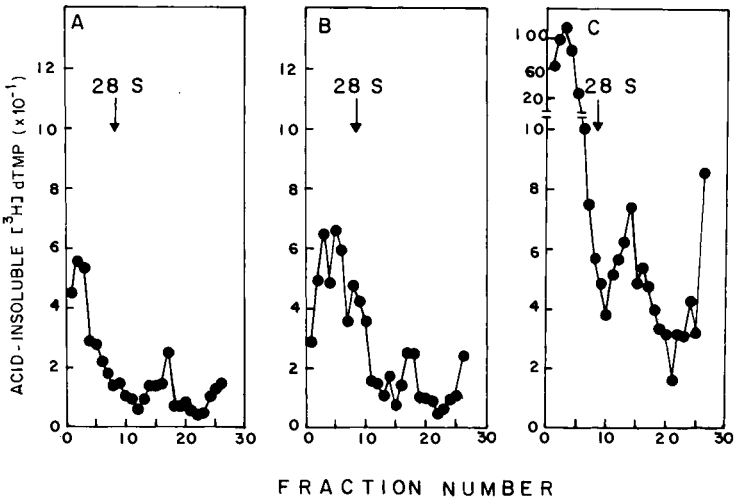

FIG. 11. Profiles of fast-sedimenting DNA obtained from the endogenously RNA-dependent DNA polymerase reaction of three different preparations of cells from a tissue culture derived from the bone marrow of a patient with acute lymphatic leukemia. The reactions were carried out according to the "simultaneous detection" technique of Schlom and Spiegelman (1971).

biochemical properties comparable to those of viruses associated with animal tumors, may be present in cells of human tumors.

VI. Conclusion

It can be concluded from the results presented and from the findings of many other investigators, that neoplastic diseases of various types in animals of a number of diverse species have a viral etiology. The oncogenic viruses etiologically associated with these neoplastic diseases exhibit morphological, biological, antigenic, biophysical, and biochemical properties which show similarities and differences. Application of techniques and concepts developed in studies of animal viruses to the problem of human cancer has resulted in an accumulation of strongly suggestive evidence that some, and possibly many, different types of human neoplastic diseases may be associated with viruses taxonomically related to tumor viruses of other species. The covert nature that seems to distinguish the putative human tumor viruses from most oncogenic RNA viruses of animals is puzzling at the present time, but the future may, and very likely will, provide the key for unlocking the secret for

the isolation of tumor viruses from human tissues. Nevertheless, sufficient inferential evidence exists at the present time which allows for a conclusion that the continuity of nature has been maintained in the viral etiology of many different types of mammalian cancer—from mice to men.

ACKNOWLEDGMENTS

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CHAPTER 15

Comparative Properties of Plant Tumors

LEON HIRTH

I. Introduction

No essential differences exist between animal and plant tumors. Indeed one of the most important characteristics of the tumor cell is its autonomy: This is common to plant and animal tumor cells. This character is, in the case of plant tumors, relatively easy to demonstrate by grafting the plant tumor on a healthy plant of the same genus. In this case a voluminous tumor is generally induced on the plant. Many other characteristics are also common, such as relatively high proliferation rate, chromosomal abnormalities, and the ability of some tumor cells to migrate in the organism and to give rise to new tumors (metastasis), but these points are generally considered less important than the ability of the tumor to escape the regulation system of the organism. Another important property of the animal tumor cells is "contact inhibition," which is

not observed with plant cells because they possess polysaccharide cell walls. The purpose of this chapter is to describe different types of plant tumors and to compare their properties.

II. Different Types of Tumors

If we omit cell proliferation (such as insect gall, proliferation due to a *Rhizobium,* and so on) which, in fact, does not really represent autonomous growth, three types of plant tumors may be described: those arising from the action of growth substances, those of genetic origin, and those induced by bacteria or viruses. These three types of tumors will be examined in detail below.

A. Tumors of Hormonal Origin

A long time ago it was discovered by Gautheret (1959) that stem pieces of carrot, for example, transplanted in a medium containing growth substances such as auxin or naphthaleneacetic acid at a suitable concentration, give rise to a disordered proliferation of the cambium cells. Under such conditions a callus is obtained which can grow indefinitely if transplanted regularly in a medium containing auxin. The morphological aspects of these calluses are very similar to those of true tumors, but when grafted on a whole plant of the same genus no proliferation occurs. This kind of disorderly proliferation can occur in many other plant tissues. When calluses obtained as described above are, after several transplantations, placed on medium without auxin cell proliferation generally stops. However, in some cases, a small number of cells begin to divide and after 3–4 weeks give rise to a voluminous callus that is able to grow in a medium without auxin (insensitive to auxin). These tissues are designed as "habituated" tissues (Fig. 1). If habituated tissues are grafted onto the stem of a plant of the same genus, tumor properties of this tissue are observed (Gautheret, 1942, 1948, 1955, 1957; Camus and Gautheret, 1948a,b). Figure 2 shows a piece of habituated tobacco tissue grafted on a stem of *N. tabacum* with the corresponding induced tumor. From these observations it can be deduced that habituated tissues have been transformed and show tumor properties. According to Gautheret (see Gautheret, 1959) tumor transformation is completed by the ability of the tissue to synthesize auxin and kinetin in a quantity that is sufficent to promote the growth of tissues, without addition of these growth substances to the medium. Until recently it was considered that transformation and prototrophy to auxin and kinetin

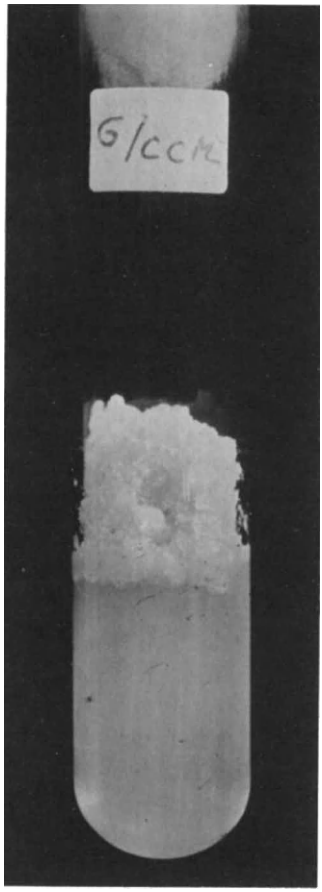


FIG. 1. Callus of "habituated" tissues of *N. tabacum* cultivated in a basic medium without growth substances.

were linked properties. But it seems that the problem is more complicated. In fact it is possible to obtain calluses of *N. glutinosa* which are able to grow intensively on a medium without auxin and kinetin (habituated tissue) and show no tumor properties when grafted. However when these tissues are transplanted in a medium rich with salts, they show a great deal of growth, and, after several transplantations in this salt-rich medium, the calluses grafted give rise to tumors. When such tissues are transplanted to a basic medium without growth substances they retain their tumor properties (Hirth and Durr, 1971). Thus prototrophy to growth substances seems to be necessary, but it is not suf-

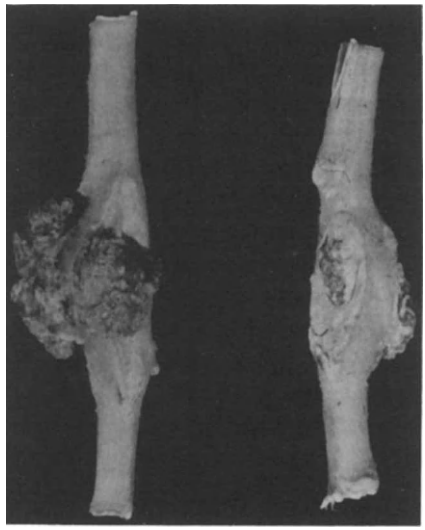


FIG. 2. A graft on a tobacco stem of a piece of "habituated" tobacco tissues (control on the right).

ficient for tumor transformation. The type of tumor transformation described above is a relatively slow process (obtained only after numerous transplantation) compared to the fast virus-induced process (i.e., case of wound tumor virus) or of *Agrobacterium tumefaciens*-induced tumors.

B. Genetic Tumors

A long time ago it was observed that tumors could be induced by crossing plants of the same genus but of different species. For example, tumors were obtained by crossing different species of *Brassica* (Kajanus, 1917), but this type of tumor has not been as well studied as those obtained by crossing *Nicotiana langsdorfii* with *N. glauca*. In the case of such a cross, hybrid tumors can be induced by wounding the stem (Fig. 3). In many cases tumors arise from natural traumatisms when leaves fall or roots sprout (Kostoff, 1930; Kehr and Smith, 1952; Schaeffer and Smith, 1963). The isolated tumor tissues grow indefinitely *in vitro* (White, 1939) in a medium without growth substances, although normal plant tissues need auxin and kinetin. The tumor tissues cultivated *in vitro* give rise to voluminous tumors by means of grafting (Izard, 1962). These tumors arise from the mixing of the genome of *N. glauca* and *N. langsdorfii* (Hitier and Izard, 1951; Kehr and Smith, 1949). The

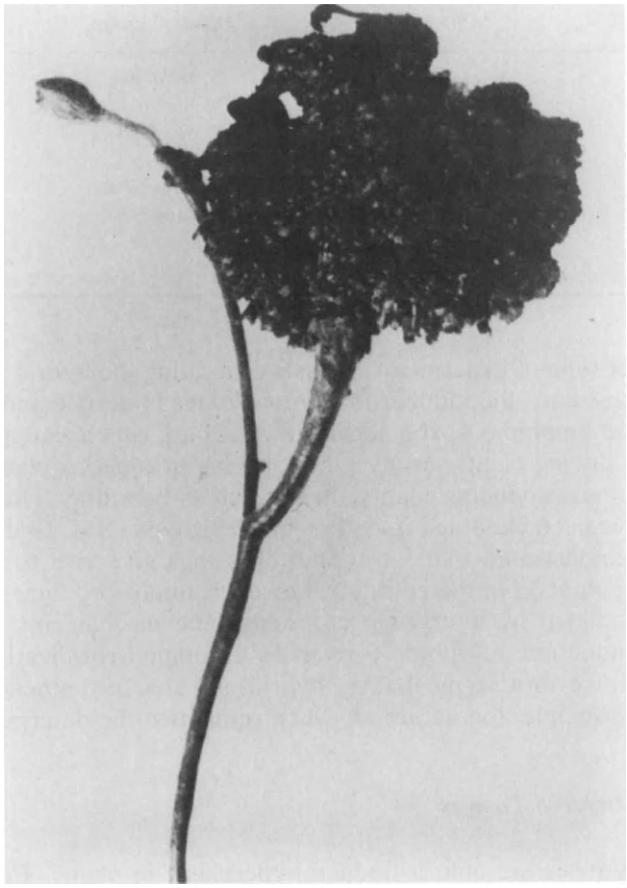


FIG. 3. Tumor obtained by the wounding of a hybrid *N. langsdorfii* × *N. glauca*.

genomes have to be intact; if one of them is incomplete the hybrid is unable to promote tumor formation, even with experimental wounding.

Some experimental evidence has demonstrated this point. It is well known that the hybrid *N. langsdorfii* × *N. glauca* (LG) is sterile, but the interspecific triploid LLG is partly fertile. When flowers of this hybrid are fertilized with pollen of *N. langsdorfii* and segregant chromosomes of *N. glauca* are obtained, such hybrids are completely devoid of tumor properties.

TABLE I

Classification of Tobacco Plants

"Plus" group	"Minus" group
N. noctiflora	*N. tabacum*
N. plumbaginifolia	*N. bigelovii*
N. longiflora	*N. suaveolens*
N. alata	*N. miersii*
N. sanderae	*N. panucilata*
N. bonariensis	*N. debneyi*
N. langsdorffii	*N. glauca*
N. forgetiana	*N. rustica*

Another type of experiment consists of treating the hybrid with colchicine. In this case the number of chromosomes is double and the hybrid is a fertile amphiploid, the fertilized seeds of which can give rise to tumor-producing plants. Many other species of tobacco plants can give rise to tumor-producing plants through cross-breeding. These tobacco plants have been classified in both + and − groups (Näf, 1958) (Table I). It was demonstrated that intragroup crossings give rise to non-tumor-producing plants. On the contrary, however, tumor-producing plants are always obtained by intergroup crossings. The mechanisms responsible for the induction of tumor properties through hybridization are unknown. However it seems that the hybrids are able to synthesize a tumor-inducing principle, the nature of which remains to be determined.

C. Virus-Induced Tumors

Many viruses are able to induce hyperplasia in plants. For example, nodules are observed in cabbage that are infected with cauliflower mosaic virus. Enations are seen in the case of plants infected with several viruses. Papillae are formed in the case of curly top disease. Many other examples could be cited, but in any case neither nodules, enations, nor papillae are able to induce tumors through grafting. They have, in fact, nothing to do with true tumors. On the other hand, a virus called "wound tumor virus" is able to induce true tumors on *Rumex, Melilotus,* and also on a number of other plants. Another type of tumor is obtained by inoculating numerous plants with *Agrobacterium tumefaciens,* but it seems, according to recent data, that a bacteriophage is present and plays an important role in tumor induction. Thus it seems logical to classify this type of tumor in the group of viral tumors.

1. Wound Tumor Disease

In nature a great number of plants develop tumors of viral origin, and the most common families showing viral tumors are Compositae, Cruciferae, Leguminous, and Polygonae. The main symptoms consist in a vein clearing and banding and in the presence of tumors on the roots (Black, 1952). Among the susceptible plants, *Rumex acetosa* and *Mellilotus alba* present the most characteristic symptoms. An extensive study of the wound tumor disease has been conducted by Black and coworkers. Kelly and Black (1949) have shown that tumors originate from the root pericycle and are a mass of parenchymatous and meristematic tissues containing some tracheids but no true vessels. This type of tumor was induced by a virus that was extracted from the tumor itself and also from the vector (see below). However, it is worth noting that stem tumors are particularly rich in virus particles, while root tumors contain less virions. However, it is easier to obtain a greater quantity of root tumors than stem tumors. Therefore virus is routinely extracted from root tumors.

The cytoplasm of tumor cells contains Feulgen-negative inclusions that are very similar to those observed in the case of animal cells infected by Reoviruses (Littau and Black, 1952). Figure 4 shows an electron micrograph of wound tumor virus that resembles an isometric virus 600 Å in diameter (Bills and Hall, 1952). The $s_{20,w}$ and the molecular weight of the particle are 500 and 16.4×10^6 daltons, respectively (Black and Markham, 1968). The particle contains a double-stranded RNA. However this RNA is not a continuous molecule, but consists of nine to ten fragments of double-stranded RNA bound together by single-stranded poly(A) sequences. Thus the wound tumor virus contains a fragmented genome. The T_m of this viral RNA is 90°–92°C; this relatively low melting point indicates a low content in G + C (19% of G + C, and 31% of A + U) (Millward and Graham, 1971). The WTV is a good antigen, and antiserum has been obtained with a very high titer. Using the fluorescent-antibody method, the cytoplasmic localization of the virus in the tumor cells was established. The presence of a viral antigen of low molecular weight was also observed in the infected cells (Black, 1965). It seems that this antigen corresponds to free protein subunits. The virus was also detected in the insect vector by means of immunofluorescence (Black *et al.*, 1958).

Some years ago (Streissle and Maramorosch, 1967), antigenic relationships between animal reovirus and wound tumor virus were suspected, but no confirmation of this result was obtained. However now it is possible to conclude that if wound tumor virus belongs to the reovirus

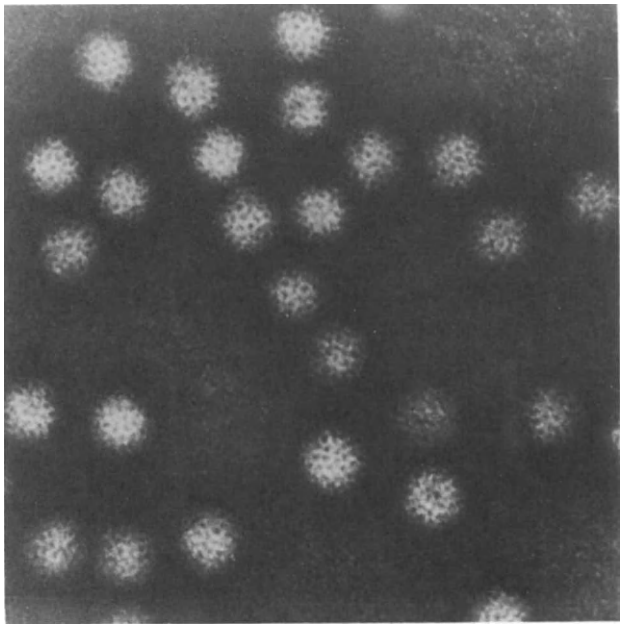


FIG. 4. Electron microscopy of wound tumor virus.

group, it seems to be serologically unrelated to the other viruses of the same group.

a. Transmission of the Virus. The virus is transmitted by grafting tumor pieces or fragments of infected plants, with or without visible tumors, onto healthy plants. Mechanical transmission of the virus is very difficult and in many cases impossible. Experimentally, the virus is transmitted by leafhoppers, such as *Agallia constricta* and *Agalliopsis novella* (Black *et al.*, 1958). It is worth noting the existence of a mutant of WTV that is transmitted neither mechanically nor by insects; this mutant is only transmitted through grafting.

The insect vector is infected by feeding on diseased plants. Experimentally, the insect can be infected by the injection of crude sap from insects or plants or by injection of purified virus suspension. The insect is able to transmit the virus only after an incubation period of 7 to 15 days following the injection. An infected insect remains a vector for the virus during its whole life. There are many indications that the virus multiplies in the vector. Recently Kimura and Black (1971, 1972) have observed the multiplication of the virus in insect tissue culture. Only a very few

number of eggs arising from infected females are able to transmit the virus to their progeny (Black, 1965).

b. Tumor Characteristics. It is worth noting that tumors appear only at places where natural or experimental cell wounding occurs in infected plants. In some cases auxin treatment of the stem of these plants is also able to induce tumor formation. These observations show that cell divisions due to scarring or to hormonal treatment are necessary for tumor induction (Black and Lee, 1957).

Tissue cultures may be obtained by subculturing primary tumors from *Rumex or Mellilotus*. The medium used is not very different from that utilized for the proliferation of habituated tissues of tobacco or carrots. It is remarkable that the growth of the tumor tissue does not need growth substances, such as auxin and kinetin. However it has been stated that tumor tissues of *Rumex* need ten times more phosphorus than other tissues in order to attain optimal growth (Nickell, 1954). After some weeks or months of subculturing the tumor tissue, the graft of fragments of these tissues on *Rumex* or *Mellilotus* generally gives rise to tumors. However, in some cases the results were negative. At this point a question arises: Is the virus still present in tissues after months or years of cultivation (Black, 1965)? Recently Streissle and Maramorosch (1969) have demonstrated that the virus content decreases gradually during cultivation in agar media. It has also been observed that the faster the multiplication of the cells, the larger is the decrease of the virus content of the tissues. Generally after 10 months of subculturing, no virus was detected either by electon microscopy or by injecting extracts of the tissues into an insect vector. However, the apparently virus-free tissue grafted on a healthy plant (in the present case, *Mellilotus alba*) gives rise to tumors, but only at the place of the graft. Wounding at other places of the stem does not cause tumors to appear. This observation demonstrates the tumor properties of the grafted tissue, but at the same time it reveals the absence of the virus in grafted tissue. Indeed when virus-containing tumors are grafted onto a healthy plant, the diffusion of the virus is observed, and many tumors are obtained by wounding the plant at different places. From the persistence of the tumor properties of the apparently virus-free cultivated tissue, Streissle (1971) deduced the presence of the viral genome in the cells. He attempted to demonstrate its presence by comparing polyacrylamide gel electrophoresis profiles of RNA's extracted from both normal and virus-free tumor tissues of *Rumex*. Some differences exist between the two, but in fact no convincing evidence for the presence of the viral genome in the tumor tissues was obtained. More work seems to be nec-

essary to prove some identity between the behavior of plant and animal oncogenic viruses.

2. *Tumors Induced by Agrobacterium tumefaciens*

Smith and Townsend (1907) have shown that tumors of *Chrysanthemum frutescens* are induced by the bacteria *A. tumefaciens*. Since then, the disease has been experimentally reproduced, and it was demonstrated that a great number of plants are able to produce tumors after being inoculated with *A. tumefaciens* (Fig. 5). Earlier studies described the appearance of the tumor at the crown of the plant, and from this the name crown gall is given to this type of plant tumor. The bacteria are easily cultivated in basic media, and several strains of *A. tumefaciens* have been described in great detail.

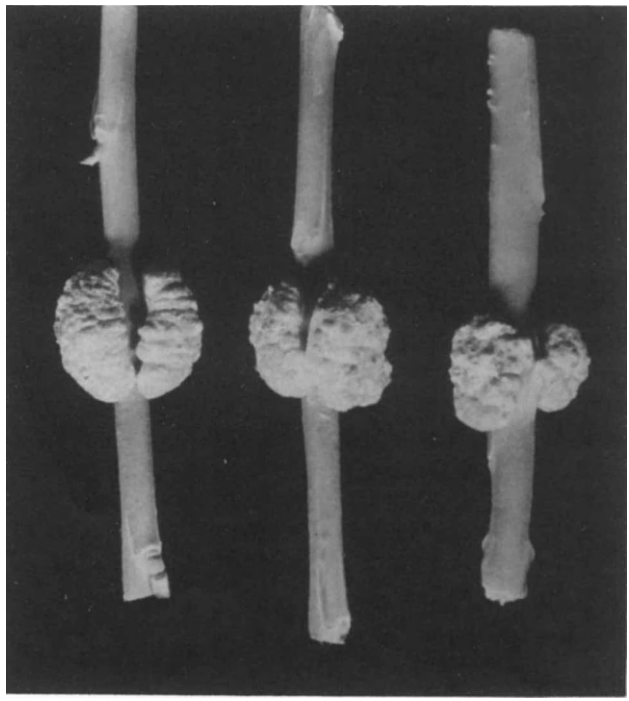


FIG. 5. Left: Tumor obtained by inoculating tobacco stem with *A. tumefaciens*. Middle: Tumor obtained by the grafting of a piece of tumoral tissue onto a tobacco stem. Right: Tumor obtained by grafting a piece of tumor tissue cultivated *in vitro* under sterile conditions for several years.

a. Conditions of Tumor Induction. Several conditions concerning both the bacteria and the plant are necessary to bring about the formation of tumors. When a plant (*Vinca rosea*) is transferred at 32°C (an inactivation temperature for the bacteria) immediately after the inoculation, no tumor is produced. When the plants previously inoculated at 32°C are transferred for varying lengths of time to 24°C at different periods after the inoculation, production of tumors is observed. The best results are obtained when the transfer at 24°C occurs 30 to 34 hours after the inoculation and for a period of 10 hours. From these observations the existence of a lag period, during which tumor transformation occurs (no matter what other conditions prevail), can be deduced. The necessity of a minimal time of contact between the plant cells and the bacteria was also demonstrated. Thus it seems that the bacteria act as the vector of a tumor-inducing principle (TIP), which when introduced into the plant cells remains active even after the inactivation of the bacteria. This principle seems to be permanently present in the transformed cells even long after the bacteria have disappeared. It is, for example, possible to cultivate crown gall tissues under sterile conditions without losing their tumor properties (tested by grafting). It is also worth noticing that fast developing tumors contain a very small number of bacteria. On the other hand, primary tumors (arising from the inoculation of *A. tumefaciens*) are able to give rise to secondary tumors (probably through tumor cell diffusion in the plant) (Gautheret, 1959). These secondary tumors never contain bacteria. This evidence strongly suggests the presence of this TIP in the virulent bacteria. The nature of this TIP will be discussed later, but it should be mentioned at this point that some strains of *A. tumefaciens* seem to be devoid of tumor-inducing ability, and they probably do not contain TIP.

b. Conditioning of the Plant Cells. The experiments reported above, and especially those showing the existence of a lag period in the induction of the tumor, suggest that the plant cells are in a particular state. A great number of investigations have shown without ambiguity that wounding plays an important role in this conditioning process (Braun, 1952; Braun and Mandle, 1948). Figure 6 reports the result of an experiment demonstrating the importance of the wound in the induction of tumors. Stems of several sensitive plants are wounded and *A. tumefaciens* is put into the wounds. At 0, 3, 6, and 12 hours, respectively, after wounding, the plants are inoculated at 32°C, and immediately afterward placed at 25°C for 24 hours, and then transferred to 32°C. Under these conditions the maximum tumor size is obtained when the bacteria are inoculated 60 hours after wounding.The size of the tumor indicates the efficiency of this inoculation. It has been demonstrated that the scarring

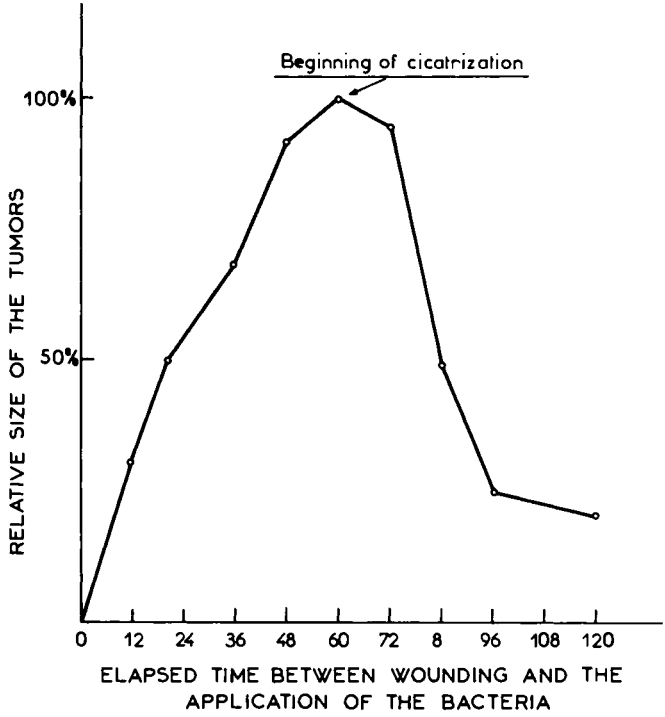


FIG. 6. Determination of the optimal time needed to condition tobacco cells (see explanation in the text).

process begins 60 hours after wounding. At this time, the plant cells seem to be in a "competent state" and able to integrate the TIP. The metabolic modifications arising during the course of the scarring process are as yet not well known; however an important production of growth hormones has already been observed. It is possible that such a production of growth hormones plays an important role in conditioning the cells, but, on the other hand, it could simply be the consequence of the cell transformation itself. This point is not yet completely understood.

Whatever the interpretation of the experiments reported above, it is clear that there are at least two steps in the tumor induction process. The first step is conditioning, which renders the cells transformable. The second step is induction, at which stage the conditioned cells "accept" the TIP and are potentially transformed. Some authors (Klein, 1965) suggest the existence of a third step, i.e., promotion, corresponding to the phenotypic expression of the transformation; in some cases this step might be inhibited by cold.

c. Comparative Cytological Properties of Normal and Transformed Cells. Some differences between normal and transformed cells have been reported (Sacristan, 1967; Sacristan and Melchers, 1969). However these differences are difficult to interpret. For example "habituated" tissues and crown gall of tobacco show chromosomal anomalies if they are compared with normal cells. However, a great variability exists between these anomalies. On the other hand, "habituated" tissues of *N. glutinosa,* devoid of tumor properties, also show chromosomal anomalies, and it is not easy to relate a particular chromosomal anomaly to tumor properties (Hirth and Durr, 1974). In the case of tumors induced by WTV, the size of the tumor cells is at least twice that of the normal cells (Black, 1965).

d. Comparative Biochemistry of Normal and Tumor Cells. An extensive study of this subject cannot be undertaken here. We have chosen to study only certain characteristic points.

i. Ability of Crown Gall to Synthesize Growth Regulators. This point has been touched upon above. Here however we wish to emphasize that it is a general property of tumor tissues of all origins. These tissues are especially able to synthesize large amounts of auxin. The synthesized quantities generally correspond to those necessary for the growth of corresponding normal tissues; this is particularly clear in the case of tumor and normal tobacco tissues. It is not yet well established whether the large amount of auxin found in tumors results from an activated synthesis of hormone or from an inhibition of the enzymes that degrade the auxin. It seems that what is true for auxin is also true for cytokinin. As already indicated above, the precise role of auxin in the conditioning and the inducing processes is not well known.

ii. Tumor Properties and Nutritional Needs. As indicated above, tumor tissues are prototrophic for growth regulators. They need no additional auxin or kinetin when cultivated *in vitro.* However they demonstrate particular needs that very often exist in direct relation to the degree of tumor transformation. Indeed, in the case of crown gall, the degree of transformation can be estimated by measuring the intensity of growth of the tissue transplanted in a basic medium that is deprived of growth factors. It is easy to obtain tumors having different degrees of transformation by inoculating with *A. tumefaciens* at different periods after wounding.

Figure 7 shows that the difference in the apparent degree of transformation may be due to the inability of the tissue to synthesize certain growth substances, which may be synthesized by the tissue showing the optimal degree of transformation (Braun, 1956, 1958). This observation

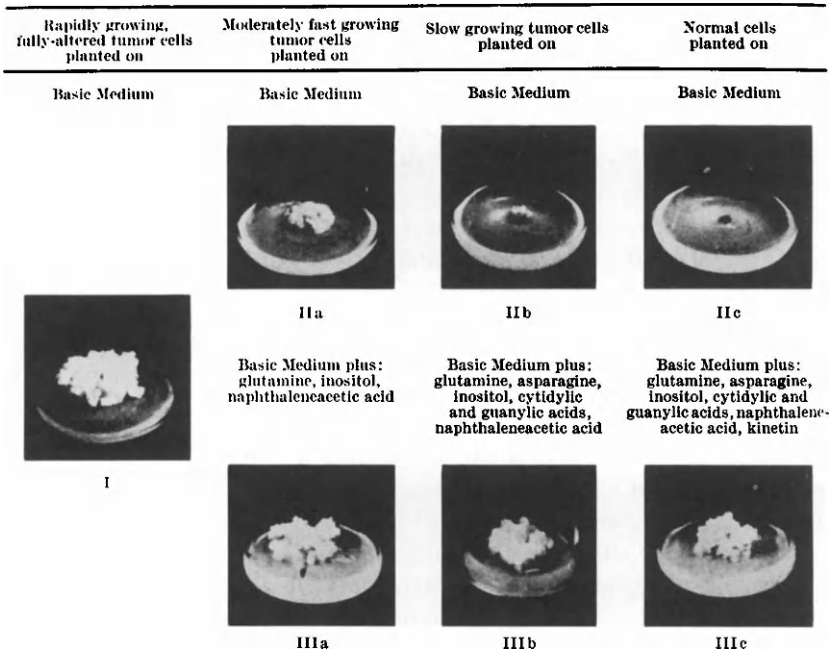


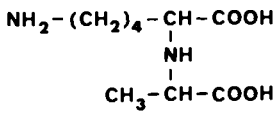
FIG. 7. Relative rates of growth of three clones of crown gall tumor tissue that show different degrees of neoplastic change planted on White's basic medium. (I) Fully altered, rapidly growing tumor cells; (IIa) moderately fast growing tumor cells; (IIb) slowly growing tumor cells; (IIc) normal cells of the type from which the tumor cells were derived. While the three clones of the tumor cells grow continuously, although at different rates on the basic culture medium, normal cells of this type do not grow on that medium. Lower pictures and legends show minimal nutritional supplements needed by the three types of tissue to achieve a growth rate comparable to that of the fully altered tumor cells according to A. C. Braun.

shows that not only is the regulation of auxin and kinetin synthesis disturbed, but also that of a great number of other growth factors. Wood and Braun (1961) have also shown that salts are able to stimulate the growth of slow-growing tumor tissues to a rate similar to that of rapidly growing tumor tissues. They demonstrated that KC1, Na_2NO_3, and Na_2PO_4 seem to favor the synthesis of growth substances needed, so that tumors that grow slowly in a basic medium can grow at a rate that is similar to that of the rapidly growing tumor tissues. This type of experi-

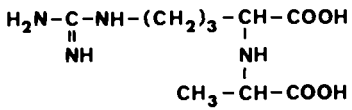
ment seems to indicate that changes in the permeability of the cell membrane and the regulation of some enzymatic activities by salts are modified. These observations prove the modification of both the genetic information and the regulation of its expression in transformed cells compared to normal cells.

In the case of virus tumors of *Rumex*, the tumor tissues cultivated *in vitro* release a large quantity of α-amylase into the medium. This is not observed when roots or stem pieces of *Rumex* are cultivated in the same medium. It is of course difficult to compare tissues and organs, but it is now admitted that tumor cells are able to release more α-amylase than normal cells. It was suggested earlier that α-amylase could be an induced enzyme, but it has been recently proved that the composition of the medium has no influence at all on either synthesis or on the release of enzyme. It seems that α-amylase release is due to modifications of the permeability of the tumor cell (Brakke and Black, 1951).

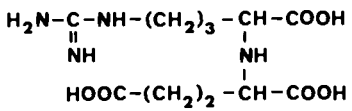
iii. Ability of the Tumor Cells to Synthesize New Substances. It was observed (Lioret, 1963, 1966) that crown gall tissues of salsify can synthesize a new amino acid: the lysopine, which is a condensation product of lysine and pyruvic acid. This condensation needs a specific dehydrogenase. This amino acid was apparently not detected in normal tissues, but was found in a great number of crown gall tumors of tissues of various origins. Later, Morel (1971) described the presence of other abnormal guanidines in crown gall tissues: octopinic acid and nopaline.



Lysopine



Octopine



Nopaline

Restricting his investigation only to nopaline and octopine Morel (1971) demonstrates that the presence of nopaline or octopine, or both is dependant upon the *A. tumefaciens* strain used to induce tumors on *Datura*. He concludes from this observation that the genetic information allowing the synthesis of these amino acids is contained in the bacterial genome, which is wholly or partly integrated into the genome of the transformed cell. However the strains of *A. tumefaciens* used in these experiments were unable to synthesize octopine and nopaline when cultivated under various conditions. They could only degrade these amino acids, and the degradation products can be utilized as a nitrogen source. Recently (Petit *et al.*, 1970) it was observed that an *A. tumefaciens* strain could induce "octopine tumor" that could degrade octopine but not nopaline; the reverse situation was also observed. Recent work done in my laboratory (Ackermann *et al.*, 1973) has demonstrated the presence of lysopine in tobacco tumor tissues irrespective of their origin. The lysopine was identified by means of a new micromass spectrometry method. A minute quantity of lysopine can be detected with this method (Fig. 8). Table II shows that nontumorous "habituated" tissues of *N. glutinosa* do not contain lysopine, but when the same tissue is transformed into tumor tissue, the presence of lysopine is detected. In this particular case, transformation is obtained without bacteria. We can conclude that genetic information concerning the synthesis of lysopine is contained in the normal cell, but is repressed. The system is derepressed however when transformation occurs. It is not evident that what seems to be true for lysopine is also true for octopine and nopaline. No attempts have been made until now to prove or disprove whether the genetic information for the synthesis of both amino acids comes from the bacteria. But whatever the controversy concerning this point may be, we will see later that more direct proof now exists confirming the integration of an exogenous genome in the genome of the transformed cell.

III. Plant Tumors: Differentiation, Dedifferentiation, and Cell Multiplication

A number of researchers have claimed that, for both animals and plants, tumor transformation is similar to a reversion of the cells to the embryonic state. Recent studies have shown that this point of view is erroneous. In fact, the main characteristic of both animal and plant tumor cells is not to conform to the correlation systems of the organism, and no relationship exists between the ability of the cells to multiply and their tumor characteristics. For example, some types of tumors multiply very slowly whereas normal cells stimulated by growth substances can

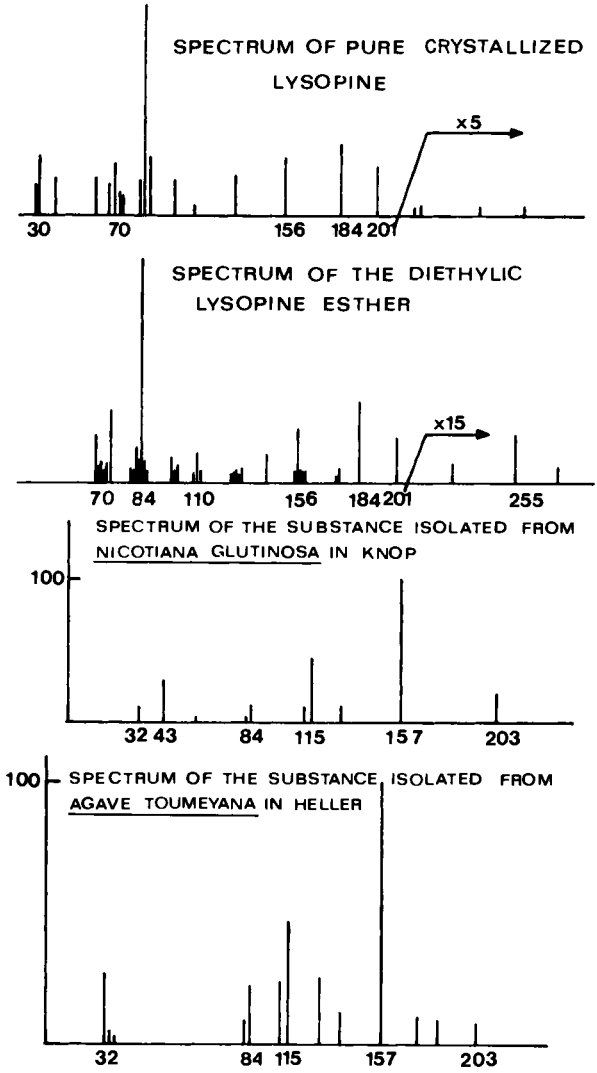


FIG. 8. Mass spectrometry spectrum of lysopine and its diethylic ester. These spectra are analogous to those obtained from tobacco tumor tissues (see Table II). Substances extracted by the same methods from nontumor tissues give a different spectrum.

grow very rapidly. This is the case for numerous normal plant tissues (carrot, salsify) growing *in vitro* in a medium containing auxin and kinetin. On the other hand, it has been recently demonstrated that tobacco crown gall tissues irradiated by X rays lose their ability to proliferate without a corresponding reduction of their tumor properties. It

TABLE II

Relationship between Tumor Properties of Plant Tissues Cultivated *in Vitro* and the Presence of Lysopine[a]

Nature of the tissues strains	Media[b]	Tumor properties	Presence of lysopine
Habituated tissues of *Nicotiana tabacum*	K	+	+
	S	+	+
	K	+	+
	S	+	+
Habituated tissues of *Nicotiana glutinosa*	K	−	−
	S	+	+
Normal tissues of *Agave toumeyana*	H	−	−

[a] For the composition of the mediums see Gautheret (1959) and Murashige and Skoog (1952).

[b] K. Knop medium; S. Skoog medium; H. Heller medium.

must be stressed however that all tumor cells are undifferentiated. The question arises whether or not plant tumor cells can reverse, when cultivated under specific conditions, and can give rise to differentiated cells having lost their tumor properties.

Braun (1951a,b) used a particular strain of tobacco crown gall tissue which has been cultivated *in vitro* and which demonstrated a relatively great ability to yield anomalous buds in a medium containing auxin and kinetin in specific proportions. He was able to recover apparently normal plants by grafting buds from this tissue. The following interpretation was given: The rapid growth of buds on a grafted plant eliminates the plasmids present in the tumor cells, which are responsible for their transformations. But this type of experiment was not convincing because tumor calluses may be a mosaic of both transformed and normal cells, and the healthy plants that recovered could have arisen from the development of the normal cells. The question was reinvestigated by Braun (1959) and in our laboratory by Lutz (1969). These authors have analyzed the behavior of plants arising from isolated cells (Fig. 9), the origin of which is tobacco crown gall tissues or "habituated tobacco tissue" having tumor properties. In both cases it was possible to recover a whole plant having a more or less abnormal morphology (Fig. 10) but whose tissues are completely devoid of tumor properties. This was particularly clear in the case of experiments of Lutz where an important number of clonal plants were obtained and analyzed; it was impossible to demonstrate the existence of tumor properties either through grafting or through cultivating their tissues in a medium without growth sub-

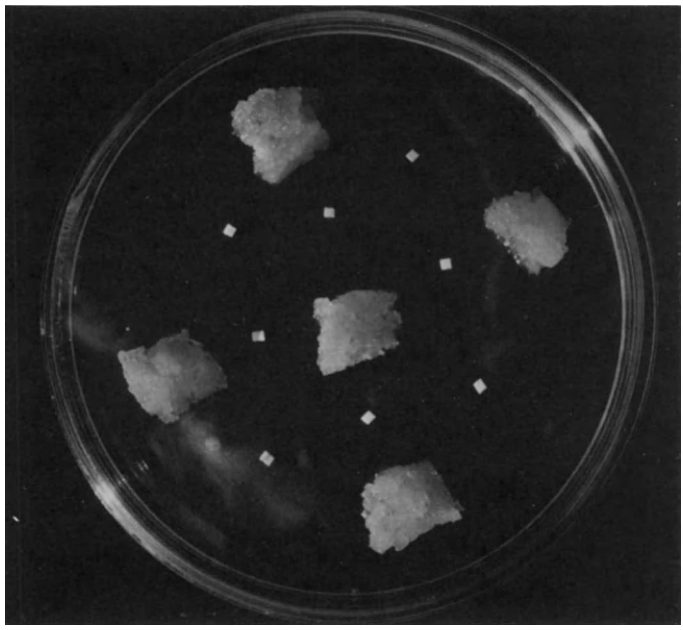


FIG. 9. This figure shows the technique utilized by Lutz to obtain cultures from isolated cells. Substances diffusing from tobacco calluses are necessary to promote the division of isolated cells.

stances. It is worth noting that tumor properties disappeared when calluses that arose from isolated cells grew very rapidly and when the differentiation of the clones occurred in a medium that was rich in salts, auxin, and kinetin. In the case of crown gall, the healing process could be explained by the mechanism involved when strains of *E. coli* that are lysogenic for λ phage are cured. This explanation apparently does not fit the case of habituated tissues. However in this case the origin of tumor transformation is not yet known.

IV. Induction and the Nature of the Tumor-Inducing Principle

We shall limit discussion to the case of crown gall, the only one, in fact, that to date has stimulated any extensive study.

It has been indicated above that grafted tumor cells bring about the development of tumors. However, it is not completely clear whether or

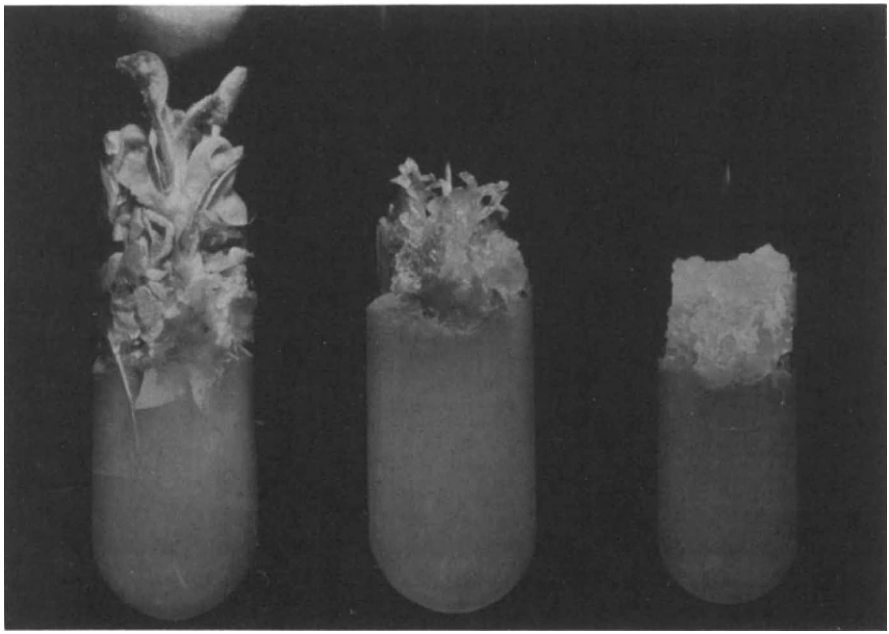


FIG. 10. On the right is shown a callus of habituated tobacco tissue of single cell origin. This callus, when transplanted on a medium containing auxin and kinetin at high concentration, gives rise to buds (in the middle). These buds develop on stems, leaves, and roots (on the left).

not the cell proliferation observed is due to the multiplication of the grafted cells or of the cells transformed by the diffusion of a TIP.

Recently an experiment by Aaron da Cunha (1969) has shown that tobacco tumor cells irradiated at proper X-ray levels were unable to multiply, but when grafted on a plant they caused multiplication of the host cells. The tissues obtained can grow in a medium deprived of auxin and kinetin. It was also observed that these tissues contained octopine. These experiments seem to prove the existence of an inducing principle that diffuses from the irradiated cells to the host cells. We have already indicated the host cells must be "competent" in order to be sensitive to the TIP. It has been suggested that TIP could be exogenous DNA capable of being integrated into the cell genome. It was difficult to determine the origin of this DNA. In addition, the assumption that crown gall is similar to a tumor induced by oncogenic animal viruses was difficult. Indeed no viruses similar to the known oncogenic viruses were discovered, either in the tumors themselves, or in the bacteria. It was

suggested some years ago (Bopp, 1961, 1962; Lipetz and Stonier, 1961) that the TIP was the bacterial DNA itself. Evidence for this was scant, consisting only in the demonstration that substances inhibiting the DNA replication also inhibited the expression of tumor properties. Two other types of experiments were performed recently. The first consisted of inoculating bacterial DNA into wounded normal tissues of salsify cultivated *in vitro*. After a lag period a large amount of cell proliferation occurred at the place of inoculation. These proliferations, when transplanted to a medium deprived of growth substances, grew very rapidly. The successive transplantations of these tissues showed that they had the characteristics of "habituated" tissues (Kovoor, 1967).

These experiments are not completely convincing for the following reasons. The quantity of inoculated DNA is high (2 to 3 mg). Under these conditions growth regulation substances can arise from the DNA. In that case, the DNA could not be directly responsible for the insensitivity to growth substances. Other types of DNA were not tested to determine whether or not they yield the same effect. Although habituated tissues often reveal tumor properties, this is not always the case. This was not taken into account in the above experiment. For this experiment to be complete, one should demonstrate that the tissues obtained were quite similar to crown gall.

The second type of experiment consisted in inoculating sensitive, wounded plants with *A. tumefaciens* DNA. The results obtained were not completely convincing, although it was shown by Stroun *et al.* (1966, 1967) that exogenous DNA can be absorbed by plants, included in the nucleus, transcribed, replicated, and probably integrated as well. On the other hand, this type of experiment could not indicate whether *A. tumefaciens* DNA was active itself or through the intermediary of the DNA of a transported virus. It is for this reason that a different biochemical approach has been used by others (Schilperoort *et al.*, 1967; Quétier *et al.*, 1969; Milo and Srivastata, 1969). The starting hypothesis was the existence of common nucleotide sequences between DNA's of tumor cells and bacterial cells. These researchers utilized the classical technique of hybridization between both types of DNA's; however in many cases such hybridization was performed between the DNA extracted from tumor cells and labeled RNA's originating from the transcription (by *E. coli* polymerase) of DNA's of various origins. Table III shows that the only RNA able to hybridize with the DNA of the transformed cells is bacterial DNA. The results obtained also indicate that only a small fraction of the bacterial genome is analogous to that of the tumor cell.

These easily reproducible experiments prove without a doubt that the

TABLE III

Hybridization of Crown Gall and Tobacco DNA with *A. tumefaciens* [^{3}H]RNA[a]

Expt No.	DNA used in the hybridization (150 μg)	*A. tumefaciens* [^{3}H]RNA (dpm)	$s_{20,w}$ of DNA used
1	Crown gall, No. 1	47,600	18
1a	Crown gall, No. 11	67,000	22
2	Tobacco (samsun)	1,950	22
2a	Tobacco (white burley)	1,330	21
3	Calf thymus	2,280	18
4	*A. tumefaciens*	180,300	19
5	No DNA	850	

[a] According to Schilperoort *et al.*, 1967.

integration of at least a part of the DNA extracted of *A. tumefaciens* into the genome of the tumor cells occurs. At this point the question what is the origin of the integrated DNA sequences arises. It could be bacterial DNA, or viral DNA present in the bacteria, or both.

In 1955, Beardsley showed that a particularly virulent strain of *A. tumefaciens* was also lysogenic. Since then, a systematic study of the different strains of *A. tumefaciens* has proved that the virulent strains were indeed lysogenic. The temperate phage, now called P58, contained in the B_6 strain has all the characteristics of a classical temperate phage. On the other hand, Parsons and Beardsley (1969) have isolated from crown gall tissues of *Helianthus annuus* and cultivated under sterile conditions during a period of 10 years, a phage that is very similar to phage P58. This observation was confirmed later by Tourneur and Morel (1970). Leff and Beardsley (1970) have recently observed that when DNA extracted from phage P58 was absorbed by *Helianthus annuus* seedlings, tumors were induced when the seedlings were wounded. Similar results were obtained with *A. tumefaciens* DNA, but the results were negative when inoculation was performed with other types of DNA. Controls seem to prove that P58 DNA alone is able to induce tumors. It is worth noting that DNA's extracted from non-virulent strains of bacteria are inefficient.

The present problem of the phages in the crown gall tissues cultivated under sterile conditions for a number of years is still a matter for discussion. Indeed, the presence of these phage particles in tissue cultures has recently been contested by Schilperoort *et al.* (1973). In fact, phage particles were never directly observed by means of electron microscopy in the extracts of tissue cultures even when concentrated. Rather they were discovered by testing the extracts with a sensitive strain of *A.*

tumefaciens, the B_6-806 strain, which is derived from B_6 strain. Under favorable conditions plaques were obtained from which P58 phages were isolated. It seems that P58 is able to multiply in B_6-806 strain. However the strain B_6-806 is lysogenic for a phage that is very similar to P58. This phage seems to be defective, and the bacteria which contains it are not immune to superinfection by P58. Schell *et al.* (1974) suggest that crown gall extracts were able to induce the multiplication of the temperate phage present in the strain B_6-806. It is worth noting that in some cases the strain B_6-806 is able to produce phages of the P58 type spontaneously.

Strains other than B_6 are able to produce temperate phages. These phages have been called omega, P_B, 2A, and LU_1. They have the same properties as P58 and are very similar to each other. All the strains of *A. tumefaciens* able to produce these phages are tumor-inducing strains. However, phage P8149 extracted from *A. radiobacter* (an non-tumorigenic bacteria) is unable to induce tumors in sensitive plants. Its morphology is different from that of the others (De Ley *et al.*, 1972).

Whatever the discussion concerning the presence of phage particles in crown gall tissue cultures, it is likely that P58 and related phages play a role in the cell transformation. But it is not clear from the experiments reported above whether or not the whole phage genome is necessary for the tumor induction, and whether the *A. tumefaciens* genome also plays a role in the process. Recently Chadha and Srivastata (1972) have demonstrated that crown gall tissues of various origins cultivated *in vitro* contained three particular proteins that are absent from the normal corresponding tissues. Two of these proteins are similar to proteins found in extracts of that bacteria that gave rise to the tumor tested. These experiments indicated that genetic information of bacterial origin exists in the transformed cells, but because the nature of the common proteins was poorly determined, it was impossible to know whether these proteins were phage or bacterial proteins. (The *A. tumefaciens* strain used was a lysogenic one.) Recently Schilperoort *et al.* (1973) have reinvestigated the problem of the nature of the integrated genome by means of the hybridization method. Table IV and Fig. 11 show that P58 DNA and *A. tumefaciens* DNA (strain A6) hybridize with DNA extracted from tobacco crown gall tissues. The interpretation of the data shows that 0.9% of crown gall DNA is analogous to the A_6 DNA, and that 0.3% of the latter is analogous to *A. radiobacter* DNA, which is not a tumorigenic bacterium (it is worth noting that a large sequence analogy exists between the DNA of A_6 and *A. radiobacter*). From the data of Table IV it is also possible to calculate that 1.8% of the crown gall DNA is similar to the DNA of P58 phage. Calculations made from a

TABLE IV

Saturation Values for Hybrid Formation between DNA from Different Kinds of Plant Tissue and [^{3}H]cRNA's Transcribed from *A. tumefaciens* A_6 DNA, *A. radiobacter* DNA, and PS8 DNA[a,b]

DNA source	[^{3}H]cRNA hybridized with DNA from		
	A. tumefacients A_6 (μg/100 μg DNA)	*A. radiobacter* (μg/100 μg DNA)	PS8 (μg/100 μg DNA)
Crown gall tissue	0.55	0.25	1.0
Normall callus tissue	0.10	0.10	0.10
Leaves/stems	0.0	0.0	0.0

[a] The hybrids contained 3 μg plant DNA per filter. The specific activity of A_6 [^{3}H]cRNA was 600 cpm/μg, of *A. radiobacter* [^{3}H]cRNA 1100 cpm/μg, and of PS8 [^{3}H]cRNA 360 cpm/μg.

[b] According to Schilperoort *et al.*, 1973.

reasonable estimation of the molecular weight of the various DNA's used have shown that approximately 1800 genome equivalents of P58 phage are present in the crown gall DNA. These results suggested that the whole P58 DNA sequence present in crown gall tissues arose from the phage genome present in the bacteria. But Table V shows that RNA transcribed from P58 DNA is unable to hybridize with A_6 DNA. This surprising result was interpreted by the above-mentioned authors as proof that the phage exists as a plasmid in the cytoplasm of the bacteria and not as an integrated genome, and that during the isolation process of the bacterial DNA the plasmid is lost. On the contrary, the

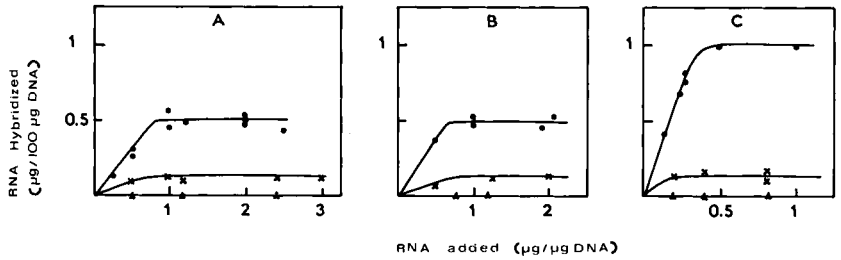


FIG. 11. Saturation curves for hybrid formation between 3 μg crown gall DNA, 3 μg leaf DNA, 3 μg stem DNA, and 3 μg normal callus tissue DNA and increasing amounts of (A) *A. tumefaciens* A_6 [^{3}H]cRNA, (B) *A. radiobacter* [^{3}H]cRNA, and (C) P58 [^{3}H]cRNA. (o—o) crown gall; (x—x) normal callus tissue DNA; (Δ—Δ) leaf DNA/stem DNA.

TABLE V

Results of Hybridization Experiments with DNA's from Different *A. tumefaciens* Strains and PS8 and PS8 [^{3}H]cRNA[a,b]

DNA source	DNA (μg)	[^{3}H]cRNA hybridized (μg/100 μg DNA)
A_6	30	0.006
B_2A_s	30	0.006
B_2A_s (PS8)	30	0.13
A_6 + 10% PS8	30 + 3	0.46
A_6 + 1% PS8	30 + 0.3	0.13
A_6 + 0.1% PS8	30 + 0.03	0.036
A_6 + 0.01% PS8	30 + 0.003	0.013
Tobacco leaves	30	0.0005

[a] In this experiment 1 μg PS8 [^{3}H]cRNA was used with a specific activity of 1200 cpm/mg. The values in this table are the averages of two assays.

[b] According to Schilperoort *et al.*, 1973.

phage DNA is integrated into the crown gall cells. This explains why hybridization experiments between phage DNA and crown gall DNA are positive and negative in the case of hybridization between phage DNA and A_6 DNA. Calculations have shown that the part of the bacterial DNA that is integrated into the genome of the tumor cell is approximately 1% of the phage DNA. (Nine hundred equivalents of phage genome for 9 equivalents of bacterial genome.) It was also determined that the integrated part of the phage genome codes for approximately two to three proteins. The integrated bacterial DNA probably consists of short repetitive sequences.

V. Conclusion

From the above considerations the following conclusions can be drawn.

1. In the case of crown gall, the integration of exogenous genetic information seems to be well demonstrated. However, in contrast with data concerning animal oncogenic viruses, this exogenous genetic information is heterogeneous and is composed partly of bacterial DNA and partly of phage DNA. It is the first time that a temperate phage is suspected to have oncogenic properties. However several points are not yet clear. For example, are the integrated nucleotide sequences of *A. tume-*

faciens necessary for the tumor transformation? If we consider the results of the experiments of Leff and Beardsley (1970), the answer seems to be negative because, according to them, the inoculation of phage DNA alone induces the formation of tumors. However too little is known about the conditions of such transformation by P58 DNA. On the other hand, contradictory results have been obtained concerning the tumor-inducing power of some strains of *A. tumefaciens*. It is now generally accepted that only the lysogenic strains of *A. tumefaciens* have tumor-inducing properties. Recently however, cured lysogenic strains of bacteria have been shown to be capable of transforming plant cells. If this observation is correct, the role of phages could be challenged. However it is possible that bacteria which are apparently cured contain a defective phage, and also that other phages, such as P58 and related phages, can induce tumor formation. But whatever the future interpretations of these facts may be, it seems that crown gall is but one example of the general phenomenon of integration of exogenous genomes into cellular ones, a reaction that may be accompanied by the appearance of the tumor properties. This is now well known in the case of animal oncogenic viruses. On the other hand, the presence of specific antigens of bacterial or viral origin in the crown gall cells shows an analogy with the presence of T antigen in the case of the animal cells transformed by SV40 virus.

2. In the case of the wound tumor disease the situation is not so clear. It has not as yet been convincingly demonstrated that the viral genome is integrated in the cell genome. Some indirect evidence exists, but the problem is as of now an unsolved one.

3. The cases of hybrid tumors and of habituated tissues are more difficult to explain. However, if we admit that tumor transformation is the consequence of epigenetic changes, we can assume that these types of tumors are not very different from those in the preceding cases; only the origin of the changes is not known.

4. Whatever its origin, tumor transformation induces important metabolic modifications. It seems that a large part of these modifications are related to the regulation of the synthesis of numerous substances, such as growth regulators and amino acids. Indeed, if we consider the case of amino acids such as lysopine it seems that the genetic information for their synthesis is contained in the plant genome and only the regulation of its synthesis is modified (see above). If we accept the point of view of Schilperoort *et al.* (1973), a very small number (two or three) of bacterial genes are integrated in many cases. It seems reasonable to assume that the integrated genes control the synthesis of those proteins that are able to modify the regulation of the cell genome. Thanks to these modifi-

cations in the regulation processes, the tumor cells in plants, like those in animals, no longer conform to the function of coordination which exists in the organism. We must therefore emphasize at this point that the main characteristic of all tumor cells is their independence from this function.

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CHAPTER 16

A New Look at the Mode of Action of Polyoma and Related Tumor Viruses

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I. Introduction

Polyoma (Py) virus, endemic in mice (Gross, 1953), was the first oncogenic virus shown to cross the species barrier and to induce a broad

spectrum of tumors not only in its natural host but also in hamsters and other rodents (Eddy, 1964, 1969; Eddy *et al.*, 1958). Subsequently, other DNA-containing viruses, such as simian virus 40 (SV40) (Defendi, 1966), endemic in monkeys, and several adenoviruses (Yabe *et al.*, 1964), isolated from men and animals, were found to induce tumors in rodents though they were apparently not oncogenic in their natural hosts.

Py and SV40 soon became favorite tools in tumor virus research, since their biological effects can be easily studied in tissue culture cells and since they contain only a very small amount of genetic information. They belong to a group of widespread tumor-inducing viruses (Fig. 1) that are similar with respect to structure and biological properties. Their genomes consist of a small molecule (3 to 5×10^6 daltons) of double-stranded, circular DNA that is infective and carries the genetic information of the virus necessary for the production of progeny virus, for the induction of tumors (di Mayorca *et al.*, 1959; Crawford, 1965; Ito, 1960; Rowson and Mahy, 1967; Weil and Vinograd, 1963), and for the "transformation" of cells in culture (Aaronson and Todaro, 1969).

The early studies on the interaction of Py virus with mouse and hamster embryo cells in tissue culture led to the hypothesis that transformation of "normal" into neoplastic (i.e., malignant) cells may involve a process that is analogous to lysogeny in bacteria (Vogt and Dulbecco, 1960, 1962, 1963), a hypothesis which was subsequently extended to SV40 and other DNA viruses. It postulates that integration of viral "cancer genes" into the genome of mammalian cells leads to specific genotypic alterations which find their phenotypic expression in new morphological properties (specific changes of the cell surface, loss of contact inhibition, release from growth controls) and that, as a consequence, these changes would confer malignancy to the "transformed" cells. The subsequent discovery of virus-specific imprints, such as new antigens (reviewed by Deichmann, 1969), virus-specific RNA (Ben-

	endemic in
Polyoma virus	mice
SV40	monkeys
Shope papilloma virus	rabbits
Canine papilloma virus	dogs
Bovine papilloma virus	cattle
Human* papilloma virus	man

* *induces warts (verruca vulgaris)*

FIG. 1. Polyoma and related viruses.

jamin, 1966), and "integrated" viral DNA (Koprowski *et al.*, 1967; Sambrook *et al.*, 1968), in some virus-transformed cell lines, years after the initial infection, tended to support the simple and attractive lysogeny hypothesis.

At the same time, however, observations began to appear in the literature which made it increasingly difficult to explain malignant transformation (i.e., the development of "normal" into cancer cells that are able to invade and destroy adjacent tissues and to form metastases) by a process akin to lysogenic conversion of bacteria. They rather suggested that some "early" viral functions, combined with a relatively slow and complex secondary process of cytogenetic variation and selection, were operational in virus-induced "malignant transformation."

Several lines of experimental observations have led us to propose the hypothesis that Py and related viruses act primarily as endogenous mitogens and that development of malignancy is an evolution of cellular properties which is not directly determined by viral genes but which is the outcome of a combination of prolonged virus-induced cell proliferation and cytogenetic instability. In this chapter we will summarize earlier and newer experimental evidence that led to this hypothesis. We will discuss the mechanism involved in the virus-specific mitogenic action, its relation to "cell transformation" in tissue culture and to tumor formation *in vivo,* and we will finally consider the potential interest of the experimental observations for certain problems in human medicine.

II. The Lysogeny Hypothesis

In their early studies on "cell transformation" in tissue culture Vogt and Dulbecco (1960, 1962, 1963) showed that infection with Py of mouse embryo cell cultures (from random-bred Swiss albino mice) within days led to a considerable fraction of the cells producing progeny virus and cell death ("lysis"). Within 4–6 weeks after infection virus production and cytopathic effects decreased; "transformed" cells appeared which within the next few weeks replaced the normal cells. Transformed cells no longer produced virus and were characterized by "loss of contact inhibition," "criss-cross growth pattern," and a pronounced tendency to form dense, multilayered cell sheets. However, no results on transplantability ("malignancy") of the transformed mouse cells were reported. Infection with Py of secondary hamster embryo cell cultures led to a comparable process of "cell transformation." However, only a very small fraction of the hamster embryo cells produced progeny

virus, the cytopathic effects were negligible, and transformed cells replaced the normal cells within 1–2 months after infection. Subcutaneous inoculation of 1 to 4 $\times$ 10^6 transformed hamster cells into adult hamsters led to the development of tumors at the site of injection. The authors concluded: "On the basis of this finding, all cells which show an unrestricted growth *in vitro* are called neoplastic." Subsequently, Vogt and Dulbecco reported the absence, in transformed cells, of detectable amounts of complete virus, infective DNA, or capsid protein and showed, furthermore, that Py-transformed cultures were apparently resistant to superinfection with Py. On the basis of these early observations they suggested that "malignant transformation" may involve a process akin to lysogeny in bacteria. Similar observations and conclusions were reported by Sachs and Medina (1961).

III. The Lysogeny Hypothesis Critically Revisited

Lysogenization, i.e., the insertion of bacteriophage DNA into the bacterial chromosome, is a rapid process that confers new genotypic properties on the lysogenized bacteria. In contrast, Py- and SV40-induced "malignant transformation" is generally a rather slow process that follows "morphological transformation" (if "malignancy" develops at all!) by weeks or months (Defendi, 1966; Kit *et al.*, 1969; Vogt and Dulbecco, 1963; Wesslen, 1970). In this context it has to be remembered that during the same lapse of time uninfected mouse, hamster, and rat embryo tissue cultures rather frequently undergo spontaneous "morphological and malignant transformation" (Sanford, 1965; Sanford and Hoemann, 1967; Sanford *et al.*, 1967; Sharon and Pollard, 1969).

Resistance of tissue culture cells transformed *in vitro* by Py (Hellström, 1962; Hellström *et al.*, 1962) or SV40 (Barbanti-Brodano *et al.*, 1970; Swetly *et al.*, 1969; Jensen and Koprowski, 1969) to superinfection with the transforming virus is an epiphenomenon not related to immunity in lysogenic bacteria; it is apparently due to the selection of cell variants resistant to the lytic effect of the transforming virus.

Uninfected cell lines, such as BHK (baby hamster kidney), which are "malignant" (as determined by their ability to form tumors in adequate hosts) can be "morphologically transformed" by infection with Py without an appreciable change in their malignant potential (Defendi *et al.*, 1963).

Uninfected mouse and hamster cells from various "normal" or "malig-

nant" cell lines are generally unable to form colonies in soft agar. Shortly after the abortive infection with Py or SV40 a fraction of the cells acquires the ability to grow in agar (Macpherson, 1973; Macpherson and Montagnier, 1964). Infection with *Mycoplasma* (Macpherson, 1973) or addition of insulin (de Asua *et al.*, 1973) to the culture medium in the agar can have the same effect. The ability of mammalian cells to form colonies in agar therefore is not synonymous with "malignant transformation."

Virus-transformed cells frequently (though not always) exhibit a "loss of contact inhibition" and, often correlated with it, the ability to grow in culture to high saturation densities. Loss of "contact inhibition" and "criss-cross" growth pattern can also be observed if "normal" (i.e., untransformed) BHK cells are grown in the presence of insulin (de Asua *et al.*, 1973). Furthermore, apparently "normal" cells can grow to relatively high saturation densities, if the pH of the culture medium is increased (Ceccarini and Eagle, 1971).

The maintenance of the "transformed phenotype" (i.e., the ability to synthesize virus-specific RNA, T antigen, and to form colonies in agar) depends on the continued presence of functional viral DNA (Oxman, 1967; Marin and Macpherson, 1969).

The aneuploid and "highly malignant" SEWA mouse cell line (originally derived from a Py-induced subcutaneous sarcoma) contains Py-induced transplantation antigen but no Py-specific RNA or T antigen detectable by the hybridization method and immunofluorescence assay presently used in our laboratory (tested 1971/1972; H. Türler and R. Weil, unpublished observations). Fusion of SEWA cells with several "low" or "high" tumor lines of L cells led to the outgrowth of hybrids, all of which contained, as a heritable property, the Py-specific transplantation antigen. Their malignant potential, however, varied from "nontumorgenic" to "highly tumorgenic." Results from karyotype analyses led to the suggestion that the malignancy of the hybrids depended rather on the constitution of the karyotype than on any virus-specific functions (Harris, 1971).

These results show that Py and SV40 can "transform" both normal and cancerous (i.e., transplantable) tissue culture cells; they also suggest that the viral genome is not required to maintain the malignant phenotype once it has been established.

In the following sections we will show that the rather confusing situation with respect to virus-induced "cell transformation" (Eagle *et al.*, 1970) can be simplified by the assumption that Py and related viruses act primarily as endogenous mitogens.

IV. The Mitogenic Effect of SV40 and Polyoma Virus in Tissue Culture Cells

A. The Mitogenic Effect of SV40 in Confluent, Primary Mouse Kidney Cell Cultures

Infection of primary MK cultures with SV40 leads within a few weeks to the appearance of colonies of "transformed" cells (Black and Rowe, 1963) that contain intranuclear, SV40-specific T antigen detectable by the immunofluorescence reaction. Kit *et al.* (1966, 1967, 1969) showed that infection with SV40 remains abortive and that it stimulates cellular DNA synthesis while no viral DNA or capsid protein are synthesized. Since no known "late" viral functions are expressed the mouse kidney–SV40 system was found to be very useful for studying the early functions of SV40 and their effects on the host cell. Detailed reports on some of these results have been published (May *et al.*, 1971, 1973; Weil *et al.*, 1973). In this chapter we will summarize the essential results, adding some new, previously unpublished observations.

1. The Properties of Confluent, Primary Mouse Kidney Cell Cultures

Since the time course of the abortive infection with SV40 and also of the lytic infection with Py virus (see below) is markedly influenced by the physiological state of the cells at the time of infection, mouse kidney (MK) cultures (Winocour, 1963) were prepared under strictly standardized conditions. In all experiments discussed here the cultures were prepared from kidneys of 10-day-old CR-1 mice and were grown to confluence in reinforced Eagle's medium containing 10% calf serum (Weil *et al.*, 1967). Comparable experimental results were obtained with MK cultures prepared from several other inbred or random-bred mouse strains (G. Pétursson and R. Weil, unpublished observations). Under the conditions used in our laboratory the cultures become confluent on the third day after seeding and at this time contain approximately 10^5 cells/cm^2. Feulgen microspectrophotometry performed on individual cells and chemical determination of total DNA content of confluent cultures show that most of the cells in uninfected, confluent MK cultures are epitheloid cells with a diploid ($2n$) DNA content which are apparently arrested in phase G_0 of the mitotic cycle. It should be noted that most of the cells in confluent MK cultures are unable, even after

trypsinization and replating, to undergo further chromatin replication and mitosis. This and other (R. Weil, unpublished) observations show that under the culture conditions used MK cells can only undergo a limited number of mitotic divisions. Most cells in confluent MK cultures are thus not "contact inhibited" but apparently irreversibly blocked in G_0.

2. *The Molecular and Cytological Events of the Abortive Infection*

Under standard conditions of infection MK cultures were generally plated in large petri dishes (94 mm diameter); 2 days after they had reached confluence, they were infected at 37°C with 0.4 ml crude viral lysate containing approximately 10^8 PFU/ml. The same results were obtained with purified viral preparations (10^8 PFU/ml). Under these conditions 50–60% of the cells present in the cultures participate in the abortive infection, as judged by the appearance of SV40-specific, intranuclear T antigen. In confluent MK cultures the time course of infection with SV40 (May *et al.*, 1971, 1973) and Py (Pétursson and Weil, 1968) is essentially the same whether serum is present in the culture medium or not. The experiments reported here were therefore performed in the absence of any added serum.

Under standard conditions of infection at 37°C, synthesis of "early" SV40-specific (messenger) RNA becomes noticeable by 3 hours after infection (May *et al.*, 1973). The rate of synthesis rapidly increases, reaches a maximum around 9–12 hours, and, within the next few hours, drops to a low level. The initial increase seems to reflect the asynchronous onset of transcription; the subsequent decrease, which can be prevented if SV40-induced cellular DNA synthesis is inhibited with cytosine arabinoside (ara-C) or 5-fluorodeoxyuridine (FUdR) is apparently due to an irreversible switch-off of transcription, linked to SV40-induced replication of the mouse cell chromatin. In all experiments a very close temporal relation between synthesis of early SV40 RNA and the subsequent appearance of intranuclear SV40-specific T antigen was observed. In addition, a linear relationship was found between total amounts of early SV40 RNA synthesized by the cultures (determined by cumulative labeling with [^{3}H]uridine), the maximum number of T antigen positive cells, and the logarithm of the plaque-forming titer (PFU/ml) of the viral preparations used for infection. The latter observation reveals a close quantitative relation between early SV40 RNA and SV40-specific T antigen. A few hours after the appear-

ance of T antigen most cells undergo SV40-induced chromatin replication (S phase), followed by normal or abnormal (tri- or multipolar) mitosis.

3. The Molecular Properties of Early SV40-Specific Messenger RNA

SV40-infected MK cultures were labeled with [^{3}H]uridine (500 μCi/ml) for 0.5, 1, or 6 hours at different times between 6 and 16 hours after infection. Total RNA was extracted with hot phenol at the end of the pulses and then sedimented through linear [15–30% (w/v)] sucrose density gradients. Fractions were collected and exhaustively hybridized with SV40 DNA fixed on membrane filters. Similar experiments were performed under conditions where SV40-induced DNA synthesis was inhibited with FUdR or ara-C, added 1 hour after infection. In all experiments the bulk of the radioactive, hybridizable early SV40 RNA sedimented as a rather uniform band with a sedimentation coefficient of about 19 S, while a smaller fraction sedimented in a band with a sedimentation coefficient of about 16 S. In short pulses (0.5 hours) very small amounts of SV40-specific RNA were found to sediment between 20 S and 26 S (May *et al.*, 1973). Although both "19 S" and "16 S" RNA are present in the nucleus, only "19 S RNA" is transported into the cytoplasm (E. May, unpublished observations). Since "19 S RNA" can be isolated from polysomes, we tentatively consider it as early SV40-specific mRNA, probably transcribed from parental viral DNA. It remains unknown whether early 19 S RNA is transcribed as such or whether it is derived by very rapid processing from larger precursor RNA molecules.

Preliminary results (E. May *et al.*, unpublished observations) from sedimentation velocity analyses in 99% DMSO gradients (see below) show that SV40-specific "19 S RNA" cosediments with 18 S ribosomal RNA (McConkey and Hopkins, 1969). This leads to an estimate of about 700,000 daltons for the molecular weight of "19 S RNA" corresponding to about 40% of the length of one strand of SV40 DNA. Early SV40-specific mRNA thus seems to be present as a single, rather large transcriptional unit that should be able to code for one or more polypeptides with a total molecular weight of about 70,000 daltons. These results agree well with the conclusion (reached by a different technique) that the early functions of SV40 are coded for by the same, contiguous segment of SV40 DNA, corresponding to 0.3–0.4 of the total length (Danna *et al.*, 1973; Khoury *et al.*, 1973; Morrow *et al.*, 1973). It is of interest that concentrated preparations of SV40-specific T antigen con-

tain a protein(s) with a molecular weight in the order of 60,000–70,000 daltons (Spira *et al.*, 1972; del Villano and Defendi, 1973).

4. *SV40-Induced Stimulation of Overall Cellular RNA Synthesis*

New results show that the abortive infection with SV40 leads to a stimulation of overall cellular RNA synthesis. It begins shortly after the appearance of T antigen but before the onset of SV40-induced cellular DNA synthesis and leads to a net increase of 15–30% in total (mainly ribosomal) RNA in the infected cultures. A comparable increase occurs if SV40-induced cellular DNA synthesis is inhibited with FUdR or ara-C. It should be noted that stimulation of SV40-induced cellular RNA synthesis is not accompanied by detectable changes in synthesis or processing of early SV40-specific RNA (E. May *et al.*, unpublished). Below we will show that the situation is very different during the lytic infection with Py virus.

The presently known events of the abortive infection with SV40 are schematically shown in Fig. 2. The striking temporal and quantitative relationship between SV40-specific early 19 S mRNA and the subsequent events of the abortive infection suggest the existence of a causative relation. Although no direct experimental evidence for this assumption exists, it is nevertheless supported by the observation that treatment

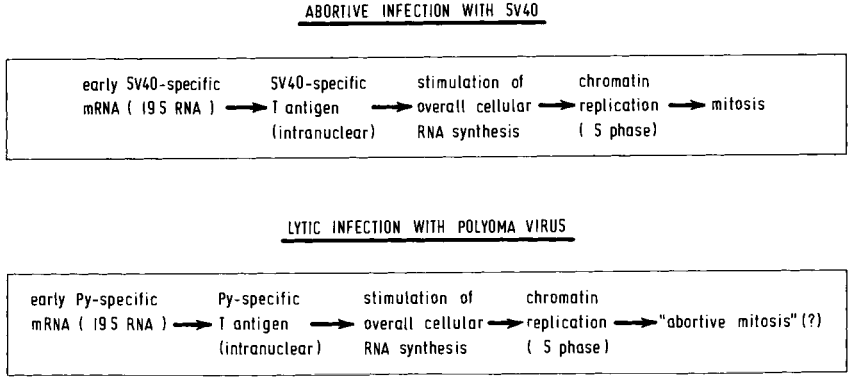


FIG. 2. Simplified scheme of the sequential events taking place in mouse kidney cells during the abortive and lytic infection with SV40 and polyoma virus, respectively. In the lytic cycle "integration" (i.e., covalent binding) of parental polyoma DNA into mouse chromosomal DNA starts only after the appearance of T antigen; replication of polyoma progeny DNA begins simultaneously with virus-induced chromation replication (not shown).

of confluent MK cultures with mouse specific interferon before (but not after) infection depresses as much as 70% of the synthesis of 19 S RNA and results in a comparable reduction (70%) both in the number of T antigen positive nuclei and in the number of cells participating in SV40-induced cellular DNA synthesis and mitosis. A similar inhibitory effect of interferon on synthesis of early virus-specific RNA was observed during the lytic infection with SV40 of monkey kidney cells (Vero cells) (Oxman and Levin, 1971). As the most simple working hypothesis that can account for the experimental observations, we postulate that SV40-specific early 19 S messenger RNA contains information necessary for the production of SV40-specific T antigen and that this antigen (or an as yet unknown early viral product whose synthesis would simply parallel the appearance of T antigen) activates or "deinhibits" a cellular regulatory element that controls chromosome replication and mitosis (Figs. 2 and 14).

5. *SV40-Induced "Transformation" of Primary Mouse Kidney Cell Cultures*

The majority of the abortively infected MK cells undergo only one cycle of SV40-induced mitosis (May *et al.*, 1971). This may be due to the irreversible switch-off of synthesis of early SV40 RNA soon after the onset of SV40-induced replication of the mouse cell chromatin (May *et al.*, 1973). However, infection with SV40 confers a continuous mitotic stimulus on a small fraction of the cells, which is apparently related to their ability to resume, after every virus-induced mitosis, synthesis of SV40-specific early 19 S RNA and of T antigen. SV40-induced mitotic activity in primary MK cultures, from the very beginning, is accompanied by a high incidence of tri- and multipolar mitoses and the rapid development of karyotypic abnormalities, comparable to those induced by SV40 in cells from other species (Moorhead and Saksela, 1965; Wolman *et al.*, 1964; Diamandopoulos *et al.*, 1969; Defendi, 1966; Penso *et al.*, 1968) and which are strikingly similar to the karyotypic and mitotic damage caused by X rays (Penso *et al.*, 1968) and chemical carcinogens (Arcos *et al.*, 1968).

Daily observation of SV40-infected MK cultures reveals an intense mitotic activity in numerous small groups of cells, all of which contain intranuclear, SV40-specific T antigen. During the first 6–8 weeks the virus-induced mitotic activity is accompanied by a high incidence of abnormal mitoses that frequently result in cell death. Thereafter the incidence of abnormal mitoses slowly decreases, and small colonies of rapidly growing, T antigen positive, highly pleomorphic and aneuploid cells

develop which apparently correspond to the colonies of transformed cells described earlier by other authors. SV40-transformed cells can now be continuously propagated *in vitro*. In 1973 we examined the SV40-specific RNA synthesized by a mouse kidney cell line "transformed" in our laboratory 4 years earlier. The cells of this line produce T antigen and contain "integrated" SV40 DNA that can be rescued (Koprowski *et al.*, 1967) by fusion with permissive CV-1 (monkey kidney) cells. The experimental results show that the virus-specific RNA has molecular properties closely similar to early SV40 RNA synthesized during the abortive infection of primary MK cultures. In the transformed cells (as in abortive infection) both SV40-specific 19 S and 16 S RNA molecules are present in the nucleus, while only 19 S RNA enters into the cytoplasm where it is associated with polysomes (E. May *et al.*, unpublished observations). The sedimentation patterns in sucrose density gradients of [^{3}H]uridine-labeled SV40 RNA extracted from the nuclei of SV40-transformed cells vary, however, with the transformed cell lines examined. The presence of intranuclear virus-specific "giant" RNA in SV40-transformed cells (Wall and Darnell, 1971) is thus not a general phenomenon.

From our observations we are led to the conclusion that SV40-induced mitotic stimulation, combined with karyotypic variation, converts primary MK cultures rather rapidly into cell lines with an unlimited potential for growth *in vitro*. Subsequently some transformed mouse cell lines become transplantable ("malignant"). The process of malignant transformation is, at least phenotypically, to some extent reminescent of the events observed during "spontaneous transformation" of tissue culture cells (Todaro and Green, 1963) or after treatment with chemical carcinogens (Berwald and Sachs, 1963; Heidelberger and Iype, 1967) and seems to involve cytogenetic variation and selection (Levan, 1969).

B. The Lytic Infection with Py Virus in Confluent, Primary Mouse Kidney Cell Cultures: An Abortive Mitosis

Most experiments reported here were performed in confluent, primary MK cultures, since essentially all cells present in such cultures participate in the lytic infection (Winocour, 1963) and produce Py progeny virus (approximately 10^5 particles/cell) (R. Weil, unpublished observations). Under standard conditions MK cultures (plated in 94 mm petri dishes) contained approximately 10^5 cells/cm^2 and were infected 2 or 3 days after confluence with 0.4 ml of crude or purified viral preparations containing approximately 10^9 PFU/ml.

1. The Asynchronous Onset of Py-Induced DNA Synthesis

a. During Infection at 37°C. Under standard conditions of infection at 37°C a small fraction of the cells (< 1%) begin Py-induced DNA synthesis around 12 hours after infection, as determined by autoradiography (Weil and Kára, 1970; Weil *et al.*, 1967, 1973). Thereafter the number of DNA-synthesizing cells rapidly increases and reaches a maximum around 25–30 hours postinfection, when 70–80% of the cells participate in Py-induced DNA synthesis (Fig. 3). The use of 10- or 100-fold higher viral titers (i.e., 10^{10} or 10^{11} PFU/ml) for infection does not significantly influence the time course of infection (R. Weil, unpublished observations).

If, however, the titer is decreased to 10^8 PFU/ml or less, Py-induced DNA synthesis begins later, the increase in the number of DNA-synthesizing cells is more asynchronous, and the maximum plateau is reached later and decreases linearly with the logarithm of the plaque-forming titer of the viral preparations used for infection. These effects seem to be related to the decreased and delayed synthesis of Py-specific early 19 S RNA and of T antigen after infection with diluted viral preparations (see below).

Infection of mouse tissue culture cells with Py leads to an increase in the activity (determined in cell-free extracts) of several enzymes involved in biosynthesis of DNA. Extending earlier studies on Py and SV40 by others (Hartwell *et al.*, 1965; Dulbecco *et al.*, 1965; Hatanaka and Dulbecco, 1967; Kit, 1967), we were led to the conclusion that in-

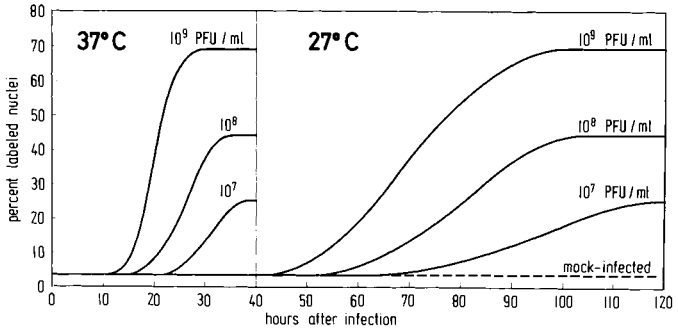
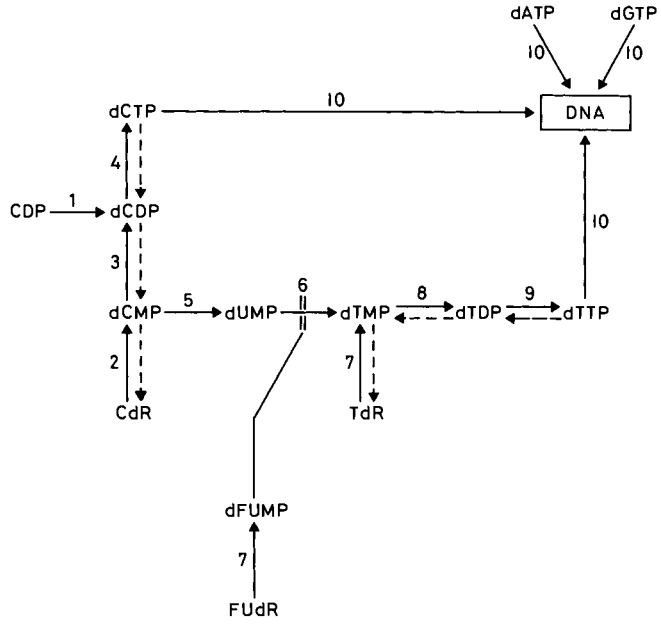


FIG. 3. Semischematic representation of the effect of the plaque-forming titer of the polyoma viral preparations on the time course of virus-induced DNA synthesis at 37° and 27°C. Primary MK cell cultures, confluent for 2 or 3 days, were infected or mock-infected. At different times after infection cultures were pulse-labeled for 1 hour with 2 μCi [^{3}H]thymidine and then subjected to autoradiography as described earlier (Weil and Kára, 1970; May *et al.*, 1971).



1	=	CDP reductase	6	=	dTMP synthetase
2	=	CdR kinase	7	=	TdR kinase
3	=	dCMP kinase	8	=	dTMP kinase
4	=	dCDP kinase	9	=	dTDP kinase
5	=	dCMP deaminase	10	=	DNA polymerase

FIG. 4. The enzymatic pathways involved in the biosynthesis of pyrimidine deoxyribonucleotides and of DNA; the site of the inhibitory action of 5-fluorodeoxyuridine (FUdR) on DNA synthesis. FUdR is converted by thymidine kinase to 5-fluorodeoxyuridine-5′-monophosphate (dFUMP), which inhibits dTMP synthetase and thus, after exhaustion of the endogenous pool of TdR-5′-phosphates, DNA synthesis. Solid arrows indicate the steps catalyzed by the enzymes indicated in the figure.

fection with Py induces a coordinate increase in the activities of the enzymes involved in the biosynthesis of pyrimidine deoxyribonucleotides and of DNA (Fig. 4) (Pétursson and Weil, 1968; Kára and Weil, 1967; Weil and Kára, 1970; Weil *et al.*, 1967). The increase in enzymatic activities under all experimental conditions tested paralleled the increase in the number of DNA synthesizing cells. From these and other experimental observations we were led to the hypothesis that an early function of Py induces an irreversible activation of the cellular DNA synthe-

sizing apparatus and that the increase in enzymatic activities, determined in cell-free extracts, reflects the asynchronous increase in the number of activated cells.

b. During Infection at 27°C. The sequence of events of the lytic infection is essentially the same at 27°C as during infection at 37°C. However, due to a cold-sensitive metabolic process ("psychrosensitive event") that is intercalated between the appearance of T antigen and virus-induced chromatin replication, the time course of infection is considerably slower at 27°C (Fig. 2). Experiments performed at 27°C were found to be particularly useful in analyzing the early events of the lytic infection (see below) (Weil and Kára, 1970; Weil *et al.*, 1965).

2. *Synchronization with 5-Fluorodeoxyuridine (FUdR) of the Onset of Py-Induced DNA Synthesis*

Owing to the asynchronous onset of DNA synthesis Py-infected MK cultures are a metabolically heterogeneous cell population; this renders it difficult, if not outright impossible, to define "early" and "late" viral functions with respect to time after infection. If FUdR (15 μg/ml) is added to the culture medium 2 hours after infection (Fig. 4), synthesis of cellular and circular Py DNA and of capsid protein are efficiently inhibited; however, the early events of the infection and the activation of the cellular DNA-synthesizing apparatus take place just as they do in normally infected parallel cultures (Hancock and Weil, 1969; Pétursson and Weil, 1968; Weil and Kára, 1970; Kára and Weil, 1967). Any time after infection the inhibitory effect of FUdR can be immediately and specifically reversed by the addition of thymidine (5 μg/ml) to the culture medium (Fig. 4). If thymidine is added around 25–30 hours after infection, i.e., when most (70–80%) cells present in the cultures are activated, Py-induced (cellular and viral) DNA synthesis starts synchronously in all activated cells within 1 minute.

3. *The Sequence of Py-Induced Replication of Mouse Cell Chromosomal DNA*

Py-infected and mock-infected MK cultures were labeled either with radioactive TdR, AdR, or CdR at different times after infection (4–30 hours at 37°C and 10–90 hours at 27°C) for periods between 30 minutes and 12 hours. If infection took place in the presence of FUdR the cultures were labeled with radioactive AdR or CdR. In a number of experiments Py-infected cultures were labeled with radioactive CdR at various

times before and after the synchronized onset of Py-induced DNA synthesis.

At the end of the pulses with the radioactive precursors total DNA was extracted either with sodium dodecyl sulfate (Pétursson and Weil, 1968), with the phenol method used in our earlier studies (Weil, 1961), or with the method used by Smith (1970). In some experiments RNA was removed by treatment with pancreatic ribonuclease. Aliquots of the DNA preparations were subjected to equilibrium centrifugation in shallow CsCl density gradients in a fixed-angle rotor under conditions that allow the separation of light satellite DNA from the bulk of the mouse cellular DNA (Flamm *et al.*, 1966; Schildkraut and Maio, 1968). Fractions were collected, and in each fraction optical absorbance at 260 nm and radioactivity determined.

The results (M. Farrell and R. Weil, unpublished observations) can be summarized as follows. Under all experimental conditions tested Py-induced chromosomal DNA synthesis started in DNA sequences rich in cytosine + guanosine, while synthesis of light satellite DNA began only 3–4 hours later. No evidence for an early replication of light satellite DNA was obtained, even if the cultures were infected with the Py strain used by Smith (1970). The absence of early replicating satellite DNA is in accordance with the report of others (Hatfield and Walker, 1973). It is at variance, however, with the claim by Smith (1970) that Py-induced DNA synthesis would begin with the replication of light satellite DNA. The sequence of Py-induced chromosomal DNA synthesis thus corresponds essentially to the sequence observed during S phase of the normal mitotic cycle in a number of cell types from different mammalian species (Bostock and Prescott, 1971; Tobia *et al.*, 1970).

4. Py-Induced Synthesis of the Chromosomal Proteins

Based on chemical analysis of chromatin isolated at different times during infection of MK cultures with Py, we were led to the conclusion that Py-induced replication of the chromosomal DNA is paralleled by the synthesis of histones and of chromosomal nonhistone proteins in proportions that are characteristic of chromatin replication during S phase of the normal mitotic cycle of uninfected mouse cells. However, if infection took place in the presence of FUdR no significant increase in histone and nonhistone proteins could be observed (Hancock and Weil, 1969). Subsequently, similar results were reported by Winocour and Robbins (1970). Lytic infection in mouse kidney cells with Py thus induces chromatin replication which, by the methods used, is indistinguishable from the S phase of the normal mitotic cycle of uninfected mouse cells.

5. *Py-Induced Appearance of Lectin Receptors on the Cell Surface Late in the Lytic Infection*

Relatively late during the lytic infection of mouse cell cultures with Py lectin receptors appear, as judged by increased agglutinability of the cells by lectins; this is prevented if Py-induced DNA synthesis is inhibited with FUdR (Benjamin and Burger, 1970). Since lectin-receptor sites also appear during mitosis of uninfected, normally growing cells (Noonan *et al.*, 1973), we consider the possibility that the cell surface changes observed during the lytic infection might be the expression of a Py-induced "abortive mitosis."

6. *Py DNA Replicates as a Nucleohistone Complex in Association with the Mouse Cell Chromatin*

The temporal and functional relation between synthesis of mouse chromosomal and Py viral DNA, considered together with the limited amount of genetic information contained in the viral genome pointed to the possibility that Py virus might use the chromosome replication machinery of the host cell for its own replication. Recent results show that two Py-specific nucleoprotein complexes, designated as complex A and B, can be isolated from mouse kidney cell cultures during the lytic infection (Seebeck and Weil, 1973). Both complexes remain associated with the host cell chromatin during the isolation procedure used; they can be dissociated, however, from the isolated chromatin by gentle homogenization in a buffer containing 0.5 *M* NaCl. Reconstruction experiments tend to rule out the possibility that the two complexes and their association with the chromatin would be experimental artifacts. Several lines of experimental evidence show that both complexes contain essentially only Py DNA, that complex A contains Py DNA molecules in different stages of replication, and that complex B contains only mature Py DNA I molecules. Complex B may correspond to the viral nucleoprotein particles previously isolated from cultures infected with Py (Green *et al.*, 1971) or SV40 (Jaenisch *et al.*, 1971). Complexes A and B were treated with formaldehyde under conditions which cross-link DNA with proteins and were then analyzed in CsCl density equilibrium gradients. The finding that both exhibit the same buoyant density (1.470 gm/cm^3) suggested that newly synthesized stretches of Py DNA are immediately complexed with proteins and that the DNA to protein ratio of the complexes remains essentially unchanged throughout replication. The proteins extracted from complex A and B in acrylamide gels exhibit essentially the same pattern as the small proteins (PV IV–VII) present in purified Py virions (Seebeck, 1974). The minor proteins present in Py

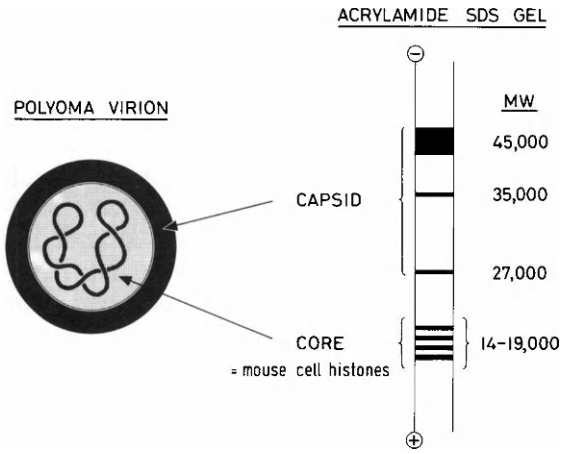


FIG. 5. Simplified and schematic representation of the structural proteins contained in polyoma virions. The indicated molecular weights are approximations. Polyoma pseudovirions enclose linear fragments of mouse chromosomal DNA instead of circular viral DNA; they contain the same 7 proteins. "Empty capsids" contain only proteins I–III.

virions are host cell histones (Hirt, 1974) most of which are newly synthesized during Py-induced chromatin replication (Seehafer and Weil, 1973). These results considered with those obtained by Consigli *et al.* (1974) led to the tentative conclusion that replicating Py DNA is immediately complexed with newly synthesized mouse cell histones and that complex B, after encapsidation by Py capsomere proteins PV I–III, becomes the "core" of Py progeny virions (Seebeck, 1974; Seebeck and Weil, 1973) (Fig. 5). Py-induced replication of the host cell chromatin may thus be necessary to provide (among other functions) the replicating Py DNA with histones. On the basis of the experimental evidence presently available we tentatively consider Py DNA as a very simplified chromosomal replication unit.

7. The Properties of "Late" Py-Specific RNA

MK cultures were labeled with [^{3}H]uridine late during the lytic infection, i.e., at different times between 25–30 hours after infection. At this time most cells are engaged in Py-induced cellular and viral DNA synthesis. Total RNA was extracted with hot phenol (Scherrer, 1969) and then sedimented through linear sucrose density gradients or subjected to electrophoresis in acrylamide gels. Individual fraction from gradients or gels were exhaustively hybridized with Py DNA fixed on membrane filters (Hudson *et al.*, 1970; Acheson *et al.*, 1971). The results from

these experiments show that Py RNA is synthesized in the form of heterogeneous "giant" RNA molecules with sedimentation coefficients as high as 80 S. Since aggregates could be ruled out, Py-specific "giant" RNA molecules are thus larger than the transcript of one strand of Py DNA (1.5×10^6 daltons) (Weil and Vinograd, 1963), which should have a sedimentation coefficient of about 26 S. New experimental results (N. Acheson, personal communication) show that at least 80–90% of the RNA present in "late" giant RNA molecules is Py-specific. Earlier observations pointed to the possibility that Py-specific giant RNA molecules may be cleaved to an RNA species with a sedimentation coefficient of 26 S and to at least two smaller RNA species with sedimentation coefficients between 16 S and 20 S. In subsequent studies Buetti (1974) labeled Py-infected cultures for 3 hours (25–28 hours after infection) with [^{3}H]uridine and then extracted cellular and viral RNA from isolated nuclei, cytoplasm, and polyribosomes. Her results show that both Py-specific "giant" RNA and 26 S RNA molecules remain in the nucleus, while the cytoplasm contains 2 species of Py-specific RNA, a major fraction (80–90%) with a sedimentation coefficient of 16 S, and a minor fraction with a sedimentation coefficient of 19 S. Since both 16 S and 19 S Py-specific RNA's are associated with polyribosomes, we tentatively consider them to be viral mRNA molecules. Sedimentation analyses in 99% DMSO gradients (using ^{14}C-labeled 16 S ribosomal RNA from *E. coli* and 18 S mouse ribosomal RNA as velocity markers) suggest a molecular weight for Py-specific 16 S RNA in the order of 500,000 daltons. The "16 S RNA" should thus contain information to code for a protein(s) with a total molecular weight of approximately 50,000 daltons. Several lines of indirect evidence suggest that the 16 S RNA is the "late" Py viral messenger that contains the information for the synthesis of the major component of Py capsid protein (PV I; molecular weight of approximately 46,000 daltons) (Seehafer and Weil, 1973). The biological significance of "19 S RNA" remains unknown. Its molecular weight is estimated to be about 700,000 daltons.

8. *Analysis of the Early Viral Functions Which Precede Py-Induced Replication of the Mouse Cell Chromatin*

a. The Time Course of Synthesis of "Early" Py-Specific RNA. Operationally we define as "early" Py-specific RNA those species of radioactive, hybridizable RNA that are synthesized either before the onset of Py-induced DNA synthesis or under conditions in which Py-induced DNA synthesis is inhibited with FUdR (added 2 hours after infection). Since the results of these studies will be published in detail elsewhere (Weil *et al.*, 1974), we will summarize here only some of the essential

observations. MK cultures were infected with Py under standard conditions (10^9 PFU/ml) in the presence or absence of FUdR. Py-infected and mock-infected cultures were labeled with [^{3}H]uridine (100–500 μCi/ml) at various times between 4 and 30 hours at 37°C and between 10 and 120 hours at 27°C for periods varying between 10 minutes and 8 hours. In a number of experiments the pulse with [^{3}H]uridine was followed by a "chase" in nonradioactive medium in the presence or absence of actinomycin D (1–2 μg/ml). The labeled cultures were extracted with hot phenol and the RNA examined by the same techniques as used previously (Acheson *et al.*, 1971; May *et al.*, 1973).

Results from more than 15 individual time course studies show that synthesis of Py-specific RNA begins several hours before the appearance of intranuclear T antigen, determined with the immunofluorescence method described by Fogel *et al.* (1967). During infection at 37°C (Fig. 6) small amounts of Py RNA can first be detected by 6–7 hours after infection, while a weak reaction for T antigen becomes detectable at the

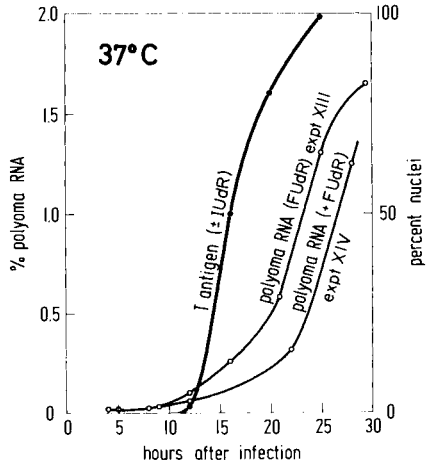


FIG. 6. Time course at 37°C of appearance of T antigen and of synthesis of "early" polyoma-specific RNA in confluent, primary mouse kidney cell cultures infected in the presence of FUdR. (Results from 2 independent experiments.) Cultures were infected with polyoma virus (10^9 PFU/ml) 2 days after confluence. Aliquots of 2 cultures each were labeled for 25 minutes with [^{3}H]uridine (250 μCi/ml, + FUdR) and then extracted with hot phenol; total RNA (11 to 35 × 10^3 cpm/μg) was exhaustively hybridized with polyoma DNA fixed on membrane filters. RNA extraction and hybridization were performed as described (Acheson *et al.*, 1971). Intranuclear T antigen was determined by immunofluorescence (Fogel *et al.*, 1967; Weil and Kára, 1970). The time course of the appearance of T antigen varies only very little from one experiment to the next. The increase, with time after infection, of the intensity of the immunofluorescence reaction is not indicated (Weil and Kára, 1970).

earliest in 0.5–1% of the cells by 10–12 hours after infection (Weil and Kára, 1970, and unpublished observations). Thereafter the rate at which Py RNA is synthesized increases rapidly; by 12 hours it reaches a level that is similar to the maximum level of early SV40-specific RNA observed during the abortive infection of MK cultures with SV40 (0.03–0.05%). Until this time (i.e., before the onset of Py-induced DNA synthesis) the increase in Py-specific RNA is the same whether infection takes place in the presence or absence of FUdR. In Py-infected cultures, even if infected in the presence of FUdR, synthesis of Py-specific RNA increases rapidly 20- to 30-fold *after* the appearance of T antigen. We excluded the possibility that this would be due to "leakiness" of the inhibition imposed by FUdR on the synthesis of Py progeny DNA. Under all experimental conditions tested during infection at 37° and 27°C, the increase in transcription coincided in time with the increase in size of the nucleoli, detectable by phase-contrast microscopy (Weil and Kára, 1970), which is apparently the morphological expression of Py-induced stimulation of overall cellular RNA synthesis occurring in those cells where the immunofluorescence reaction for T antigen has reached a certain level (see below).

During infection at 27°C (in the presence of FUdR) small amounts of Py-specific RNA can be detected at the earliest by 17–20 hours after infection, i.e., about 10 hours before the appearance of T antigen. Again a marked increase in synthesis of early Py-specific RNA coincides in time with Py-induced stimulation of cellular RNA synthesis (Fig. 7).

b. Evidence for Synthesis of at Least Two Species of Early Py-Specific RNA During the Lytic Infection. Cumulative labeling with [^{3}H]uridine and pulse-chase experiments show that the turnover of early Py-specific RNA ("19 S RNA") synthesized *before* the onset of Py-induced stimulation of cellular RNA synthesis (see below) is very similar to the slow turnover of SV40-specific early 19 S RNA (May *et al.*, 1973). In contrast, Py-specific RNA synthesized *after* the induction of cellular RNA synthesis ("early giant RNA") turns over rather rapidly and behaves like "late" Py-specific giant RNA.

i. Py-Specific Early 19 S RNA. MK cultures were labeled with [^{3}H]uridine for varying lengths between 0.5 to 5 hours and at different times between 6 and 12 hours at 37°C and 17–40 hours at 27°C after infection, i.e., before the onset of Py-induced cellular RNA synthesis. Total RNA was extracted with hot phenol and then analyzed by the same methods used earlier (Acheson *et al.*, 1971; May *et al.*, 1973). In all experiments the bulk of Py-specific RNA present sedimented in linear (10–30%) sucrose density gradients as a relatively uniform band

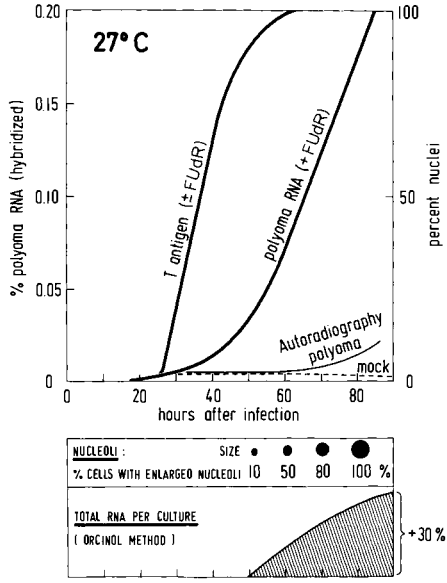


FIG. 7. Semischematic representation of the time course at 27°C of the lytic infection with polyoma virus in the presence of FUdR. Primary MK cultures were infected with polyoma virus (10^9 PFU/ml) 4 days after confluence; aliquots of 2 cultures each were labeled for 1 hour with [^{3}H]uridine (200 μCi/ml + FUdR) and then extracted with hot phenol. [^{3}H]RNA was hybridized with polyoma DNA fixed on membrane filters. From polyoma-infected and mock-infected parallel cultures total RNA was extracted and quantitatively estimated as described in Fig. 11. The RNA content of mock-infected cultures is taken as 100%. The number of T antigen-positive cells was determined as in Fig. 6. The increase, with time after infection, of the intensity of immunofluorescence is not indicated. The number of DNA-synthesizing cells was determined by autoradiography performed on cultures labeled for 1 hour with [^{3}H] thymidine (2 μCi/ml).

with a sedimentation coefficient of 19 S, while a variable but generally smaller fraction sedimented faster (Fig. 8). Sedimentation through 99% DMSO gradients revealed a similar pattern; however, the main band of Py-specific RNA ("19 S RNA") cosedimented under these conditions with cellular 18 S ribosomal RNA (Fig. 9). Estimated from its sedimentation behavior the molecular weight of "early 19 S RNA" should be in the order of 700,000 daltons; this would correspond to the transcript of about 45% of the information contained in one strand of circular Py DNA.

Synthesis of Py-specific early 19 S RNA is not significantly influenced if protein synthesis is inhibited by the addition of cycloheximide (25 μg/ml) to the culture medium prior to the onset of transcription

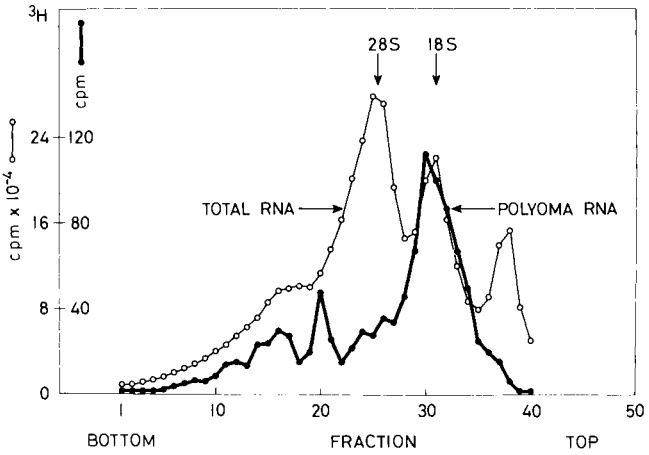


FIG. 8. Sedimentation velocity analysis in a linear [15–30%(w/w)] sucrose density gradient of polyoma-specific "early 19 S RNA" (synthesized from 7 to 11 hours after infection at 37°C). Three MK cell cultures, confluent for 2 days, were infected with polyoma virus (10^9 PFU/ml) at 37°C in the presence of FUdR. Polyoma-infected and mock-infected (not shown) cultures were labeled from 7 to 11 hours after infection with [^{3}H]uridine (250 μCi/ml + FUdR); total RNA was extracted with hot phenol. 100 μg of [^{3}H]RNA (68,300 cpm/μg) in 100 μl of 0.1 × SSC (sodium saline citrate; 0.15 *M* NaCl, 0.015 Na citrate, pH 7.1) were layered on top of a 15–30% (w/w) sucrose density gradient (4.4 ml; sucrose in 10 m*M* triethanol amine–50 m*M* NaCl–1 m*M* EDTA). The sample was centrifuged at 54,000 rpm for 105 minutes at 22°C in a Spinco SW56 rotor. Samples were collected from the bottom of the tube and aliquots of 10 μl per fraction were spotted on filter papers, dried, and washed in ice cold 5% TCA; the remainder of each fraction was adjusted to a concentration of 4 × SSC. To each fraction 2 filters containing 0.2 μg denatured, (single-stranded) polyoma DNA per filter and one blank filter were added; hybridization was performed at 65°C for 48 hours. Thereafter the filters were treated with ribonuclease, washed, dried, and counted in a toluene based scintillation fluid, under the conditions used earlier (Acheson *et al.*, 1971). [^{3}H]RNA from polyoma-infected cultures (labeled from 7–11 hours) contained 0.014% polyoma specific RNA while RNA from mock-infected controls showed a background hybridization of about 0.001%. Total RNA (○——○) represents the total ^{3}H cpm per fraction; polyoma RNA (●——●) corresponds to the ^{3}H cpm per fraction hybridized to polyoma DNA.

(C. Salomon and R. Weil, unpublished). This and the observations on "integration" of Py DNA during the lytic infection (see below) suggest that "early 19 S RNA" is transcribed in the nucleus from free (i.e., not "integrated") parental Py DNA by a DNA-dependent RNA polymerase of the host cell. The early functions of SV40 and Py, during both the lytic and abortive infection (see below), seem thus to be contained in a single, rather large transcriptional unit ("early 19 S mRNA"), which subsequently appears in the polyribosomes. The sum of the molecular weights of "early" and "late" Py-specific mRNA (approximately

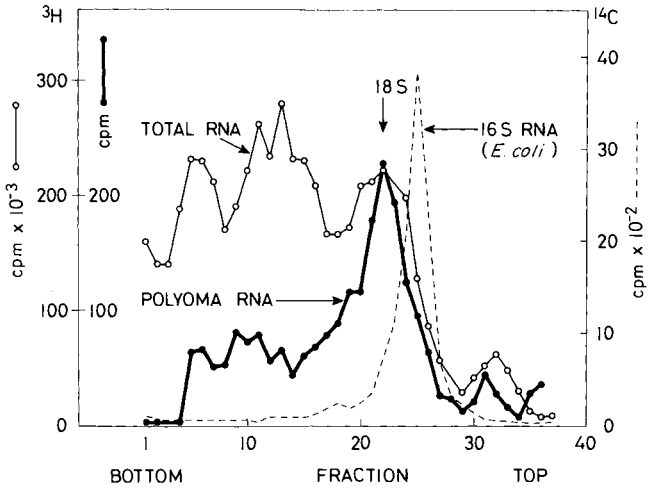


FIG. 9. Sedimentation velocity analysis of polyoma-specific "early 19 S RNA" under denaturing conditions in a DMSO-chloral hydrate gradient. Three MK cell cultures, confluent for 3 days, were infected with polyoma virus (10^9 PFU/ml) at 27°C in the presence of FUdR; they were labeled from 25–30 hours after infection with [^{3}H]uridine (200 μCi/ml + FUdR); total RNA was extracted with hot phenol. 113 μg [^{3}H]RNA (60,500 cpm/μg) were dissolved in 1 ml of a DMSO solution [0.25 ml of EDTA-Na_2, 0.25 ml DMSO (dimethyl sulfoxide Merck-Schuchardt) and 0.5 ml *N,N*-dimethyl formamide Merck]; 5 μl of ^{14}C-labeled 16S *E. coli* ribosomal RNA (35,000 cpm) were added as sedimentation marker. In other experiments ^{14}C-labeled mouse cell RNA (see Fig. 13) was used as sedimentation marker (not shown). The sample was layered on top of a linear DMSO–chloral hydrate (37 ml) gradient (0–10% chloral hydrate in DMSO, containing 1% (v/v) water–1 m*M* EDTA-Na_2), prepared according to I. Maxwell (unpublished), and was centrifuged at 27,000 rpm for 28 hours at 25°C in a Spinco SW27 rotor. Fractions of approximately 1 ml each were collected from the top. Aliquots of 50 μl of each fraction were spotted on filter papers, dried, and washed in ice cold 5% TCA. To the remainder of each fraction 40 μg yeast RNA and 3 volumes absolute ethanol (0.1 *M* sodium acetate, pH 5.1) were added and total RNA was precipitated in ice water (3 hours). Pellets were washed in ice cold 70% EtOH–0.1 *M* sodium acetate, and then resuspended in 100 μl 0.1 × SSC. Hybridization and counting were carried out as described in the text of Fig. 8. Total RNA (○——○) and marker RNA (– – – –) represent total ^{3}H or ^{14}C cpm per fraction; polyoma RNA (●——●) corresponds to ^{3}H cpm per fraction hybridized to polyoma DNA.

700,000 and 500,000 daltons, respectively) is less than the molecular weight expected for the transcript of a strand of Py DNA (1.5×10^6 daltons). This suggests that either our estimates of the molecular weights of early and late virus-specific mRNA are too low, or that the band of "16 S RNA" might comprise two distinct species of mRNA (Buetti, 1974).

ii. Py-Specific Early Giant RNA. The bulk of Py-specific RNA extracted from cultures *after* the onset of Py-induced cellular RNA syn-

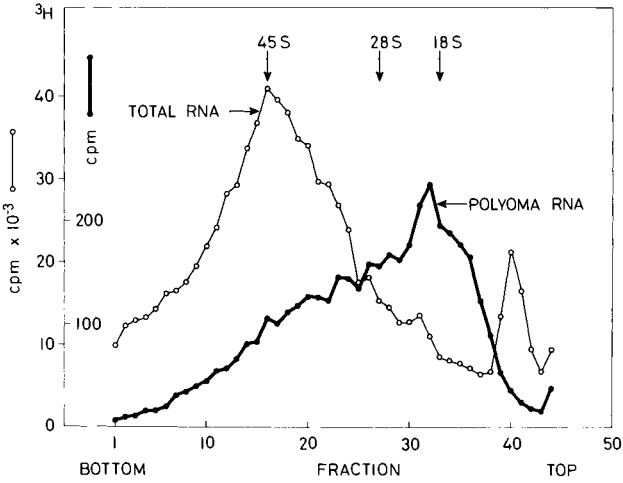


FIG. 10. Sedimentation velocity analysis in a linear [15–30% (w/w)] sucrose density gradient of polyoma-specific "early giant RNA" (synthesized 24 hours after infection at 37°C). Three MK cell cultures, confluent for 2 days, were infected with polyoma virus (10^9 PFU/ml) at 37°C in the presence of FUdR. The cultures were labeled for 10 minutes at 24 hours after infection with [^{3}H]uridine (500 μCi/ml + FUdR). Total RNA was extracted with hot phenol. 100 μg of [^{3}H]RNA (12,400 cpm/μg) in 100 μl of 0.1 × SSC were sedimented and the individual fractions analyzed under the conditions described in Fig. 8. As sedimentation marker 5 μl (30,000 cpm) ^{14}C- labeled total mouse cell RNA was used. {[^{14}C]RNA was extracted with hot phenol from uninfected, growing MK cell cultures, labeled for 24 hours with [^{14}C]uridine (4 μCi/ml).} Total RNA (○——○) represents total ^{3}H cpm per fraction; polyoma RNA (●——●) represents ^{3}H cpm per fraction hybridized to polyoma DNA.

thesis has a sedimentation behavior that is very similar to that of "late" Py-specific giant RNA (Acheson *et al.*, 1971) (Fig. 10). The results from pulse-chase experiments performed either in the presence or absence of actinomycin D (1–2 μg/ml) show that early giant RNA molecules are synthesized in the nucleus and that they turn over rather rapidly; they also point to the possibility that early giant RNA may be processed into smaller RNA molecules that form a relatively broad band in sucrose gradients with a sedimentation coefficient around 19 S and which may contain the minor species of polysome-associated Py-specific mRNA isolated by Buetti late during normal lytic infection.

9. *Py-Induced Stimulation of Overall Cellular RNA Synthesis*

In a large number of experiments performed during the last few years we observed that phenol extracts from MK cultures infected under stan-

dard conditions, (in the presence or absence of FUdR) at 37°C for about 24–30 hours and at 27°C for 60–72 hours (or longer), generally contained 30% more RNA than mock-infected parallel cultures as determined by optical absorbance at 260 nm and by the orcinol method. Sedimentation velocity analyses in linear (10–30%) sucrose density gradients reveal that the increase in total RNA is largely due to an increase in ribosomal 28 S and 18 S RNA. Labeling with [^{3}H]uridine shows however that all major species of cellular RNA in Py-infected cultures incorporate radioactive [^{3}H]uridine at a moderately increased rate, suggesting that infection stimulates overall cellular RNA synthesis.

These results did not exclude the possibility that Py-infected and mock-infected cultures actually contain the same amounts of total RNA but that the hot phenol method extracts ribosomal RNA more efficiently from infected cultures. We therefore determined total RNA in more than 10 independent experiments (at different times during infection at 37° or 27°C) by a chemical method based on that reported by Schneider (1957) and estimated the ribose content by the orcinol method. In all experiments a small but significant increase in total RNA content could be detected in the infected cultures shortly after the begin of the nucleolar increase, i.e., around 15–17 hours after infection at 37°C (Fig. 11) and around 48 hours at 27°C (C. Salomon and R. Weil, unpublished). The RNA content of infected cultures reached a maximum around 24 hours after infection at 37°C and around 72 hours at 27°C. At these times Py-infected cultures contain regularly 30% (±5%) more RNA than mock-infected parallel cultures. It should be stressed that essentially the same increase in total RNA occurs whether infection takes place in the presence or absence of FUdR.

The increase in total, mainly ribosomal, RNA thus begins shortly after the appearance of T antigen but before the onset of Py-induced DNA synthesis, and it is independent of the latter. The close temporal relation between the appearance of T antigen and the subsequent increase in nucleolar size points to a causative relation between T antigen (or another, as yet unknown early viral function) and Py-induced stimulation of cellular RNA synthesis.

10. Effect of Some Variables on the Time Course of the Lytic Infection with Py

a. The Infective Titer of the Py Viral Preparations Used for Infection. The same results are obtained with crude viral lysates and highly purified viral preparations if they contain the same plaque-forming titer (PFU/ml). Infection with preparations containing 10^8 PFU/ml or less, results in a decrease and a delay in the synthesis of Py-specific early 19

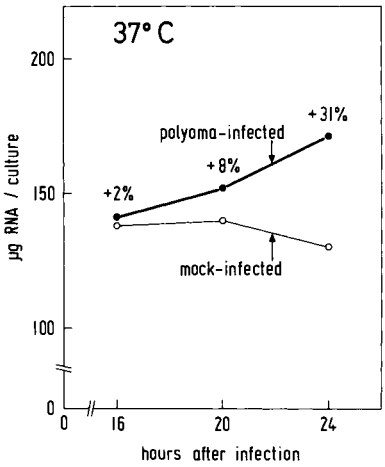


FIG. 11. Polyoma-induced increase in total cellular RNA in primary mouse kidney cell cultures infected under standard conditions at 37°C in the presence of FUdR. Six large (94 mm diameter) petri dishes, confluent for 2 days, were infected with polyoma virus (8×10^8 PFU/ml) and 6 parallel cultures were mock-infected. At 16, 20, and 24 hours after infection 2 polyoma-infected and 2 mock-infected cultures were treated as follows. The cultures were put on ice, washed with 5 ml ice-cold PBS (isotonic phosphate-buffered saline). Thereafter 5 ml ice cold 5% TCA (trichloroacetic acid) were added to each dish. After incubation on ice for 20 minutes the cells were scraped off and the suspension centrifuged for 15 minutes at 3,000 rpm in an HB4 Sorvall rotor at 4°C. The supernatant was discarded and the pellet resuspended in 5 ml ice cold 5% TCA, and again centrifuged as described. The resulting pellet was suspended in 2 ml 1 *N* KOH and then incubated at 37°C for approximately 20 hours which was sufficient to hydrolyze the RNA. Then the tubes were put on ice and 400 µl HCl and 250 µl 50% TCA were added to each tube. The samples were kept on ice for 30 minutes and centrifuged at 3000 rpm for 15 minutes in an HB4 Sorvall rotor at 4°C. The supernatant was removed and from each tube aliquots of 0.5 and 1.0 ml were immediately assayed for ribose content by the orcinol method.

S RNA and of T antigen, as compared to infection with 10^9 PFU/ml or more. The maximum number of cells that will synthesize T antigen decreases approximately linearly with the logarithm of the plaque-forming titer used for infection. As expected, the increase in nucleolar size also occurs later, i.e., again at the time when T antigen has reached a certain level. The onset of Py-induced DNA synthesis (determined by autoradiography) is thus delayed and more asynchronous (Fig. 3). The increased asynchrony of the time course of Py-induced DNA synthesis seems to be linked to the delayed and decreased synthesis of early Py RNA. These observations are also important for the interpretation of the results obtained with "T antigen-negative" mutants of Py virus (see below).

b. Infection of Mouse Kidney and Mouse Embryo Cell Cultures with Temperature-Sensitive "T Antigen-Negative" Mutants of Py Virus. In six independent experiments we infected primary MK cultures and secondary mouse embryo cell cultures with Py viral preparations (1 to 5×10^7 PFU/ml) of temperature-sensitive "T antigen-negative" mutants [ts-a, ts-10, ts-25, ts-616] obtained from Dr. W. Eckhart (Oxman *et al.*, 1972) at the nonpermissive temperature ($39.7° \pm 0.3°C$).

In all experiments parallel cultures were either mock-infected or infected with wild-type Py used in our laboratory and diluted to the same low infective titers (1 to 5×10^7 PFU/ml) as the mutant virus. The use of low infective titers is necessary, since the temperature-sensitive mutants are "leaky" if the cultures are infected with higher titers (10^8 PFU/ml or more)!

The results from these experiments can be summarized as follows. As judged by the use of three different antisera (prepared in hamsters or rabbits) all four mutants tested induce the production of Py-specific T antigen at the nonpermissive temperature (Fig. 12), although at a slower rate (delay of about 12 hours) than that observed in parallel cultures infected with wild-type Py virus. Similarly, the beginning of the nucleolar increase and the onset of Py-induced DNA synthesis are delayed by about 12 hours (or more) in cultures infected with the mutants. These results suggest that the mutation may be responsible for a decrease in the rate at which T antigen is synthesized at the nonpermissive temperature but that T antigen (or a product synthesized in parallel), once it has reached a critical concentration, induces cellular RNA synthesis and the replication of mouse cell chromatin just as does wild-type T antigen. The markedly decreased production of progeny virus after infection with these mutants at the nonpermissive temperature (Oxman *et al.*, 1972; Eckhart, 1969) points to the possibility that T antigen may provide a function(s) necessary for synthesis of viral progeny DNA which would be impaired in some of the temperature-sensitive "T antigen-negative" mutants.

c. The Physiological State of the Cultures at the Time of Infection. Cell density (cells/cm^2) and time after confluence exert little if any effect on the synthesis of Py-specific early 19 S RNA and on the appearance of T antigen. However, both factors influence the host cell response toward the infection. If "crowded" MK cultures (containing 3×10^5 or more cells/cm^2) are infected at 37°C the increase in the nucleolar size and Py-induced DNA synthesis are delayed and are more asynchronous. The influence of the length of time of confluence before infection is particularly noticeable if MK cultures are infected at 27°C; if MK cultures are infected *one day* after confluence the nucleolar increase

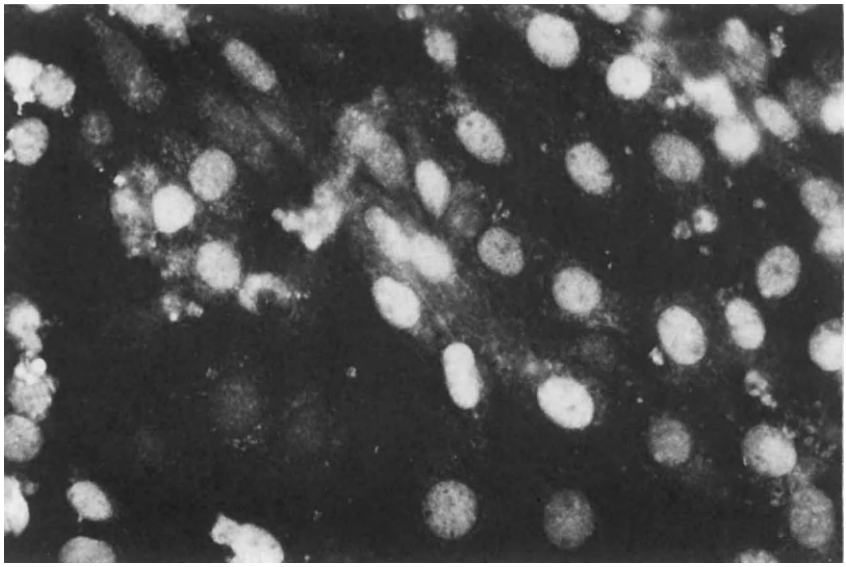


FIG. 12. Evidence that ts-a, a "T antigen-negative," temperature-sensitive polyoma mutant, produces T antigen at the nonpermissive temperature. Secondary mouse embryo cultures were infected with ts-a (5×10^7 PFU/ml) at 39.5°C; coverslips were fixed 45 hours after infection to determine T antigen by immunofluorescence. In this experiment about 40% of the nuclei present in the culture gave a positive reaction while mock-infected control cultures were completely negative.

and Py-induced cellular DNA synthesis begin about 12 hours earlier than in parallel cultures infected 3 days after confluence (Weil and Kára, 1970). If cell density, time after confluence, and the infective titers are controlled the time course of the infection is predictable and very reproducible.

C. The Abortive Infection with Py of Hamster Embryo Cultures

Py induces an abortive infection in hamster embryo tissue culture cells which leads to mitotic stimulation and to "cell transformation" while only very few, if any cells, synthesize viral DNA and capsid protein (Vogt and Dulbecco, 1960, 1962, 1963). To determine the molecular properties of Py-specific RNA synthesized during the abortive infection we infected secondary hamster embryo cultures, plated in large (94 mm diameter) petri dishes that contain about 10^7 cells with Py virus (0.4

ml, 10^9 PFU/ml). After adsorption the cultures were covered with 10 ml culture medium. In experiments where relatively small (young) embryos had been used to prepare the cultures a distinct mitotic stimulation could be observed 2 or 3 days after infection. In these experiments 25–50% of the cells contained Py-specific T antigen, determined around 20–24 hours after infection, while essentially no cells (<0.01%) contained capsid protein. In experiments where older embryos were used to prepare the cultures only 1–5% of the cells became positive for T antigen and virtually no virus-induced mitotic stimulation could be observed.

In four independent experiments we determined the amounts of radioactive, hybridizable Py-specific RNA synthesized in cultures prepared from younger (about 7 mm) or older (12 mm or more) hamster embryos. Py-infected and mock-infected cultures were labeled with [^{3}H]uridine (250 μCi/ml) for 2–3 hours at different times between 19–26 hours after infection (either in the presence or absence of FUdR). After the pulses total RNA was extracted with hot phenol and then exhaustively hybridized with Py DNA fixed on membrane filters. In two experiments where 25 and 50%, respectively, of the cells contained T antigen, small but significant amounts of radioactive Py-specific RNA could be detected (approximately 0.010% in Py-infected and 0.001% in mock-infected controls). In contrast, cultures with only 1–5% T antigen-positive cells did not contain detectable amounts of Py RNA. In two independent experiments total RNA from Py-infected hamster embryo cultures with 25 or 50% T antigen-positive cells was sedimented through linear [10–30% (w/v)] sucrose density gradients; individual fractions were collected and then exhaustively hybridized with Py DNA fixed on membrane filters (Fig. 13). In both experiments the bulk of Py-specific RNA sedimented as a rather uniform band with a sedimentation coefficient of about 19 S. A minor fraction of Py-specific RNA formed a band with a sedimentation coefficient of about 16 S, while no faster-sedimenting virus-specific RNA could be detected. The sedimentation behavior of Py-specific RNA synthesized during the abortive infection of hamster cells with Py is thus strikingly similar to that of SV40-specific early RNA synthesized during the abortive infection of MK cultures with SV40. These observations are compatible with the hypothesis that parental Py DNA in hamster embryo cells directs the synthesis of "early 19 S mRNA" and the production of T antigen and thus is able to induce mitotic stimulation but that in (most) hamster cells the factors necessary for the synthesis of Py progeny DNA are not available.

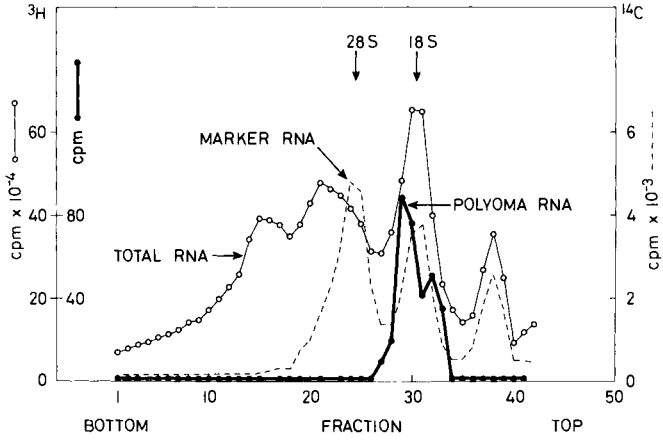


FIG. 13. Sedimentation velocity analysis in a linear [15–30%(w/w)] sucrose density gradient of polyoma-specific RNA synthesized during the abortive infection with polyoma virus of secondary hamster embryo cell cultures. Cultures were infected at 37°C with polyoma virus (10^9 PFU/ml) and labeled from 24 to 26.5 hours after infection with [^{3}H]uridine (250 μCi/ml). Total RNA was extracted with hot phenol. 100 μg of [^{3}H]RNA (96,800 cpm/μg) were sedimented and subsequently hybridized with polyoma DNA fixed on membrane filters under the conditions described in Fig. 8. As sedimentation marker (100,000 cmp) ^{14}C-labeled mouse cell RNA was added (see text to Fig. 10). Total RNA (○——○) and marker RNA (---) represent total ^{3}H or ^{14}C cpm per fraction. Polyoma RNA (●——●) represents ^{3}H cpm per fraction hybridized to polyma DNA. The sedimentation pattern of ^{3}H-labeled total RNA from mock-infected parallel cultures was indistinguishable from that shown in this figure.

V. Polyoma-Induced Tumor Formation Revisited

Despite the vast literature on Py-induced tumors (Eddy, 1969), we repeated some of the experiments performed earlier by other authors. In collaboration with F. Chatelanat (Department of Pathology, University of Geneva) we performed a detailed study by light and electron microscopy of the effects of the infection with Py in newborn hamsters and rabbits. The results obtained generally confirmed the earlier experimental observations. However, our observations show that Py induces a widespread, intense mitotic stimulation that leads to a rapid outgrowth of relatively undifferentiated cells in numerous organs and tissues. These results suggest that many Py-induced tumors, diagnosed in earlier studies as sarcomas and other forms of cancer, are actually hyperplasias ("benign tumors"). The primary tumors induced by Py seem, at least to some extent, analogous to warts induced by the papilloma viruses. In agreement with earlier reports (Defendi *et al.*, 1967) we found that all

cells in Py-induced (subcutaneous) tumors contained intranuclear Py-specific T antigen. These observations suggest that the mitogenic effect of Py observed in tissue culture cells may also be operational *in vivo*, leading in newborn hamsters and rabbits to widespread, intense mitotic stimulation and, in some areas, to the development of hyperplasias. In contrast, the development of cancer, if it develops at all, appears to be the result of complex secondary events similar to "tumor progression" (Levan, 1969; Koller, 1972) observed to occur spontaneously or as the result of chemical or physical carcinogens. Subcutaneous infection of newborn hamsters with the temperature-sensitive Py mutant ts-a (50 μl; 2 to 5 $\times$ 10^7 PFU/ml) within 3–4 weeks induces rapidly growing tumors in the inner organs, particularly in the heart (F. Chatelanat and R. Weil, unpublished). This observation is at variance with that of Fried (1965). In newborn hamsters infected subcutaneously with ts-a this author mainly observed the development of subcutaneous tumors. On the basis of this observation he suggested that "malignant transformation" with ts-a would occur more readily subcutaneously because of the lower temperature in this region. Our own observations show, however, that mainly subcutaneous tumors develop if newborn hamsters are infected with dilute (wild-type) Py viral preparations, containing 10^7 PFU/ml or less.

VI. The Shope Papilloma Virus (SPV)

Within a few days after infection SPV induces an intense mitotic stimulation in the epithelium of the skin of the domestic rabbit (Rous and Beard, 1934; McMichael *et al.*, 1963), while little if any progeny virus is produced. Although the molecular events of the infection with SPV remain unknown, we consider the possibility that they are similar to those observed during the abortive infection with SV40 or Py. SPV-induced papillomas either regress or develop within the next few months into very large masses, which generally consist of well-differentiated, diploid and noninvasive epithelial cells and thus are hyperplasias. However, 9 to 12 months or later after infection, dysplastic ("precancerous") regions develop rather frequently within the papillomas; they contain karyotypically and morphologically abnormal cells and tend to progress to invasive skin carcinomas (McMichael *et al.*, 1963). Rous and Beard (1934) inoculated cell suspensions, prepared from very small warts (1–2 weeks after infection), into various inner organs (such as the liver) of the same rabbit; they observed that the inocula, which consisted of "benign" wart cells, developed within 1–2 months into large, invasive, anaplastic and metastasizing epithelial carcinomas.

These observations suggest that SPV acts in the epithelium of the skin of the domestic rabbit as an endogenous mitogen, conferring on the abortively infected cells a high degree of mitotic autonomy, leading *in situ* (i.e., in the skin) primarily to the development of hyperplasias. 'Malignant transformation,' i.e., progression to cancer, seems to be the result of secondary processes which are preceded or paralleled by karyotypic variation and selection which occurs much more rapidly if the wart cells are allowed to grow in tissues other than the skin.

VII. A Comparison between Viral and Chemical Carcinogenesis

A short discussion of chemical carcinogenesis is included since it reveals some similarities between "malignant transformation" induced by chemical carcinogens (Berwald and Sachs, 1963; Huggins *et al.*, 1959, 1961; Arcos *et al.*, 1968; Heidelberger and Iype, 1967; Mondal and Heidelberger, 1970; Bielschowsky and Bielschowsky, 1961; Farber, 1968, 1973) and oncogenic DNA viruses.

One of the most striking early effects of chemical carcinogens in liver, mammary gland, and skin is a marked mitotic stimulation in the target tissues which can only partially be explained as a repair reaction. It begins rather early after treatment and within a few weeks leads to the development of hyperplastic nodules in liver and mammary gland and to papillomas (hyperplasias) in the skin. Carcinogen-induced stimulation of cellular DNA synthesis, mitosis, and development of squamous metaplasia can also be observed *in vitro*, among others, in rat mammary tissue grown in organ culture in the presence of 7,12-dimethylbenz[a]anthracene, a potent inducer of mammary cancer in the rat (Koyama *et al.*, 1972).

The development of hyperplasias in the animal generally requires the continued exposure of the target tissues to the chemical carcinogen and is generally accompanied by karyotypic alterations. If the carcinogen is removed, further growth stops and the hyperplasias may even regress. However, if exposure to the carcinogen is continued dysplastic ("precancerous") regions develop where the cells generally exhibit morphological, karyotypic, and functional alterations to a varying degree. Eventually a "point of no return" is reached; the mitotic activity of some cells present in the dysplastic areas has become autonomous and no longer requires the exposure to the carcinogen. Since these cells are invasive and are generally able to form metastases, they can be regarded as "cancer" cells. These observations suggest that during chemical carcinogenesis a prolonged period of mitotic stimulation, accompanied by

cytogenetic variation and selection, ("tumor progression") (Levan, 1969), sets the stage for the development of increasingly autonomous cellular variants, some of which eventually can overcome the homeostatic controls of the animal. Clinical and histopathological observations suggest that "tumor progression" may also play an important role in the development of certain malignancies in man, such as cancer of the cervix (Marchant, 1969; Koller, 1972).

VIII. Discussion and Conclusions

A. The Mitogenic Effect of SV40 and Py in Mouse and Hamster Tissue Culture Cells

Most experiments reported here were performed in confluent, primary MK cultures, which consist mainly of epitheloid cells that are irreversibly arrested in phase G_0 of the mitotic cycle. In such cultures SV40 induces an abortive infection leading to normal or abnormal (tri- or multipolar) mitosis, while no viral progeny DNA or capsid protein are synthesized. In contrast, Py induces a lytic infection, which leads to an "abortive mitosis," accompanied by the production of progeny virus followed by cell death. The sequence of known "early events" during the abortive and lytic infection is analogous (Figs. 2 and 14). The events of the abortive infection with Py of hamster embryo cells seem to be similar to those observed during the abortive infection of primary MK cultures with SV40.

Both in the abortive and lytic infection with SV40 and Py the early viral functions seem to be transcribed in the nucleus as a single, rather large transcriptional unit (virus-specific "early 19 S RNA"). From the sedimentation behavior in 99% DMSO gradients its molecular weight was estimated to be in the order of 700,000 daltons, corresponding to the transcript of about 40% of one strand of circular Py or SV40 DNA. This is rather similar to the estimates on the size of the "early region" of SV40 DNA reported by several groups (Morrow *et al.*, 1973; Khoury *et al.*, 1973; Kelly and Lewis, 1973) but is considerably below the molecular weight (900,000 daltons) reported by Weinberg *et al.* (1972) for SV40-specific 19 S RNA.

Since newer results (E. May *et al.*, unpublished) show that SV40- and Py-specific early "19 S RNA" can be isolated from polysomes, we consider it as early virus-specific mRNA. Judging from its molecular weight, 19 S mRNA may contain the information to specify one or more polypeptides with a total molecular weight of about 70,000 daltons. The close temporal and quantitative relationship between early 19 S mRNA

and virus-specific T antigen suggests that the early viral mRNA contains the information necessary for the production of T antigen (see, however, discussion in Weil and Kára, 1970). Although preparations enriched in SV40-specific T antigen contain a protein(s) with a molecular weight in the order of 60,000 to 70,000 daltons (Spira *et al.*, 1972; del Villano and Defendi, 1973), further studies seem to be necessary in order to clarify whether this protein(s) actually corresponds to (or is contained in) T antigen.

Virus-induced stimulation of overall (mainly ribosomal) cellular RNA synthesis, which leads to a 30% increase in total RNA (per cell), is the earliest presently known host cell response both in the abortive and lytic infection with SV40 and Py, respectively. It begins shortly after the appearance of T antigen but before the onset of virus-induced chromatin replication and also occurs if DNA synthesis is inhibited with FUdR (Figs. 2, 7, and 11).

The results reported here suggest that virus-induced cellular RNA synthesis may comprise the cold-sensitive metabolic process ("psychrosensitive event") that is intercalated between the appearance of T antigen and virus-induced chromatin replication (Weil and Kára, 1970; Weil *et al.*, 1967; May *et al.*, 1971, 1973).

As a working hypothesis we propose that early viral mRNA ("early 19 S RNA"), synthesized both during the lytic and abortive infection with SV40 and Py, respectively, directs the synthesis of a virus-specific mitogen (which might correspond to T antigen or an other, as yet unknown, early viral product synthesized in parallel) and that this mitogen, after reaching a critical concentration, interacts with a cellular regulatory element that controls chromatin replication and mitosis, and thus triggers the host cell automatism (cellular RNA synthesis–S phase–normal or abortive mitosis) (Fig. 14). Several lines of experimental evidence point to the possibility that at least some of the virus-induced alterations of cell membranes, such as the appearance of lectin binding sites (Pollack and Burger, 1969; Noonan *et al.*, 1973; Benjamin and Burger, 1970; Inbar and Sachs, 1970; Lis and Sharon, 1973) may be secondary events.

At present it remains unknown why the infection of mouse cells with SV40 remains abortive while the same cells are able to reproduce Py virus. Py-induced chromatin replication appears to be prerequisite for the production of progeny virus, since Py DNA replicates as a nucleohistone in close association with the mouse cell chromatin and apparently makes use of the chromosome replication machinery of the host cell for its own synthesis (Seebeck and Weil, 1973; Seebeck, 1974). Although virus-induced chromatin replication may be necessary it is, however, an insufficient condition for synthesis of viral progeny DNA,

and additional requirements have to be met. Türler observed that during the lytic infection of mouse cells covalent binding of parental Py DNA molecules ("integration") occurs only *after* the appearance of T antigen and precedes or coincides in time with the activation of the cellular DNA-synthesizing apparatus. "Integration occurs whether mouse cell cultures are normally infected or infected in the presence of FUdR (Weil *et al.*, 1973; Türler, 1974). "Integration" of parental viral DNA can also be observed during the lytic infection with SV40 (Hirai *et al.*, 1971) and with adenovirus type 2 (Burger and Doerfler, 1973). "Integration" of Py and SV40 DNA early during the lytic infection may thus be one of the additional requirements for synthesis of viral progeny DNA. It remains unknown whether "integration" corresponds to a linear insertion of the viral DNA into the chromosomal DNA or rather to some other form of covalent linkage (to chromosomal replication sites?). As a working hypothesis we consider the possibility that an early viral function (T antigen?) is involved in the activation or induction of an endonuclease with "double-hit" kinetics (Cuzin *et al.*, 1973, and Chapter 6) which would introduce breaks into the mouse chromosomal and viral DNA and thus set the stage for multiple interactions and exchanges between viral and cellular DNA. Such an enzyme might also be involved in the excision of nucleohistone fragments (Seehafer and Weil, 1973) from the mouse cell chromatin and thus be involved in the production of pseudovirions (Michel *et al.*, 1967; Türler, 1974).

B. The Relation of the Mitogenic Effect to Virus-Induced "Cell Transformation" in Vitro

The absence of well-defined and reliable morphological or physiological criteria to define "virus-transformed" cells (see Eagle *et al.*, 1970) compounded by the synonymous and frequently indiscriminate use of the terms "transformed," "neoplastic," and "malignant" has led to conceptual confusion with respect to the term virus-induced "cell transformation." The situation can be simplified by the following assumptions.

1. Py, SV40 and probably also the related papilloma viruses act primarily as endogenous mitogens.
2. In tissue culture Py and SV40 confer a mitogenic stimulus both on "normal" and "cancerous" cells. The increased potential for growth *in vitro*, i.e., the ability of (at least some) virus-transformed cells to form colonies in soft agar and to grow in the presence of low levels of serum, seem to be related to the virus-specific mitogenic effect.
3. Virus-specific mitotic stimulation is frequently accompanied by virus-induced mitotic and karyotypic abnormalities, leading to cy-

togenetic instability (Moorhead and Saksela, 1964/1965; Wolman *et al.*, 1964; Diamandopoulos *et al.*, 1969; May *et al.*, 1971; Weil *et al.*, 1973). This is particularly striking after the abortive infection with SV40, where the first wave of virus-induced mitoses already exhibits a broad spectrum of abnormalities that are similar to those induced by X rays (Penso *et al.*, 1968) and by chemical carcinogens (Arcos *et al.*, 1968). The combination of virus-specific mitotic stimulation and virus-induced cytogenetic instability converts diploid primary cultures and cell strains within a few weeks into aneuploid cell lines with an unlimited potential for growth *in vitro*.

4. The virus-induced mitotic stimulus may already be lost after the first virus-induced mitosis; in primary MK cultures abortively infected with SV40, this loss seems to be linked to the (irreversible) switch-off of synthesis of early SV40-specific 19 S RNA soon after the onset of virus-induced chromatin replication (May *et al.*, 1973).

5. Several lines of experimental evidence suggest that virus-induced mitogenic stimulation does *not* require the "integration" of viral DNA; the assumption that the mitogenic effect is expressed without "integration" may explain the phenomenon of *"abortive transformation"* (Oxman, 1967; Stoker and Dulbecco, 1969). The "transformed phenotype" (operationally defined by the ability of abortively infected cells to synthesize virus-specific RNA and T antigen and to form colonies in agar or in the presence of low levels of serum) is lost after a small number of virus-induced mitoses, and the cells revert to a "normal" growth pattern. The results obtained by P. Berg (personal communication) suggest that, in the instances tested, the reversion was accompanied by the loss of (SV40) viral DNA from the abortively transformed cells.

6. As a relatively rare event, the "transformed phenotype" is maintained indefinitely, a situation generally referred to as "stable transformation." It seems to depend on the continuous presence of "integrated" functional viral DNA, which is carried, as a heritable property, from one cell generation to the next. At least in some SV40-transformed cell lines the viral genome can be rescued, even years after the original abortive infection, by fusion with permissive (e.g., monkey) cells that apparently contain a factor(s) necessary for the synthesis of viral progeny DNA (Wever *et al.*, 1970; Croce and Koprowski, 1973). We tentatively assume that "stable transformation" is the outcome of an aborted lytic infection that is blocked at some stage after "integration" and before the replication of viral progeny DNA. According to this view the "transformed phenotype" is the result of the continued expression of an early viral gene(s) directing the synthesis of early 19 S messenger RNA,

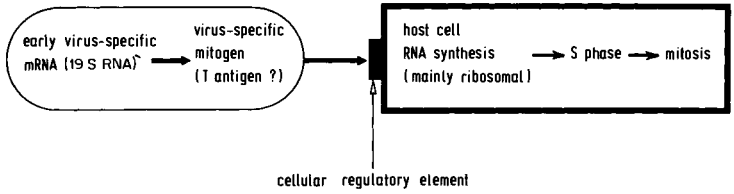


FIG. 14. Scheme of the mitogenic action of SV40 and polyoma virus.

which in its turn contains the information for the production of the "early" viral polypeptide(s). Virus-induced "cell transformation" would then be the complex result of a combination of prolonged virus-induced mitotic stimulation and cytogenetic variation and selection; obviously, its final outcome will be influenced by the procedures used to select for "transformed cells" (Stanners, 1963; Stanners *et al.*, 1963; Eagle *et al.*, 1970) and whether viral DNA is maintained or lost.

7. Reversion of the "transformed phenotype" can be the result of the loss of "integrated" viral DNA from the transformed cells, e.g., by the elimination of chromosomes (Weiss, 1970; Marin and Macpherson, 1969). Rather frequently Py- and SV40-transformed cells revert to a "normal" growth pattern (Pollack and Burger, 1969; Rabinowitz and Sachs, 1970; Hitotsumachi *et al.*, 1971) but maintain functional, "integrated" viral DNA (which can be "rescued") and continue to synthesize virus-specific RNA and T antigen. The "flat revertants" described by Pollack belong into this group (Pollack *et al.*, 1970). At least in some instances this type of "reversion" seems to be the result of cellular changes (Renger and Basilico, 1972) that apparently render the cells unable to respond to the virus-specific stimulus (Fig. 14).

8. The presence of "integrated" Py or SV40 viral DNA seems not required to maintain 'malignancy' (i.e., transplantability) once it has been established.

C. The Relation of the Mitogenic Effect of Py and Related Viruses to Tumor Formation in Vivo

In animals, Py and related viruses also seem to act primarily as endogenous mitogens, leading to the development of rapidly growing hyperplasias ("benign tumors"). The process involved in the transition of the hyperplasias into true cancer (which may, although need not, take place) remains unknown; to some extent it is reminiscent of "tumor progression" observed during chemical or spontaneous carcinogenesis.

We presently feel that "malignant transformation" induced by Py and related viruses may represent a special case of the more general phenomenon of carcinogenesis on the basis of cytogenetic variation and selection (Levan, 1969).

D. The Potential Interest for Human Medicine of the Experimental Observations with Py and Related Viruses

Many experimental studies on cancer in man are based on the assumption that integrated viral or derepressed cellular "cancer genes" are responsible both for development and maintenance of malignancy.

So far, the search for viral imprints in human cancer has either yielded negative or inconclusive results (Gallo *et al.*, 1970; Bowen *et al.*, 1973), with the exception of Burkitt's lymphoma and the nasopharynx carcinoma. In the latter case, evidence for the continued presence of EBV (Epstein-Barr virus) DNA in Burkitt's lymphoma cells was obtained (zur Hausen, 1972). However, the majority of the cell-associated viral DNA molecules are not "integrated" but seem to replicate, in an episomelike fashion, in parallel with the host cell chromatin (Nonoyama and Pagano, 1972, 1973). Newer results suggest that some viral DNA molecules may be linked to chromosomal DNA by alkali-labile bonds (Adams *et al.*, 1973).

The experimental observations with Py and SV40 make it appear likely that the continued presence of viral genes is not required to maintain malignancy, once it has been established. We should therefore also consider the possibility that in man viruses might induce malignancies by a "hit and run" effect (Fig. 15). Localized chronic infections (e.g. with herpesviruses) (Marchant, 1969); Royston and Aurelian, 1970) could lead, by a combination of prolonged virus-induced mitotic stimulation and karyotypic variation (Koller, 1972), to localized dysplasia ("precancerosis") that would either regress or, possibly years later, develop into cancer by "tumor progression." If all viral imprints would be lost (e.g., by elimination of chromosomes) the resulting virus-induced cancers would be indistinguishable from malignancies arising either spontaneously or as a result of chemical or physical carcinogens.

The assumption that "malignant transformation" by DNA viruses is a secondary phenomenon may also be relevant for the discussion of the relation between EBV, infectious mononucleosis (Stites and Leikola, 1971; zur Hausen, 1972; Henle and Henle, 1972, 1973), and Burkitt's lymphoma. Several lines of evidence strongly suggest that EBV plays an etiological role in the development of infectious mononucleosis. This (generally) benign, febrile disease is characterized by a very marked

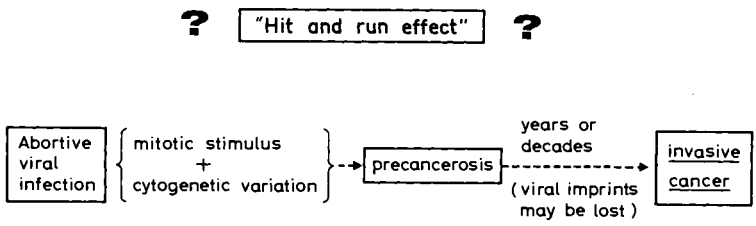


FIG. 15. "Hit and run effect."

increase in atypical lymphocytes in the peripheral blood, lymph nodes, spleen, and bone marrow and can be regarded as a reversible hyperplasia of the lymphatic system. Since EBV was shown to induce cellular DNA synthesis in human leukocytes (Gerber and Hoyer, 1971), we assume (as a working hypothesis) that the abortive infection with EBV confers a mitotic stimulus *in vivo* on (immature ?) lymphocytes, which results in a marked hyperplasia of the lymphatic system. The presence of virus-specific proteins in the abortively infected lymphocytes subsequently provokes an immunological reaction that leads to the destruction of the lymphocytes. Under certain conditions, however (genetic factors, malaria ?), the virus-induced mitotic stimulus, possibly accompanied by a process of cytogenetic variation and selection, might lead to the development of cell clones able to break through the homeostatic controls and then develop into malignant lymphomas.

If the cancer problem is examined from a broader point of view than that offered by virology and molecular biology alone, one cannot avoid the impression that "cancer" may not be a disease *sui generis* but rather a syndrome, i.e., the phenotypic expression of various disturbances of the regulatory systems involved in mitosis, differentiation, and homeostasis.

Since the virological and molecular approaches to malignancy are still in an early stage, it would be presumptuous to make predictions on the significance of viruses in the etiology of cancer in man. Though it appears likely that viruses may also play a role in man in the development of at least certain types of cancer, the assumption that all malignancies in man are simply the phenotypic expression of integrated viral "cancer genes" or of activated "oncogenes" (Huebner and Todaro, 1969) seems, at present, to be overoptimistic.

On the basis of the considerations discussed in this chapter (and in more detail in a forthcoming review; Weil, 1974) we feel that the long-range potential of Py and related viruses for biological research and medicine resides in their usefulness as tools to study regulation of mi-

tosis, transcription, and processing of mammalian mRNA and possibly as model systems to gain insight into the mechanisms involved in embryonic determination and differentiation.

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CHAPTER 17

Immunological Patterns of Virus-Transformed Neoplastic Cells

GEORGE KLEIN

Two kinds of virally determined functions can be identified in virally transformed cells by immunological methods. One type of antigen is connected with the productive viral cycle, but not *necessarily* with the transformed cells. This can be illustrated by the gs antigens in C type virus systems. They may, but do not have to be, present in the neoplastic cell. This facultative presence suggests that (a) the viral function reflected by the antigen is *not* necessary for neoplastic growth and (b) it *is* compatible with continued cell proliferation. The latter follows from the fact that C type viruses do not kill the cell that makes them. The situation is quite different with all the known DNA viruses. Oncogenic papova-, adeno-, and herpesviruses damage their host cells irreversibly as soon as they enter the productive cycle and ultimately kill them. For a lymphotropic, transforming herpesvirus, EBV (Epstein-Barr virus), it has been shown that the appearance of the virally determined early antigen complex (EA) already signals an irreversible shutdown of host cell macromolecular synthesis (Gergely *et al.*, 1971). It is a logical consequence of this fact that the proliferating, EBV-transformed lymphoblastoid cell lines either do not make any viral anitgens at all ("nonproducer lines"), or, if they do, they make it by throwing off abortive "side lines" that enter the productive cycle and are thereby

automatically eliminated from the mainstream of cell proliferation. In these and other DNA virus-transformed producer lines, virally determined antigens can be found that are *not* compatible with continued cell proliferation. These antigens may or may not be included eventually in the viral particle.

The second large category of viral or virally determined products is regularly associated with the virally transformed cells and is compatible with continued cell multiplication. There are two main types: intranuclear antigens and membrane antigens. The following discussion will focus on these antigens, with particular emphasis on some systems within our personal experience.

I. Intranuclear Antigens

The best known examples are the T antigens of the polyoma-, SV40-, and adenovirus-transformed cells. While the chemical nature of the T antigens is still not well defined, it is clear that they are nuclear sap antigens that enter the cytoplasm on division, are not included in the virion, and appear as a very early function during the viral cycle (Weil *et al.*, Chapter 16). Each virus induces T antigens of a different specificity, but the same in all target cells, irrespective of species. Also, SV40 is known to induce the same T antigen in monkey cells during the lytic cycle, and in nonpermissively transformed mouse, hamster, or human cells. Although there is no definite proof, it is therefore most reasonable to assume that the specificity of the T antigen is coded by the viral, and not the cellular, genome.

So far, it has not been possible to dissociate T antigens from the neoplastic transformation. Although it has been claimed that T antigen-negative SV40-transformed lines exist (Butel *et al.*, 1972), later work showed that these lines lacked every known SV40-specific function (Ting and Herberman, 1971), including viral mRNA and DNA (Levine *et al.*, 1970). Their relation to SV40 is therefore strongly in doubt.

While it is thus possible and even likely that the virally induced T antigen reflects a *necessary* transformation function, it does not follow that it would be also sufficient. Numerous revertants have been isolated from transformed lines. They resemble nontransformed cells *in vitro* and/or *in vivo*, in spite of the fact that they continue to make T antigen (MacPherson, 1970). Reversion was invariably associated with considerable changes in chromosomal constitution. It has been suggested (Hitotsumachi *et al.*, 1971; Yamamoto *et al.*, 1973) that the balance of chro-

mosomes influencing the expression of the transformed state in a positive or in a negative direction is of crucial importance in promoting or suppressing the neoplastic potential determined by the viral genome. The influence of both viral and cellular functions on the expression of the neoplastic state has been strikingly documented by recent studies on temperature-sensitive mutants. On the one hand, *viral* ts mutants could be isolated that were temperature sensitive for the transformation function (Sambrook, 1972). It is also possible, however, to isolate temperature-sensitive *cellular* mutants (Renger and Basilico, 1972). In the latter case, the ts function responsible for the control of the transformed phenotype was coded by the cellular, and not the viral, genome. The latter point was conclusively proved by the isolation of wild-type virus from the temperature-sensitive transformants.

An essentially analogous picture was obtained by a quite different approach, somatic cell hybridization (Klein *et al.*, 1971; Klein and Harris, 1972; Wiener *et al.*, 1971). Fusion of polyoma-transformed cells with normal cells suppressed malignant behavior (Klein *et al.*, 1971; Wiener *et al.*, 1971), whereas the expression of the polyoma-specified T and transplantation antigen was maintained (Klein and Harris, 1972; Meyer *et al.*, 1974). Fully malignant behavior reappeared after a number of chromosomes, derived from the normal parent, had been lost. This supports the idea of chromosomally determined suppressor elements in normal cells.

Recently, we have discovered yet another type of nuclear antigen (Reedman and Klein, 1973). Epstein-Barr virus-transformed lymphoblastoid lines that do not produce virus (designated as nonproducers) do not contain any of the "classical" EBV antigens, detected by direct or indirect fluorescence and designated as MA (membrane antigen), EA (early antigen), and VCA (viral capsid antigen), respectively (Klein, 1973). They were known to contain a soluble, EBV-associated antigen, detected by complement fixation, however. It may be recalled, in this connection, that T antigens have been originally detected by complement fixation (Black *et al.*, 1963). The EBV-determined CF antigen was not demonstrable by direct or indirect fluorescence, presumably for quantitative reasons. Anticomplement fluorescence is known to amplify fluorescent reactions, however (Goldwasser and Shepard, 1958; Hinuma *et al.*, 1962). When applied to the EBV system, it revealed the existence of an unusually brilliant nuclear antigen, present in all human or simian lymphoblastoid lines that carried the EBV genome and absent from lines that lacked the genome (Reedman and Klein, 1973). The antigen has been designated EBNA (EBV-determined nuclear antigen). EBNA is thus, at least superficially, analogous to the T antigens induced by the

small oncogenic DNA viruses. It differs, however, in one, very interesting respect. Whereas T antigens are localized in the nuclear sap and enter the cytoplasm on mitosis, EBNA is entirely confined to the chromosomes. It represents, in other words, a virally determined or virally changed chromosomal protein.

We have recently studied the behavior of EBNA in somatic cell hybrids derived from the fusion of an EBV-carrying human lymphoblastoid cell and the A9 mouse fibroblast line. In a series of independently derived hybrids (Allderdice *et al.*, 1973) the frequency of EBNA-positive nuclei was proportional to the average number of EBV genome equivalents, detected by cRNA–DNA hybridization (Klein *et al.*, 1974). As human chromosomes were lost during continuous propagation *in vitro*, the frequency of EBNA-positive nuclei diminished and finally disappeared. As long as EBNA was present, it could be demonstrated in all chromosomes, mouse and human, within the positive metaphase plates, whereas negative plates were completely negative. This suggests that EBNA can attach to chromosomes, irrespective of species, as long as those human chromosomes are present that carry EBV DNA.

The function of EBNA is unknown. It is tempting to speculate that it may be related to the control of viral transcription. Suppression of viral transcription and/or translation is an essential prerequisite for a nonproductive, proliferative, and therefore potentially oncogenic association (Klein, 1973). Alternatively, EBNA may play a role in stabilizing the complex structure of 50–100 viral genomes, 10^8 MW each, known to be linearly associated with the cellular DNA through a protease-resistant but alkali-labile linkage (Adams *et al.*, 1973). It will be obviously very important to learn about the chemical nature of EBNA, and particularly about the question of whether it has any specific binding affinity for DNA.

There is no information about the question of whether EBNA is essential for the EBV-induced transformation of the human lymphoblastoid cell. However, the regular association of the antigen with all EBV-transformed lines so far investigated suggests that this is probably the case. It is particularly interesting, in this regard, that simian lymphoblastoid lines, transformed by herpesvirus saimiri, do not contain EBNA, whereas EBV-transformed simian lines do. Tumor biopsy cells of African Burkitt's lymphoma patients regularly contain EBV DNA and also EBNA, whereas cells of other lymphomas or leukemias that occur in EBV-seropositive patients contain neither EBV DNA nor EBNA (zur Hausen *et al.*, 1970; Klein *et al.*, 1973b; Reedman and Klein, 1973).

These studies confirm and amplify the conclusion, drawn from the study of experimental DNA virus-induced tumors, that intranuclear, virally determined antigens represent important, regular and readily recognizable "footprints" of the viral genome in the transformed cell. One may therefore ask the question whether disease associated nuclear antigens can be found in tumors of unknown origin, including human tumors, and whether such findings can help in tracing new tumor-associated viruses.

We have recently encountered a previously unknown nuclear antigen that may belong to this category, in the course of an unsuccessful search for EBNA in various leukemias (Klein *et al.*, 1974). EBNA could not be found in CLL, ALL, AML, and CML cells. The serum of the leukemic cell donor patient was regularly included in the anticomplement fluorescence tests. No positive reactions were seen in any of the CLL or CML specimens with EBNA-positive reference sera or the autochthonous patient serum. Too few ALL cases have been tested to allow a safe judgment. In certain AML specimens, a brilliant, coarsely granular nuclear antigen was detected with the autochthonous or allogeneic AML sera, however. We have tentatively designated the antigen as LANA (leukemia-associated nuclear antigen). LANA is mainly associated with the AML myeloblasts. The frequency of LANA-positive cells is closely related to the blast count. When CML cases have entered acute blast crisis, LANA appeared, whereas in cases with the opposite development, from AML to a more differentiated, myelocyte type, LANA disappeared from the peripheral blood although serum antibodies directed against it persisted. LANA was not found in CLL or CML cells, in normal peripheral white cells, or in infectious mononucleosis. Surprisingly, it was detected in two African lymphoma biopsies that did not contain EBV DNA and/or EBNA, unlike the usual type of African Burkitt's lymphoma.

AML patients' sera contained antibodies to LANA in 76% of the cases, whereas ALL, CML, and CLL sera were positive in 27, 20, and 10% of the cases, respectively. BL sera were positive in 17%. None of 61 normal, healthy donors had antibodies to LANA, although two of them contained nonspecifically reacting antinuclear antibody.

Conceivably, LANA could be a myeloblast-associated, cell type specific antigen. Preliminary evidence indicates, however, that LANA also occurs in solid lymphomas and in PHA transformed normal lymphocytes. If confirmed, this would suggest that it may be a nuclear component, specifically associated with or preferentially expressed in blast cells of both the lymphoid and the myeloid series.

II. Virus-Induced Membrane Antigen Changes

One of the earliest, virally induced common denominator of virus transformed cells was TSTA, the tumor-specific transplantation antigen (for review, see Klein, 1968). Habel (1971) and Sjögren *et al.* (1961) reported simultaneously that polyoma-induced mouse tumors carried a common antigen, not present on the polyoma virus particle, that could induce a rejection reaction in syngeneic mice. The antigen was shared by all polyoma-induced tumors but not by tumors induced by other viruses. The question whether this transplantation antigen is identical with the surface (S) antigens that can be demonstrated by humoral antibody reactions, such as membrane immunofluorescence, radioiodinated antibody binding, or *in vitro* cytotoxicity, has still not been settled (cf. Butel *et al.*, 1972). In the light of later developments that showed the presence of the transplantation antigen on virally infected normal or abortively transformed cells, the designation TATA (tumor-associated transplantation antigen) appears preferable to TSTA.

Numerous TATA-type antigens have been identified in other virus-induced tumors, including both RNA and DNA virus systems (Deichman, 1969; Pasternak, 1969). In the nonbudding papova- and adenovirus systems TATA is not present on the virus particle. In the enveloped, C type virus systems this is not equally clear, although there are reports suggesting specific virally induced cell membrane alterations, not shared with viral envelope antigens, in the avian (Kurth and Bauer, 1972) and feline (Essex, 1974) C type virus systems.

Several questions can be raised in relation to the virally induced transplantation antigens. The following appear particularly important: Do all virus-induced tumors express new transplantation antigens? Does the transplantation antigen reflect a membrane alteration directly involved in the neoplastic change? What is the role of the virally induced membrane change in relation to the immune surveillance mechanism?

Concerning the first question, it is widely believed that *all* virus-induced tumors have new membrane antigens, which are common for each etiological group. Very recently it has been shown, however, that some transformed cells do not have any detectable membrane changes either by rejection reactions or by humoral antibody tests, in spite of the fact that they carry the genome of the transforming virus (Stephenson and Aaronson, 1972; Strouk *et al.*, 1972). This may depend, however, on the inability of the host to recognize a virally induced membrane change as a foreign antigen, rather than on the absence of such a change.

This dilemma will be discussed below, since it is closely related to the question of immune surveillance.

In the tumors where a membrane change can be demonstrated by immunological methods, at least some approaches are open toward the second question. Small oncogenic DNA viruses like polyoma may serve as an interesting model, since their genome is so small that the chance for a meaningful association between a regular, virally determined cellular change and the change responsible for the neoplastic transformation is relatively great.

Support for an intimate association between the antigenic change and the neoplastic transformation was gained when it was found (Sjögren, 1964) that polyoma tumors maintain their specific TATA in spite of prolonged negative selection against antigenic cells by passage in preimmunized mice.

The concept that the polyoma-induced TATA reflects an essential membrane change for neoplastic behavior is attractive for several reasons. It is widely believed that membrane receptors are profoundly involved in growth control. Cell contact-dependent signals involved in the repression and derepression of DNA synthesis are probably triggered by membrane receptors. Membrane receptors are also involved in transmitting most hormonal signals that stimulate or restrict cell proliferation. A membrane change that is regularly associated with a certain kind of virally induced neoplastic transformation is therefore a reasonable candidate for the role of *the* key change that has put the normal receptor system out of function. Damage to the relevant receptor may unclutch a free-wheeling cycle of DNA synthesis.

This picture is also compatible with the strategy of the virus. The polyoma genome can only code for a maximum of 7–10 proteins. At least 3 or 4 are needed for the production of the virus particle, which is *not* made in the transformed cell. These functions are therefore either repressed or missing (if the transformed cell carries a defective virus). Only a few functions remain that can change the cell. The T antigen is one of them, and the membrane-localized TATA is another. Either of these could be regarded as a reasonable candidate for the transforming function, in view of their regular association with all polyoma-transformed cells. The membrane antigen might be favored on the basis of the postulated importance of membrane functions in growth control.

What is the status of TATA in revertants isolated from polyoma-transformed cells or in hybrids between polyoma-induced tumors and normal cells that show a suppression of malignant behavior? There is more or less indirect evidence that TATA is maintained in both situa-

tions (Butel *et al.*, 1972; Klein and Harris, 1972; MacPherson, 1970; Meyer *et al.*, 1974) in spite of considerable chromosomal changes in the revertants, and the presence of normal cell-derived chromosomes in the hybrids.

A consideration of the situation in the somatic hybrids is particularly pertinent. The ability of normal cells or of established, low malignant cell lines to suppress the neoplastic behavior of highly malignant tumor cells upon somatic hybridization has been demonstrated for chemically induced tumors, DNA (polyoma) virus-induced tumors, RNA (Moloney) virus-induced lymphomas, and "spontaneous" tumors (Klein *et al.*, 1971; Wiener *et al.*, 1971, 1973). It is thus a fairly general phenomenon. Superficially at least, it is analogous to the "extinction" of differentiated characteristics that occur in most (but not all) combinations where highly differentiated cells are fused with cells that lack the corresponding differentiation traits. It is true for both differentiation and malignancy, furthermore, that extinction in the complete or nearly complete somatic cell hybrids is followed by "reexpression" of the extinguished trait, after a sufficient number of partner cell-derived chromosomes had been lost. In Ephrussi's terminology, the "epigenotype" of the differentiated and, by analogy, of the malignant cell is maintained during the period of phenotypic extinction.

In the polyoma–normal cell somatic hybrid, the full maintenance of the T and the TATA antigens can be interpreted to mean that these traits are not strictly linked to the malignant phenotype, or, to put it differently, that the presence of these antigens does not make a cell automatically malignant. A possible explanation is that the normal genotype introduces normally responding growth control receptors. It could still be true that TATA represents a strategically changed receptor in the polyoma tumor cell, but in the presence of other, normally responding receptors, this would not be expressed more than recessive lethals in a heterozygote. A similar argument was used to explain the behavior of revertants (Yamamoto *et al.*, 1973).

Membrane antigen changes are important not only as footprints of viral genomes, potentially helpful in etiological searches, or as clues for the understanding of malignant behavior at the cell level, but also as instruments for the understanding and practical exploration of immune surveillance.

First, it has to be emphasized that the same membrane change, induced by an oncogenic virus, may be "regarded" as highly foreign by the immune system of one species, while remaining virtually unrecognized in another. This can be illustrated by the interaction of *Herpesvirus saimiri* with different host cells.

It is known (Deinhardt, 1973) that this virus is completely harmless in its natural host, the squirrel monkey, but induces 100% fatal malignant lymphoproliferative disease in two experimental hosts, the marmoset and the owl monkey. As far as it is known, it enters an essentially similar virus–cell relationship in all three species, but the immune response of the squirrel monkey is more prompt and regular (Klein *et al.*, 1973). It seems that the natural host has been selected for a high, genetically determined immune responsiveness that can nip potentially neoplastic cells in the bud. The experimental hosts are, on the other hand, so poor in recognizing the antigenicity of the transformed cells that even vaccination attempts have been unsuccessful so far (Ablashi *et al.*, 1973). The same situation seems to apply to another lymphotropic herpesvirus, Marek's disease virus (MDV) in chickens (Nazerian, 1973). Genetically resistant birds develop relatively benign, self-limiting disease, whereas susceptible chicks die regularly with malignant disease. The resistance of the former can be abolished by neonatal thymectomy, suggesting that the genetic mechanism operates through the immune system or, more specifically, its T cell-dependent compartments. Susceptible birds can be vaccinated with an apathogenic, antigenically cross-reactive turkey herpesvirus (HVT) (Hilleman, Chapter 19; Nazerian, 1973). Vaccination prevents malignant disease in the majority of the infected birds, but does not reduce virus shedding from the feather follicle. The natural chicken host can thus prevent the outgrowth of the virally transformed neoplastic cell. If it is a genetically low responder, its immune system has to be alerted by vaccination in time to do so. In high responders, this is not necessary since they can mount an efficient response without intervention.

Recent advances in the study of genetic mechanisms that influence immune responsiveness against certain, defined antigens has shown the existence of specific determinants (*Ir* genes) that exert the most profound influence on the ability of the host to respond (e.g., Benacerraf *et al.*, 1971; McDevitt *et al.*, 1972). Some *Ir* genes act at the T, others at the B cell level. It is likely that all immune responses are under the influence of specific *Ir* genes. It is reasonable to assume that all natural species, which harbor potentially oncogenic viruses in their normal habitat, have been selected for high responsiveness against the membrane changes induced by these viruses on potentially neoplastic, transformed cells. Polyoma is one of the best examples. The virus-induced TATA is a powerful immunogen in adult mice (Sjögren *et al.*, 1961). Inoculation of mice more than a few days old does not induce tumors; on the contrary, it leads to effective resistance against the implantation of established, syngeneic polyoma-induced tumor cells. In nature, the virus is not on-

cogenic, because most adults have antibodies (Huebner, 1963). Suckling mothers transmit antibody to their young, providing protection during the critical neonatal period. Later, active infection does not lead to any tumors under normal circumstances, as already mentioned. In immunosuppressed adults, however, virus infection can induce tumors (Gaugas *et al.*, 1973; Law, 1970). Something similar may happen in immunosuppressed human recipients of kidney transplants (Penn and Starzl, 1972). It follows that such patients may be the most interesting source of human viruses with an oncogenic potential.

Another naturally occurring virus with a high oncogenic potential is the Gross leukemia virus. In most animals, it does not cause leukemia under normal circumstances. In some strains it can do so at old age or after X irradiation. The laboratory strains from which it had been isolated carry a special *H-2* linked susceptibility gene (Lilly and Pincus, 1973), localized in the same (*H-2.K*) region as an important *Ir* gene and possibly identical with it. This susceptibility gene, designated *Rgv-1*, has been derived from the AKR strain, specifically bred for high leukemia incidence by continuous inbreeding and selection.

The opposite case can be made for the $H\text{-}2^b$ linked resistance factor that protects mice against the oncogenic effect of the mammary tumor virus (MTV) (Mühlbock and Dux, 1971). This gene has been derived from the C57Bl strain, selected for a low incidence of mammary carcinoma.

It thus appears that specific, genetically determined responsiveness has evolved to deal with common events of potential neoplastic transformation, occurring upon the exposure of the natural host to ubiquitous and potentially oncogenic viruses. This does not necessarily mean, however, that surveillance can deal with *any* membrane change associated with a neoplastic cell. The complete lack of protection of the marmoset and the owl monkey against *Herpesvirus saimiri*-induced lymphoma has been already mentioned. Even more relevant are the chemically induced tumors that show no demonstrable antigenicity in the ordinary immunization–rejection type of test (Baldwin, 1973). Although the versatility of the immune system and its ability to respond to new antigenic configurations that it could not have encountered during evolution is notorious, it obviously has its limits. Since tumors induced by chemical carcinogens have individually distinct antigens that include, in all probability, a vast variety of potential new specificities, it is not surprising that an unprepared immune system cannot deal with all of them.

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CHAPTER 18

The Immune Response to Oncornavirus Infections

MYRON ESSEX

I. Introduction

Of the many oncogenic viruses that have been described in recent years, virtually all fall into one of three general classification groups: the enveloped double-stranded DNA herpesviruses, the enveloped single-stranded RNA oncornaviruses or leukoviruses, and the naked double-stranded DNA papova- and adenovirus groups. All either have DNA in the virion or, in the case of the oncornaviruses, undergo a stage of reverse transcription to generate DNA copies while infecting the cell. This provides a logical baseline for the premise that a virus, to be oncogenic, must be able to interact with the DNA of the cell in a potentially stable fashion.

Although it may be true that a virus must possess and/or generate DNA to be oncogenic, this characteristic alone does not instill such properties on the virus. A comparison of the relative similarity of nucleic acid base ratios between the most oncogenic adenoviruses (types 12, 18, 31) and mammalian cells with the corresponding lack of such a relationship for otherwise similar but nononcogenic adenoviruses shows that a particular type of DNA is obviously necessary for virus–cell interaction at this level (Green and Lacy, 1965).

Most viruses in the herpes and papova–adeno groups are cytopathic rather than oncogenic under normal circumstances. Only certain representatives of these groups appear to have oncogenic capabilities at all, and even these more frequently involve the usual cytopathic response. Additionally, only those cells that are infected in a cytocidal manner produce progeny virus, while transformed cells produce no virus. The oncornaviruses differ strikingly from herpes and papova–adeno groups in being noncytopathic, but rather existing in most cases in a noncytocidal steady-state association with the virus-producing cell.

Of the many RNA tumor viruses thus far described, all fit the rather rigid criteria of oncornavirus classification (Nowinski *et al.*, 1970; Melnick, 1971); they are enveloped, bud from the cell membrane, have a size range of 80–120 nm, and contain reverse transcriptase and a high molecular weight (70 S) RNA. As with the other groups, however, many oncornaviruses appear to be nononcogenic.

Table I lists some of the most important representatives of the virus groups listed above. Although most of these agents have been shown to be tumorigenic under certain conditions in a given strain or species of animal, and/or capable of transforming certain cells in culture, most have not been found to be oncogenic in the outbred free-living species that might harbor the agent in nature. In the case of the papovavirus SV40, efficient transformation occurs in cultured cells from several species and tumors can be regularly induced in baby hamsters. The virus apparently is only found under natural conditions in monkeys, however. A somewhat similar situation occurs with *Herpes saimiri* in squirrel monkeys, *Herpes ateles* in spider monkeys, and polyoma virus in wild mice. Each is apparently nonocogenic for the respective species of isolation, but very oncogenic for other species (marmoset and other primates for *Herpes saimiri* and *H. ateles*, and inbred mice and hamsters for polyoma). Rather than representing a purely chance situation it appears likely that this reflects selection pressure to develop resistance within the species naturally harboring the virus. Considerable evidence suggests that in the case of *Herpes saimiri* and mouse polyoma this

TABLE I

Oncogenic Viruses

Virus group	Representative viruses	Oncogenicity	
		Natural host	Experimental hosts
Papova–adeno (naked, DNA)	Mouse polyoma,	− (feral mouse)	+ (hamster, mouse)
	SV40,	− (monkey)	+ (hamster)
	Adeno 12, 18, 31	− (man)	+ (hamster)
Herpes (enveloped, DNA)	Marek's disease virus,	+ (chicken)	+ (chicken)
	Herpes saimiri,	− (squirrel monkey)	+ (marmoset, others)
	Herpes ateles,	− (spider monkey)	+ (marmoset, others)
	Epstein-Barr virus	? (man)	−
Oncorna (enveloped, RNA)	AvLV	+ (chicken)	−
	AvSV	+ (chicken)	+ (rat, mouse)
	FeLV	+ (cat)	+ (dog)
	FeSV	+ (cat)	+ (many sp.)[a]
	MuLV	+ (laboratory mouse)	+ (rat)
	MuSV	+ (laboratory mouse)	+ (rat, hamster)

[a] Rat, rabbit, dog, pig, sheep, and monkey.

selection pressure resulted in successful surveillance of tumor development by the immune response (Klein, 1973). It thus follows that as virologists and cancer biologists we should avoid categorization of many of these agents as being of significance only to the molecular biologist.

In the case of oncornaviruses, the early experiments resulted in discoveries of truly oncogenic agents (Ellerman and Bang, 1908; Rous, 1910; Gross, 1951; Graffi *et al.*, 1955; Friend, 1957; Moloney, 1960; Rauscher, 1962; Jarrett *et al.*, 1964; Snyder and Theilen, 1969). This usually occurred for obvious reasons; the host candidates were animals with tumors or inbred strains of mice selected on the basis of a high degree of malignancy expression. It eventually became apparent, after the advent of electron microscopy, group-specific antigen serology, and various cell culture "rescue" techniques, that oncornavirus strains fit a spectrum ranging from those such as the Snyder-Theilen feline sarcoma virus (FeSV), the various strains of Rous sarcoma (RSV), and Rauscher and Friend murine leukemia viruses (MuLV) on the most oncogenic side to many endogenous viruses and "C type particles" that were apparently nononcogenic, at least in the classical sense. Recent studies by advocates of the oncogene hypothesis of Huebner and Todaro (1969) have

elegantly demonstrated the presence of endogenous oncornaviruses from strains of mice with low incidences of cancer and from cell cultures derived from normal mouse and chicken embryos (Todaro and Huebner, 1972; Huebner and Gilden, 1972). With few exceptions however (Armstrong *et al.*, 1973) these agents appear to have little or no oncogenic activity. Many may thus represent laboratory strains created largely by mouse geneticists or commercial poultry breeders who selected animal strains on the obvious basis of health and reproductive vigor within the limits of the inbreeding framework. As we might expect that continued association of such viruses with increasingly similar individuals in a given host species could lead to a loss of oncogenicity, such an association might also lead to a decreasing dependency for horizontal virus spread. This line of reasoning suggests that vertical virus transmission would follow the degree of inbreeding, eventually resulting in stable genetic transmission of virus genomes in inbred strains. Along with the development of decreasingly oncogenic viruses, these hosts apparently also responded by developing genetic resistance to infection by various groups of oncornaviruses. This was probably mediated by the loss of cell surface attachment sites and can be illustrated by the classification of chicken cells according to their infectability by subgroup A, B, and C viruses (Vogt, 1970; Crittenden and Briles, 1971). In combination with the herpes and polyoma situations described above, this illustrates the two major categories for satisfactory perpetuation of a host–parasite relationship: (a) the selection of a more resistant host and (b) the selection of a less pathogenic parasite.

With the assumption that chromosomal virus transmission occurs in some instances, thought patterns geared to classical virus "infection" followed by an immune response to the foreign agent must obviously be altered. Under such conditions the host should be immunologically unresponsive to virus-coded antigens, or at least all such antigens that are expressed during embryogenesis. Although earlier studies based on less sensitive serologic procedures supported an absolute tolerance (Huebner and Todaro, 1969), most recent studies do not (Oldstone *et al.*, 1972; Olsen and Yohn, 1972; Weber and Yohn, 1972; Dougherty, 1973). In addition, studies with the feline agents—the only group of proven oncogenic oncornaviruses thus far isolated from and associated with spontaneous tumors of an outbred mammalian species—have yielded results that do not deviate significantly from patterns of transmission seen with oncogenic herpes- and papovaviruses and most pathogenic nononcogenic viruses of all classification groups (Jarrett, 1972; Essex *et al.*, 1973a).

Oncornaviruses or oncornavirus-like particles have been observed in other species or cell cultures derived from them. These include man (Dmochowski *et al.*, 1965; Priori *et al.*, 1971; McAllister *et al.*, 1972), monkeys (Theilen *et al.*, 1971; Wolfe *et al.*, 1971; Kalter *et al.*, 1973), cattle (Miller *et al.*, 1969; Kawakami *et al.*, 1970; Stock and Ferrer, 1972), swine (Howard *et al.*, 1968), guinea pigs (Nadel *et al.*, 1967; Hsiung, 1972) rats (Weinstein and Moloney, 1965; Klement *et al.*, 1971), hamsters (Stenback *et al.*, 1968; Kelloff *et al.*, 1970), and snakes (Zeigel and Clark, 1969). Since most of these have not yet been shown to be oncogenic and the true origins of some are unclear, they will not be discussed.

The immune response to both the virus and the new cell surface or turmor-specific antigens associated with oncornavirus infection will be considered. The issue of whether certain of the tumor cell surface antigens may be the same as embryonic antigens will not be considered. The evidence that these antigens are specifically induced by the viruses is substantial. It is recognized that this induction could be the result of derepression of cell genes. If so, it is a virus-specific derepression, and a derepression of an embryonic function that is common to animals of different species and even classes.

In this chapter considerable emphasis will be given to the feline oncornaviruses, both because the author has studied them and because they represent an instance of horizontal infection of outbred animals under natural conditions. Endogenous viruses antigenically identical to the oncogenic feline viruses have also not yet been found in cat embryos, although another class of distantly related (and possibly nononcogenic?) endogenous oncornaviruses, exemplified by the RD 114 agent, have been found in nonproducer cat embryo cell cultures (Fischinger *et al.*, 1973; Livingston and Todaro, 1973).

Most studies with the oncornavirus immune response followed either artificial virus infections (injections) or transplants of virus-induced tumors. Many were also done in unnatural hosts, and the significance of this must be recognized. The type of immune response mounted by a mammal given a transplanted tumor that was originally induced with a subgroup A avian virus tells us much about how that mammal may respond to certain classes of tumors, but relatively little about how free-living jungle fowl may respond to the same virus when infected.

The immune response to most cytopathogenic viruses plays a major role in controlling infection and overcoming disease. It is important for us, as cancer biologists and virologists, to learn as much as possible about the response following infection with oncogenic viruses.

II. Antigens of Avian, Feline, and Murine C Type Oncornaviruses

A. General Virus Structure

Three morphologic types of oncornaviruses have been described: A, B, and C (Bernhard, 1960). The electron microscopic appearance of these viruses has received much attention (de Harven, 1968; Laird *et al.*, 1968; Dougherty and Rickard, 1970; Nowinski *et al.*, 1970; Luftig and Kilham, 1971; Dalton, 1972a,b; Nermut *et al.*, 1972). The B type is represented by the mammary tumor virus of mice, which will not be discussed here. The C type represents the typical extracellular particles found in chickens, cats, and mice with hematopoietic or sarcomatous tumors, and in some healthy individuals of these and other species. The A particle is believed to be the precursor budding form which "matures" and condenses to the B or C form after exposure to the extracellular environment (de Harven, 1968).

Oncornaviruses from different species appear quite similar in size and morphology, but distinct differences can be seen if sufficiently high resolution is used. These differences are usually only apparent when comparing viruses isolated from different species, although two morphologic types of feline agents have been described (Dalton, 1972a; Stephens *et al.*, 1972). All morphologic differences between groups of C type viruses appear to be related to the envelope structures, not the nucleocapsid (Sarkar *et al.*, 1971). The fine differences between virion envelope morphology are independent of the species or cell type from which the virus emerges (Dalton, 1972a).

Virus proteins can be detected in the cytoplasm where they are produced, but assembly of the particles takes place at the budding site at cell or vacuolar membranes. Since the viruses appear to form as part of the cell membrane itself, certain cell proteins may be incorporated into the virus envelope (Mommaerts *et al.*, 1954; Beard *et al.*, 1957; Dorfman *et al.*, 1972). This process may be selective, as evidenced by a decrease or complete lack of many widely distributed cell membrane antigens on the virion envelope (Aoki *et al.*, 1970a; Dorfman *et al.*, 1972).

C type viruses have a total diameter of 85–120 nm. The envelope may contain knobs or spikes (Dougherty and Rickard, 1970; Nermut *et al.*, 1972). Inside the membrane is an electron-luscent space, and inside the space an electron-dense core of 60 to 75 nm. The core represents a condensed helical nucleocapsid (Sarkar *et al.*, 1971). The viruses have a buoyant density of 1.14–1.17 gm/cm^3 in sucrose (O'Connor *et al.*, 1964;

Kawakami *et al.*, 1967) and internal proteins of very similar size and structure (Bolognesi and Bauer, 1970; Fleissner, 1971; Hung *et al.*, 1971; Oroszlan *et al.*, 1971b; Shafer *et al.*, 1971; Nowinski *et al.*, 1972). They all also contain reverse transcriptase, a protein that is antigenically distinct for different groups of oncornaviruses (Scolnick *et al.*, 1972).

B. Virus Envelope Antigens

At least two antigens are found in the virus envelope. They have been estimated at 70,000 and 100,000 daltons (Duesberg *et al.*, 1970; Fleissner, 1971; Nowinski *et al.*, 1972). The proteins differ slightly between different strains of viruses when analyzed by SDS–polyacrylamide gel electrophoresis (Robinson *et al.*, 1970). They are glycoprotein in nature. As antigens, they are virus-specific. They may be concentrated in the knob or spike areas of the virus envelope, and play an important role in specific adsorption of the virus to the cell surface. The envelope proteins are essential for infectivity. Relative or complete genetic resistance to virus infection at the cellular level may be due to a lack of cell receptors for a particular virus. Saturation of these receptors probably also accounts for the interference observed between viruses of the same type that would otherwise infect (Rubin, 1960; Vogt and Ishizaki, 1966; Sarma and Log, 1971). Avian and mammalian groups of viruses can also become phenotypically mixed when infecting the same cell (Hanafusa *et al.*, 1964; Fischinger and O'Connor, 1969; Gilden *et al.*, 1972). In other words, when FeLV and MuSV are present in the same cell, the progeny will contain viruses with FeLV envelopes and MuSV cores, viruses with MuSV or MuLV envelopes and FeLV cores, and the usual FeLV and MuSV agents. These hybrid viruses will then have the infectivity spectrum of the envelope type, but will of course only produce true virus of the nucleic acid core. This can lead to great confusion when attempting to type virus pools that may have been maintained in uncharacterized laboratory animals or cell cultures. This "hybridization" occurs between and within the mouse and cat virus groups, but has not yet been described between mammalian and avian viruses. Antibody directed to virus envelope causes virus neutralization, and this procedure can be used to separate both subgroups and individual types. Unless the viruses are first treated with such enzymes as neuraminidase and phospholipase (Shafer and Szanto, 1969), they do not spontaneously agglutinate erythrocytes (Vogt, 1965).

The avian oncornaviruses have been divided into A, B, C, D, and E subgroups. This classification is based on viral interference and cell

receptor patterns (Vogt, 1970). Of the two major antigenic determinants in the virus envelope, one probably cross reacts between all viruses within a given subgroup, while the other would be specific for each virus type (Duff and Vogt, 1969; Bauer and Graf, 1969). Neither should cross react between subgroups, except possibly for subgroups B and D. Virus interference is also subgroup specific, although cross interference occurs between B and D subgroups, presumably because one of the envelope antigens is similar or common for these two (Bauer and Graf, 1969). Most field isolates of avian viruses are subgroup A (Vogt, 1970).

Feline oncornaviruses have been similarly divided into subgroups A, B, and C on the basis of interference and virus neurtalization (Sarma and Log, 1971). Cat cells that show differential susceptibility to given subgroups have not yet been described, but they probably exist because it has been shown that canine and human cells are resistant to infection by A subgroup viruses but not B and C subgroup viruses (Jarrett *et al.*, 1972; O. Jarrett, personal communication). Many field isolates are combinations of two subgroup viruses and the Kawakami-Theilen FeLV (Theilen *et al.*, 1969) is a mixture of all three (P. S. Sarma, personal communication). The subgroups isolated from field cases are related to geographical areas to some extent; differences have been noted between European and United States isolates.

The characterization of envelope antigens of the murine oncornaviruses has been more difficult than with the avian or feline systems for two major reasons. First, most if not all laboratory mice and cultures derived from them contain one or more endogenous viruses. These endogenous viruses often have envelope characteristics that may be different from any of the standard oncogenic murine viruses (Fischinger *et al.*, 1972; Gomard *et al.*, 1973). These agents also contaminate many virus pools, which serves to confuse studies based on interference patterns. Secondly, mice were thought to be immunologically tolerant to the virion envelope of Gross virus, the prototype. This is apparently not true tolerance, but rather a state of antigen excess and persistent viremia, so that free antibody cannot be detected (Oldstone *et al.*, 1972). While neutralizing antibody can be produced to any of these viruses in rats or rabbits, the antiserum obtained will neutralize all mouse oncornaviruses that have been tested, including Gross, Friend, Rauscher, Graffi, and Moloney types (Geering *et al.*, 1966; McCoy *et al.*, 1968; Levy *et al.*, 1969; Micheel *et al.*, 1972). This indicates that heterologous species recognize a common envelope antigen that mice do not.

The recent study by Gomard *et al.* (1973) circumvented this problem by using intrathymic injections of virus into mice. Based on these studies it appears that at least four subgroups exist: (a) Friend, Moloney,

Rauscher, Friend-LLV, Rich, and Buffet viruses; (b) Graffi, Mazurenko, and Stepina-Zilber viruses; (c) Gross virus, the AKR virus, and Kirsten virus; and (d) Tennant virus (Gomard *et al.*, 1973). The L cell virus and many other endogenous viruses as well as the myeloma cell C type particles and the NZB mouse virus probably fall in none of the above categories (Aoki *et al.*, 1972; Micheel *et al.*, 1972; Shafer *et al.*, 1972; Gomard *et al.*, 1973). In addition to the group- and subgroup-specific antigens above, less immunogenic type-specific antigens probably exist for each virus.

It should be stated that the viral envelope antigens of mouse oncornaviruses do not correspond to either the Gross positive or negative (Friend, Molony, Rauscher) tumor cell surface antigens (Levy *et al.*, 1969; Gomard *et al.*, 1973). Neither do they correspond to the N-B tropism based on resistance of NIH-Swiss and BALB/c mice (Lilly and Pincus, 1973) to infection (Gomard *et al.*, 1973). The N-B tropism is both less stable and less qualitative than the resistance of certain avian cells to infection by AvLV subgroups. As with other oncornaviruses, it appears that murine virus envelope antigens do not usually overlap virus-induced cell surface antigens. Table II lists the major virus envelope and core antigen systems of the three major groups of oncornaviruses.

TABLE II

Primary Oncornavirus Envelope and Core Antigens

Oncornavirus group	Envelope antigens[a]	Core antigens
Avian	(1) subgroup specific[b] A, B, C, D, or E	(1) group specific gs 1 (2) group specific gs 3
Feline	(1) subgroup specific	(1) group specific gs 1 (2) group specific[c] gs 3
Murine	(1) subgroup specific (a) Friend, Moloney Rauscher, others (b) Graffi, others (c) Gross, AKR virus, others (d) Tennant virus (e) L-cell particle, others	(1) group specific gs 1 (2) group specific[c] gs 3

[a] Other group- or type-specific antigens that are less immunogenic probably exist.
[b] Some cross reaction between B, D, and E subgroups.
[c] Interspecies cross reaction between feline and murine groups.

C. Virus Core Antigens

The virus core antigens are small basic proteins (Nowinski *et al.*, 1972). They are generally called group-specific antigens because they are usually common for all avian, murine, or feline viruses. The only significant exception to the rule thus far appears to be RD 114, an apparently endogenous virus of some cat cells that does not cross react completely with prototype FeLV (Oroszlan *et al.*, 1972). Either five (for the AvLV) or four (for the FeLV and MuLV) different core proteins have been described of approximate molecular weights 30,000, 19,000, 15,000, 12,000, and 10,000. The avian and mammalian proteins have very similar molecular weights, suggesting a common origin (Bolognesi and Bauer, 1970; Fleissner, 1971; Oroszlan *et al.*, 1971b; Shafer *et al.*, 1971; Nowinski *et al.*, 1972).

The group-specific core (gs) antigens best studied serologically are gs 1 or gs a (MW 30,000) and gs 3 or gs b (MW 15,000). The gs 3 is also called the interspecies antigen because it has been shown to cross react between all mammalian viruses (mouse, cat, hamster, and rat) but not between mammalian and avian viruses (Geering *et al.*, 1968, 1970; Hardy *et al.*, 1970; Gilden *et al.*, 1971). The gs 1 is species-specific, and it may exist on the same molecule as gs 3 (Gilden *et al.*, 1971; Oroszlan *et al.*, 1971b). It is also the strongest immunogen, and this may cause an overshadowing of the immune response to gs 3 (Oroszlan *et al.*, 1971a). The gs antigens produced by the virus are not affected by the cell or host. The gs produced by AvLV in chickens is the same as that produced by Rous sarcoma virus in mammalian tumors (Bauer and Janda, 1967).

RD 114, the oncornavirus first detected when human tumor cells were passed through fetal cats (McAllister *et al.*, 1972), contains gs 1 that is different from the gs 1 of other FeLV (Orszlan *et al.*, 1972). This is the only instance thus far where viruses with non-cross-reacting gs 1 have been isolated from a single species. It is not yet known if this virus ever causes spontaneous tumors in cats, or if it just represents an endogenous nononcogenic deviant (Fischinger *et al.*, 1973; Livingston and Todaro, 1973). It apparently does not produce progeny virus in feline cells of the types that have been checked, but it will infect primate cells in a productive fashion (Livingston and Todaro, 1973).

The gs antigens can be readily detected in the cytoplasm of producer cells using fixed-cell immunofluorescent techniques (Cook *et al.*, 1966; Yoshida *et al.*, 1966; Ubertini *et al.*, 1971; Hardy *et al.*, 1973a). It has also been suggested that they may be deposited on the cell membrane (Maruyama and Dmochowski, 1969; Yoshiki *et al.*, 1973).

Specific host gene regulation of expression of the gs antigens has been demonstrated in inbred mice (Taylor *et al.*, 1971) and chickens (Payne and Chubb, 1968). They are often detected in the absence of infectious virus particles in mouse and chicken embryos and in oncornavirus-induced sarcomas in heterologous species (Dougherty *et al.*, 1967; Payne and Chubb, 1968; Huebner *et al.*. 1970; Allen and Sarma, 1972; Gilden and Oroszlan, 1972; Todaro and Huebner, 1972; Huebner. 1973). Induction of "switched-off" gs antigen often follows treatment of cells with such chemicals as bromodeoxyuridine (Klement *et al.*, 1971; Lowy *et al.*, 1971; Todaro and Huebner, 1972). The antigen may also be spontaneously released following long-term cultivation of cells *in vitro* (Todaro and Huebner, 1972; Huebner, 1973), and alternate expression of the virus between *in vivo* passages has been observed (Ioachim *et al.*, 1972).

III. Tumor Cell Membrane Antigens Associated with Oncornavirus Infections

A. General Characteristics of Tumor Cell Membrane Antigens

Tumor-specific cell membrane antigens or tumor-associated antigens have been described for a wide variety of tumors, such as SV40- and polyoma virus-induced tumors (Klein, 1968; Tevethia *et al.*, 1968; Sjögren and Bansal, 1971), Burkitt's lymphoma (which is suspected to be caused by a herpesvirus) (Epstein, 1970; Klein, 1971), spontaneous human tumors of many histopathological types (Hellström *et al.*, 1971), tumors induced with chemicals such as methylcholanthrene (Klein, 1968; Prehn, 1968; Baldwin *et al.*, 1971), as well as oncornavirus-induced tumors. In general, the antigens associated with virus-induced tumors are consistent for the etiological agent regardless of the host species, while the strongest antigens on tumors induced with chemicals are individually specific. Some virus-induced tumor cell membrane antigens, such as those induced by SV40 and polyoma, which cross react with embryonic antigens, may be due to virus-specific derepression of a cell antigen (Pearson and Freeman, 1968; Coggin *et al.*, 1971). In the case of papova–adeno- and herpesviruses, tumor cell membrane antigens are usually expressed by the cell when neither whole virus nor virus structural proteins are produced. This, along with the observation that DNA synthesis is usually not required, indicates that expression of these antigens is an early function of virus infection (Deichman, 1969; Tevethia *et al.*, 1968; Klein, 1971; Sjögren and Bansal, 1971; Rapp and Crouch, 1971; Gergeley *et al.*, 1971; Klein *et al.*, 1972).

The immune response to these antigens is related to protection against tumor growth. Most tumor cell membrane antigens do not show cross reactivity with virus envelope antigens, but the membrane antigen induced by Epstein-Barr virus may be an exception (Bremberg *et al.*, 1969; Pearson *et al.*, 1970; Silvestre *et al.*, 1971). The most commonly used procedures for demonstrating tumor cell membrane antigens *in vitro* are cytotoxicity, membrane immunofluorescence, and immunoelectron microscopy. Tumor transplantation *in vivo* is used whenever histocompatible hosts are involved.

Studies involving the relationship of the expression of tumor cell membrane antigens to the stage of the cell cycle have been done (Cikes, 1971; Cikes and Friberg, 1971; Cikes and Klein, 1972). It was shown that the amount of antigen expression on murine lymphoma cells and MuLV-infected monolayer cultures was inversely proportional to cell volume and growth rate. DNA inhibitors, such as hydroxyurea, mitomycin C, and ara C, did not interfere with the expression of this antigen, although inhibitors of protein synthesis, such as puromycin and cyclohexamide, did. The significance of these observations regarding the *in vivo* expression of tumor cell membrane antigens and the possible control of tumor growth due to an immune response directed at these antigens is not known.

Attempts to characterize oncornavirus-induced cell membrane antigens are confused by the fact that most species that become successfully infected with these viruses produce antibody to both virus envelope and cell membrane antigens. Since most serum samples contain both activities, this often leads to misconception about the cross reactivity of the two antigen classes. As a result, early studies generally supported cross reactivity, while more recent experiments using more sophisticated procedures and serum from non-virus-producer tumor-bearing hosts suggest that significant cross reactivity between viral envelope and tumor cell membrane antigens usually does not occur (see below).

B. Avian Oncornavirus-Associated Cell Membrane Antigens

Avian oncornavirus-induced cell surface antigens have been demonstrated on chicken, rat, mouse, and hamster cells (Bubenik and Bauer, 1967; Bauer *et al.*, 1969; Jonsson, 1971; Kryukova *et al.*, 1971; Kurth and Bauer, 1972a,b; Gelderblom *et al.*, 1972; Meyers *et al.*, 1972; Gelderblom and Bauer, 1973). It is clear that one major group-specific cell membrane antigen exists that is induced by all subgroups of avian viruses. It is the strongest of the tumor cell membrane antigens (Kurth

and Bauer, 1972b). The antigen has been shown by the *in vitro* assay for cytotoxicity, membrane immunofluorescence, and immunoferritin labeling as well as by tumor challenge *in vivo*. It can probably be specified by either Rous sarcoma virus or helper AvLV in the same pools, but it is believed to be restricted to transformed cells or tumor cells (Kurth and Bauer, 1972a; Gelderblom *et al.*, 1972). It is the same antigen that is expressed on cells of Rous sarcoma virus-induced mammalian tumors (Jonsson and Sjögren, 1965; Jonsson, 1971; Bauer *et al.*, 1969; Kurth and Bauer, 1972a; Gelderblom and Bauer, 1973). It does not overlap with viral envelope antigens as evidenced by the lack of correlation between antibody reactions to this antigen and virus neutralization (Jonsson and Sjögren, 1965; Gelderblom *et al.*, 1972; Meyers *et al.*, 1972). Expression of the antigen is also unrelated to virus production by the cells (Kurth and Bauer, 1972a).

A second subgroup-specific antigen is also detectable on virus producer cells. This overlaps activity with the virus envelope and may simply represent areas of budding virus particles (Gelderblom *et al.*, 1972; Kurth and Bauer, 1972b).

C. Feline Oncornavirus-Associated Cell Membrane Antigens

Feline oncornavirus-associated cell surface antigens have been demonstrated on cat, human, monkey, dog, and pig cells using either biopsied tumor cells or cultured cells infected *in vitro* (Essex *et al.*, 1971b, 1972, 1973b; Oshiro *et al.*, 1971; Boone *et al.*, 1973; Slauson, 1973). The antigens have been studied using membrane immunofluorescence, ^{125}I labeling, cytotoxicity, and immunoferritin labeling. Evidence has also been obtained for the expression of this antigen in feline oncornavirus tumor-bearing animals of several species, including cats, dogs, monkeys, pigs, goats, sheep, and cattle (Essex *et al.*, 1971a,b, 1972, 1973a,b, 1974; Oshiro *et al.*, 1971; Boone *et al.*, 1973; Cotter *et al.*, 1974; Essex and Snyder, 1973; Jarrett *et al.*, 1973a,b; L. D. Pearson *et al.*, 1973). The standard feline target cell for assay of the antigen is an FeLV producer cell line developed by Theilen *et al.* (1969). This line produces all three major subgroups of FeLV (Sarma and Log, 1971). Serum taken from animals exposed to all major sources of FeLV (Kawakami-Theilen, Rickard, and Jarrett strains) and FeSV (Snyder-Theilen and Gardner-Arnstein strains), as well as serum from animals exposed to many field strains, will react with the target cell.

Studies to determine if this antigen is group- or subgroup-specific have not yet been done. It is likely, however, on the basis of other similarities

with the avian and murine oncornavirus systems, that at least one of the predominant antigens visualized by immunofluorescence will prove to be group-specific. Such similarities include observations that neither neutralizing antibody directed to the virion envelope nor antibody to the group-specific core antigen appear to cross react with the major cell membrane antigen (Jarrett *et al.*, 1973b; Essex *et al.*, 1974; W. D. Hardy, Jr., personal communication; Olsen *et al.*, 1974). In addition, we have found that removal of antibody to the virus envelope (all subgroups) from highly active antisera does not decrease specific reactivity for the cell membrane antigen. Although the immunoferritin study of Oshiro *et al.* (1971) showed labeling of both budding virus and virus-free areas of the cell membrane, it is very likely that the serum used contained a mixture of both neutralizing antibody and antibody to any additional antigens on tumor cells.

We have found that the major feline oncornavirus-associated cell membrane antigen is distinct from the major murine and avian or simian oncornavirus-induced antigens (Essex *et al.*, 1973b; M. Essex, unpublished observations). It also does not cross react with the Epstein-Barr virus-induced membrane antigen when both may infect the same human lymphoblastoid cells (Essex *et al.*, 1972). We also do not know if the feline virus-induced cell membrane antigen is expressed on cells that become productively infected but not transformed. We have successfully induced the antigen in many cultured human, canine, and feline lymphoblastoid and fibroblastoid cells by infecting with either FeSV or FeLV, but most if not all of these lines were already "transformed" in the sense of demonstrating immortality for the line *in vitro*. We have noticed that the degree of expression of the antigen increases with the length of time the cells have been in culture following virus infection. For example, only 10% of the cells in a highly sensitive human lymphoblast culture are antigen-positive 10 days after exposure to virus. After several months, but without any additional exogenous virus, the number of antigen positive cells rises to 80–90%, and each cell stains more intensely (Essex *et al.*, 1972; M. Essex, unpublished observations).

Using rabbit antiserum to FeLV, a common cell membrane antigen was demonstrated by immunoelectron microscopy and membrane immunofluorescence for all tumor cells and cultured fibroblasts infected with murine and feline oncornaviruses (Yoshiki *et al.*, 1973). The immunoferritin procedure showed the antigen was not virus envelope protein but that it was specifically deposited in discrete spots away from budding particles. The authors suggested that it may represent the interspecies gs 3 viral core antigen expressed free on the cell membrane. A

previous report by Maruyama and Dmochowski (1969) may be a related observation.

D. Murine Oncornavirus-Associated Cell Membrane Antigens

The expression of murine oncornavirus-associated cell membrane antigens has been demonstrated in virus-infected mouse cell cultures, and in tumor-bearing mice (Klein and Klein, 1964; Klein, 1968; Old *et al.*, 1968; Pasternak, 1969; Aoki, 1971; Herberman, 1972; Leclerc *et al.*, 1972). Because virus neutralizing antibodies could not be detected in Gross virus-infected mice, the murine viruses were originally classified on the basis of their oncornavirus-associated cell membrane antigens as being G+ or G−. The typical G+ cells were Gross virus related, and the G− cells were Friend, Moloney, Rauscher, or Graffi (FMRGi) virus related. It was first thought that the grouping for virus envelope antigens would follow this system for cell membrane antigens. As mentioned in Section II,B, the elegant studies of Eckner and Steeves (1972) and Gomard *et al.* (1973) have shown that this is no so. Graffi and Moloney viruses, for example, which share the same oncornavirus-induced cell membrane antigen FMRGi, do not show cross virus neutralization. A second major cell membrane antigen that is common for all murine oncornaviruses was described recently (Herberman, 1972; Micheel *et al.*, 1972; Leclerc *et al.*, 1972). It is not known how this common antigen may be related to the antigen shown to be related to both feline and murine viruses by Yoshiki *et al.* (1973).

As with the avian and feline systems, the major murine oncornavirus-induced cell membrane antigens do not appear to overlap the antigens of the virus envelope. Immunoferritin labeled antibody to the cell membrane antigen does not label virus particles (Aoki *et al.*, 1970a; Aoki, 1971), and virus neutralizing antibodies are distinct from antibodies directed to this antigen (Old *et al.*, 1968; Levy *et al.*, 1969; Law and Ting, 1970; Micheel *et al.*, 1972). The examination of hybrid cell pairs that had either very low or very high expression of the Moloney virus-induced cell membrane antigen yielded a similar result; the expression of tumor cell membrane antigen varied independently with virus production (Fenyo *et al.*, 1968, 1971; Fenyo, 1971). Evidence that certain murine oncornavirus-associated cell membrane antigens may overlap embryonic antigens have also been published (Hanna *et al.*, 1971; Ishimoto and Ito, 1972).

The question of whether defective murine sarcoma viruses may induce cell membrane antigens at all, or any that may be different from

those induced by murine leukemia viruses, was frequently asked, but difficult to resolve, due to the constant presence of MuLV helper in MuSV stocks. It was already known that most MuSV-induced tumors contained oncornavirus-associated cell membrane antigens that were specific for MuLV (Chuat *et al.*, 1969). The development of sarcoma virus-positive, leukemia virus-negative cloned cell lines provided a new approach to this question (Aaronson *et al.*, 1970; Bassin *et al.*, 1970). It became apparent that MuSV specific antigens equal in intensity to the MuLV directed antigens were not induced by MuSV (Stephenson and Aaronson, 1972; Strouk *et al.*, 1972), but a weakly antigenic minor antigen that is MuSV specific has been found by Aoki *et al.* (1973).

Additional classes of tumor cell membrane antigens, the G_{IX} system (Stockert *et al.*, 1971), the T L system (Boyse *et al.*, 1966), the E system (Aoki *et al.*, 1970b), and G_L and G_T (Nowinski and Peters, 1973) have been described. These are presumed to be cell-coded alloantigens. In some strains the expression of these antigens may be related to virus infection or leukemia induction, but since they are probably not virus directed, they will not be discussed here.

Soluble antigens in plasma and body fluids of AKR and C 58 mice have also been studied (Aoki *et al.*, 1972). These include viral group- and type-specific antigens as well as Gross virus-associated cell membrane antigens and G_{IX} cell membrane antigens (Aoki *et al.*, 1972). In regard to the Gross antigens occurring both in plasma and at the cell membrane, it is not known if this is due to turnover of the membrane with sloughing into body fluids or to adsorption of a soluble antigen to the cell membrane.

Table III lists the principal avian, feline, and murine oncornavirus-associated cell membrane antigens.

TABLE III

Avian, Feline, and Murine Oncornavirus-Associated Cell Membrane Antigens

Virus group	Primary antigens	Demonstrated by
Avian	Group specific[a]	Membrane fluorescence, cytotoxicity, ferritin labeling, *in vivo* challenge
Feline[b]	Group specific	Membrane fluorescence, cytotoxicity, ferritin labeling, ^{125}I labeling, *in vivo* challenge
Murine[b]	Subgroup specific[c]	Membrane fluorescence, cytotoxicity, ferritin labeling, *in vivo* challenge

[a] Less immunogenic subgroup-specific antigens probably also exist.

[b] Additional interspecies group-specific antigen also described for feline and murine systems.

[c] Subgroups not same as those based on virus envelope antigens.

IV. Immune Response to Avian Oncornavirus Infections

Infection of an animal with oncornaviruses can occur as a result of transmission by any of three routes: (a) horizontal, which is the predominant route for most cytopathic viruses; (b) vertical extrachromosomal or congenital-transplacental or milk-borne infection of the neonate by the mother; and (c) chromosomal transmission of "switched-off" viral genes as part of the usual inherited genetic material of all individuals within the given species. The last, of course, can only be referred to as "virus transmission" by virtue of the fact that these genes can code for the production of typical virus particles if the environment became suitable. Considerable evidence indicates that all three of these routes of infection occur in avian and murine species (Dougherty, 1968; Mirand and Mirand, 1969; Burmester and Purchase, 1970; Huebner, 1973). The important issue, however, is which of these routes represents the predominant transmission cycle for those oncornaviruses that cause cancer, as opposed to those serologically and morphologically identical oncornaviruses that appear to have lost oncogenic potential.

The route of infection should, to some extent, determine both the nature and magnitude of the immune responses mounted to the virus itself and to virus-induced cell membrane antigens. If the viral genome is genetically transmitted and the genes coding for viral proteins are expressed during embryogenesis, we should expect these proteins to be recognized as self, and immune tolerance should result. This could also occur with cogenital extrachromosomal infection, assuming that the infection occurred prior to the development of immune tolerance. Although most early work suggested that chickens were immunologically tolerant to virus core (gs) antigens (Dougherty, 1968), recent work shows that this is not so (Rabotti and Blackham, 1970; Roth *et al.*, 1971; Weber and Yohn, 1972; Dougherty, 1973). The development of antibodies in some chickens to gs antigen could be due to either a break in tolerance or to a lack of tolerance in the first place in certain birds. Antibodies directed to the virus core antigens play no significant role in virus elimination, since they can neither neutralize the virus nor penetrate producer cells to inhibit virus production. Only in the unlikely event that these antigens also compose a sensitive portion of the cell membrane could the antibody also attack tumor cells.

Antibody to the virus envelope probably plays a very significant role in limiting viremia in chickens that become infected horizontally (Dougherty, 1968). This probably represents the majority of chickens housed under commercial flock conditions (Purchase, 1969). Most of

these birds have persisting high titers of virus neutralizing antibody but experience only transient viremia. Antibody is not detectable during the viremia, but rapidly follows the disappearance of virus. Adult female chickens pass this antibody to their progeny in the egg yolk (Rubin *et al.*, 1961). Significant levels may then persist until the birds are several weeks old and possibly then beyond the stage of high risk for tumor development if exposed to exogenous virus (Witter *et al.*, 1966). Virus neutralizing antibody can presumably inhibit tumor development only by decreasing the amount of virus to which the bird is exposed, thus decreasing the frequency of cell transformation events, and not by controlling the replication of transformed cells. The virus neutralizing antibody response would therefore probably be of greater significance with inefficient leukemia-inducing oncornaviruses as opposed to the more efficient sarcoma-inducing oncornaviruses.

A smaller percentage of chickens in most flocks have persisting viremia (Purchase, 1969). These birds regularly excrete virus in the saliva and feces (Rubin *et al.*, 1961) and congenitally infect their progeny. They are also the individuals most likely to develop tumors, especially if the immune response to the cell membrane antigens becomes depressed. Evidence supporting genetic transmission of avian oncornaviruses is also excellent (Huebner and Gilden, 1972). If the agents transmitted in this manner are oncogenic, only the immune response to the tumor cell membrane antigen would probably be functional, and the breakdown of this response could be the most important determinant of tumor development. A recent study of Malaysian jungle fowl suggests that genetic transmission occurs even under such "natural" conditions, but that the vertically transmitted viruses represent the poorly oncogenic or nononcogenic virus subgroups (Weiss and Biggs, 1972). The same study found no evidence for genetic transmission of A or B subgroup viruses. Since the naturally occurring oncogenic leukemia viruses all appear to be in the A or B subgroups (Burmester and Purchase, 1970), it will be important in future studies to determine the subgroup status of viruses that appear to be exclusively transmitted in an endogenous or genetic manner.

Depending on dose of virus and the the age and the genetic background of chickens given RSV, many develop tumors that subsequently regress. The regression is believed to be related to the immune response directed at virus-associated cell membrane antigens. Infiltration of regressing tumors with mononuclear cells has been observed (Dougherty, 1968), and the development of cell-mediated immunity has been detected in regressor chickens using the cell-mediated cytotoxicity and colony inhibition assays (Sjögren and Jonsson, 1970; Kurth and Bauer,

1972b). Significant humoral antibody titers have also been found in regressor chickens but not in those with progressing tumors (Radzichovskaja, 1968). Cell-mediated immunity was apparently active in both progressors and regressors (Sjögren and Jonsson, 1970). This suggested that "blocking factors," such as circulating sloughed tumor cell membrane antigens with or without complexed antibody, may interfere with cell-mediated immunity. Successful immunization of chickens to challenge by RSV has been accomplished (Sigel *et al.*, 1971; Meyers *et al.*, 1972). Either subtumorigenic doses of RSV or other avian oncornaviruses were suitable immunogens. Protection was directed at common cell membrane antigens for all virus subgroups. Although the type of immune response involved was not studied, it seems likely that both humoral antibodies and cell-mediated immunity may be important.

Several studies have concentrated on the immune response of mice and rats to Rous sarcoma virus-induced tumors (Jonsson and Sjögren, 1966; Bauer *et al.*, 1969; Bellone and Pollard, 1970; Jonsson, 1971; Sjögren and Borum, 1971; Kurth and Bauer, 1972a). Transplantation immunity against Rous tumor isografts was clearly demonstrated in mice that (a) received live virus injections but developed no tumors or (b) were pretreated with irradiated tumor cells. The mice usually developed no virus neutralizing antibody, but were not tested for antibody to cell membrane antigens. Others (Kurth and Bauer, 1972a) have shown that similarly immune mice also develop high titers of cytotoxic antibody to the cell membrane antigen. Cell-mediated immunity was also found in rats bearing Rous sarcoma virus-induced tumors (Jonsson, 1971; Sjögren and Borum, 1971). Blocking serum activity was found in the progressor animals (Sjögren and Borum, 1971). With the exception of the work of Radzichovskaja (1968), very little emphasis has been placed on the study of the humoral antibody response of chickens to the avian oncornavirus-associated cell membrane antigens. Two possible reasons for this come to mind: (a) the disappointment of early workers who found that high neutralizing antibody titers were not associated with regression, not realizing that different antigen systems were involved, and (b) the discovery that antibody, at least when complexed with sloughed cell membrane antigen, may interfere with the cell-mediated response that might otherwise successfully attack the tumor. It is quite possible that the latter phenomenom is more related to the "sloughability" of the tumor than to the nature of the antibody. Since recent work with mouse and cat oncornavirus-induced tumors indicate a positive correlation between the presence of humoral antibody to tumor cell membrane antigens and tumor regression or blocking of tumor development (see Sections V and VI), similar studies with the avian system may be useful.

V. Immune Response to Feline Oncornavirus Infections

Almost all the current seroepidemiologic studies with femine oncornaviruses suggest that horizontal transmission of virus between cats represents the most important infection route (Essex *et al.*, 1971a, 1973a,b; Jarrett, 1972; Cotter *et al.*, 1973; Hardy *et al.*, 1973a,b, 1974; Jarrett *et al.*, 1973a,b; Hardy *et al.*, 1974). The first suggestions that horizontal transmission might be important in this species came when observations of clusters of leukemia in apparently unrelated cats were reported (Schneider *et al.*, 1967; Brody *et al.*, 1970; Jarrett, 1971; Cotter *et al.*, 1974). No evidence has yet suggested that either chromosomal or extrachromosomal vertical transmission of oncogenic oncornaviruses occurs in cats to any significant degree, but C type particles have occasionally been seen in cat embryos (Schneider, 1971), and RD 114 virus can be activated from some cat embryo cell cultures (Fischinger *et al.*, 1973; Livingston and Todaro, 1973). The RD 114 virus however, is not a typical feline oncornavirus (see Section II).

Several procedures were used to demonstrate horizontal virus transmission under both laboratory and field conditions. When uninfected laboratory cats of all ages came in contact with oncornavirus-infected cats they rapidly developed antibody to the feline oncornavirus-associated cell membrane antigen (Essex *et al.*, 1971a, 1973a,b; Jarrett, 1972; Essex and Snyder, 1973; Jarrett *et al.*, 1973b). Most also developed viremia, at least temporarily, and had morphologic evidence of C type viruses in blood platelets and bone marrow (Jarrett *et al.*, 1973b). A smaller yet significant percentage developed clinical leukemia. Hardy *et al.* (1973a,b) demonstrated that horizontal transmission of virus frequently occurred under field conditions. When a leukemic cat was found in any particular household, examination of the healthy feline housemates indicated that 39% were viremic, as compared to fewer than 1% of the street controls or cats from leukemia-free households. This study was based on 154 cats from 78 cat leukemia households and about 1200 controls representing both stray street cats and cats from leukemia-free houses. This has been confirmed and extended to include the examination of cats from the same categories but a different geographical area (Cotter *et al.*, 1973; Essex *et al.*, 1974). We have now examined (in collaboration with Hardy) about 120 cats from two particular leukemia clusters. About 46% of the healthy cats in the two households are persistently viremic. Most of the nonviremic cats have high antibody titers to the feline oncornavirus-associated cell membrane antigen, so that more than 90% of the cats in these houses had evidence of recent

virus exposure. Neither the antibody response nor the presence of viremia appeared to be directly related to genetic background for the cats in these houses. The incidences of leukemia, viremia, and high antibody titers (4 to 256) were similar for three different breeds of cats housed together. The high frequency of viremia and leukemia seen among cats from cluster environments is probably due to exposure to larger virus doses at a relatively young age. Viremic cats excrete virus in saliva, respiratory secretions, urine, and feces (Dougherty *et al.*, 1969; Gardner *et al.*, 1971; Jarrett *et al.*, 1973b). The most significant route for excretion may be respiratory secretions, since almost all infected cats have virus budding from respiratory epithelial surfaces (Jarrett *et al.*, 1973b). Hoover *et al.* (1972) have shown that FeLV is oncogenic when administered by intranasal instillation.

Cats exposed to FeLV or FeSV under either natural or experimental conditions do not exhibit immune tolerance to either the major virus antigens or the virus-associated cell membrane antigens (Olsen and Yohn, 1972; de Noronha *et al.*, 1972).

The discovery that the magnitude of the humoral immune response directed to the virus-associated cell membrane antigen was directly correlated with tumor growth following exposure to FeSV (see below) led us to examine the relationship between leukemia development and the humoral antibody response. Cats exposed to FeLV injections that developed leukemia never appeared to develop high antibody titers to the cell membrane antigen. On the other hand, cats which developed high antibody titers (greater than 4), following either natural or artificial exposure to FeLV, did not develop leukemia (Jarrett *et al.*, 1973b; Essex *et al.*, 1974; W. F. H. Jarrett, personal communication). We have now checked the antibody titers of 63 cases of spontaneous feline leukemia and found that they also show a deficient humoral immune response to the cell membrane antigen.

Experiments have also been done which show that FeLV may induce thymic atrophy and immunosuppression (Anderson *et al.*, 1971; Perryman *et al.*, 1972). Observations of higher than expected frequencies of other infectious diseases in FeLV viremic cats may be a result of immunosuppression under natural conditions (Cotter *et al.*, 1973, 1974; Essex and Snyder, 1973; Essex *et al.*, 1973a).

The humoral immune response to FeSV-induced tumors in cats was also studied (Essex *et al.*, 1971a,b, 1973b; Essex and Snyder, 1973). Cats that developed progressively growing sarcomas never developed high antibody titers to the feline oncornavirus-associated cell membrane antigen. Cats that developed no tumors following virus challenge, or developed tumors that subsequently regressed, had high antibody titers

(4 to 256). Kittens that received antibody passively as neonates from immune mothers were able to resist the development of tumors following challenge with at least 100 to 1000 times the lethal dose of FeSV (Essex *et al.*, 1971a, 1973a,b). Although it is possible that virus-directed antibodies present in the same serum samples may have played an important role in the prevention of tumors in some cats, it is very unlikely that virus neutralizing antibody plays a role in tumor regression. This seems apparent because the immune response to the virus-induced cell membrane antigen is distinct and independent from the immune response to either virus core (gs) or virus envelope antigens. It was found that some cats remain persistently viremic with an apparently inefficient virus neutralizing antibody response but maintain high antibody titers to the cell membrane antigen and a disease-free status (Jarrett *et al.*, 1973b; W. D. Hardy, Jr., personal communication; Essex *et al.*, 1974). We also observed that antibody titers developing to the virus core antigens bore no relation to the development of antibody titers to the cell membrane antigen (Olsen *et al.*, 1974). Studies of the role of cell-mediated immunity in the regression or prevention of feline oncornavirus-induced tumors in cats have not been published, although a mononuclear cell infiltrate has been observed in regressing tumors

TABLE IV

Humoral Antibody Response of Cats from Several Categories to Feline Oncornavirus-Associated Cell Membrane Antigen

Group	Number examined	Titer of antibody[a]	
		Range	Geometric mean
(1) FeSV injected			
(a) Progressors	42	0–4	0.6
(b) Regressors	25	4–256	18.5
(c) No tumor developed	8	8–128	29.8
(2) FeLV injected			
(a) Leukemia	7	0–1	0.2
(b) No tumor	2	8–64	22.7
(3) Spontaneous leukemias	63	0–16	1.2
(4) Laboratory contact controls[b]	23	0–128	7.9
(5) Naturally exposed but healthy cats in cluster leukemia households[c]	119	0–256	12.5
(6) Street controls	96	0–256	1.0

[a] Reciprocal of highest twofold serum dilution still positive by membrane fluorescence.
[b] Exposed to either FeLV or FeSV injected littermates.
[c] Most cats in this category with low titers were viremic.

(Snyder, 1971). It is quite possible that both cell-mediated immunity and humoral antibody directed to the tumor cell play an important role in the feline system, as apparently they do with murine oncornavirus-induced tumors. It seems very unlikely that humoral antibody plays a deleterious role in feline tumor development, since high circulating antibody levels are only found in regressor or tumor-free cats, never in progressor tumor-bearing animals. The reverse would be expected if antibody was primarily acting to block or interfere with the cell-mediated immune response. Table IV summarizes the antibody response to the feline oncornavirus-associated cell membrane antigen for cats from several categories. An immune response to the feline oncornavirus-associated cell membrane antigen has been found in monkeys (Essex *et al.*, 1972), dogs (Slauson, 1973; Essex *et al.*, 1973b) and pigs (L. D. Pearson *et al.*, 1973) exposed to FeSV. Dogs and pigs developed high antibody titers when tumors regressed, but this relationship was not evident with the monkeys examined. Cell-mediated cytotoxicity was also detected in regressor dogs.

VI. Immune Response to Murine Oncornavirus Infections

As opposed to the situation with avian and feline oncornaviruses, the only significant transmission route for murine oncornaviruses in laboratory strains of mice appears to be vertical (Rich, 1968; Gross, 1970; Huebner and Gilden, 1972). In strains of mice that have a high incidence of leukemia this transmission appears to be genetic rather than extrachromosomal. This suggests that when artificial selection is based on expression of a malignant disease, such as leukemia, the development of a stable pattern for transmission of viral genes may indeed occur. The possibility remains, however, that the model may not represent what occurs when either nature takes its own course of selection or when inbred strains are selected for reasons other than the expression of malignancy. These selection processes might instead follow the classical guidelines for "survival of the fittest." Under such circumstances we might expect proportionally much less host resistance against evolutionary pressure to "genetically transmit" only C type virus genomes that have lost their oncogenic potential. This could become further complicated by at least two additional issues that may be related. First, the possibility that certain proteins coded by the viral genome, such as the viral core gs antigens, might have an overlapping function in normal embryogenesis (Hirshorn *et al.*, 1973; Huebner and Gilden, 1972). If this could be demonstrated, it would obviously substantiate the case for

true genetic transmission. A second point is the possibility that the apparent Mendelian expression of cancer being due to direct inheritance of C type virus genes may in effect be due to very specific genetic control over classes of immune response genes affecting host response to oncornaviruses or oncornavirus-induced antigens (Benacerraf *et al.*, 1971; Tennant, 1972; Klein, 1973). Such circumstances might make it possible for extrachromosomal vertical (transplacental or congenital) transmission of viruses to be misinterpreted as chromosomal.

Evidence with strains having a low spontaneous incidence of leukemia are easier to interpret as extrachromosomal rather than true genetic. For the most commonly studied murine oncornaviruses (Gross, Moloney, Friend, Rauscher), the transmission is primarily maternal. Although both transplacental and congenital colostral infection occurs, transmission through the milk appeared to be most common, since leukemia incidence could be greatly reduced by foster nursing. It has been shown that infected mice excrete the virus (Mirand and Mirand, 1969; Myers *et al.*, 1970), but this is believed to be of little or no significance in laboratory strains (Myers *et al.*, 1970). Studies with oncornaviruses of wild mice, such as those by Gardner *et al.* (1973), will be very important in determining the infection and transmission routes under natural conditions.

The relative absence of horizontal transmission in laboratory mice, as opposed to cats and chickens, would lead to the prediction that mice would be less likely to mount an effective immune response to the virus due to immunologic tolerance. Observations of an apparently inefficient antibody response of mice to virus core antigens tend to support this hypothesis. That mice may exhibit immunologic tolerance to the virus core antigen is probably not significant for preventing tumor development, however. Only those immune responses directed at the virus envelope or tumor cell membrane antigens would be expected to interfere with oncogenesis, and these appear to be functioning to at least some degree. Evidence for this is the fact that mice succumb more rapidly to oncornavirus infections when artificially immunosuppressed (Allison and Law, 1968; Law and Chang, 1971; Law, 1972; Larson *et al.*, 1972; Zisblatt and Lilly, 1972). That oncornaviruses themselves can induce immunosuppression could be a very important factor in disease pathogenesis (Notkins *et al.*, 1970; Dent, 1972).

Mice injected or naturally infected with leukemogenic viruses experience persisting viremia that can exist in the absence of pathology for long periods, especially in the case of the less acute thymic lymphomas. At some stage prior to the emergence of evident gross pathology, mononuclear cells with virus-associated cell membrane antigens can be de-

tected. In the case of lymphoma induced by Moloney virus and erythroblastic leukemias induced by Friend or Rauscher viruses, membrane antigen-positive cells are found localized in the spleen (Wirtz *et al.*, 1969; Cerny *et al.*, 1973). In the case of Friend disease, we found that 50% or more of the mononuclear spleen cells were membrane antigen-positive by at least 4 days after virus injection (Cerny and Essex, 1974). The number of spleen cells positive for Friend virus-associated cell membrane antigen then rises to 80% or more by the time disease signs are pronounced. In the case of "regressing" Friend leukemia (Rich *et al.*, 1969, 1971; Rich and Clymer, 1971) the splenic enlargement diminishes back to normal and the antigen-positive cells largely disappear (Cerny *et al.*, 1973).

Mice immunosuppressed by treatment with anti-lymphocyte serum show increased susceptibility to MuLV and MuSV oncogenesis (Law, 1972; Larson *et al.*, 1972; Zisblatt and Lilly, 1972). Tumor frequency is higher and latent periods shorter in mice so treated. Law (1972) found that such an immunosuppression-mediated increased tumor susceptibility could not be reversed by immunotherapy with either hyperimmune cells or serum in the case of Moloney virus-induced lymphomas. G. R. Pearson *et al.* (1973), on the other hand, found that passive administration of MuSV regressor serum could provide effective immunotherapy for mice with transplanted Moloney leukemia virus-induced tumors, when single injections of serum were given anytime from 3 days before to 2 days after tumor challenge. Multiple injections of hyperimmune serum during the latent period for tumor development resulted in up to 90% successful prevention of early death from lymphoma. Evidence for development of a cell-mediated immune response for oncornavirus-induced mouse lymphomas has also been found (Fass and Fefer, 1972; Leclerc *et al.*, 1972; McCoy *et al.*, 1972). The development of cell-mediated cytotoxicity was observed to occur at about the time tumors became palpable. A decline in the number of immune cells occurred if the tumors progressed. It was suggested that such an apparent decline could be due to possible masking of immune cells *in vivo* by blocking factors.

Several studies have involved the immune response of mice to Rauscher virus-induced lymphomas or transplants of Rauscher virus-induced tumors (Mayyasi *et al.*, 1968; Barski *et al.*, 1972; McCoy *et al.*, 1972; Youn *et al.*, 1973). These investigators agreed that the cell-mediated immune response became ineffective *in vivo* at the time tumors are most actively progressing. This defective functioning is specific, since the same mice can mount a normal immune response to transplanted syngeneic polyoma cells while they are unable to resist

Rauscher tumor transplants (McCoy *et al.*, 1972). Disagreement exists over the possible reasons for this failure. One study suggested that a particular type of antibody might be the blocker that interferes with cell-mediated immunity because such antibody was found in progressor animals (McCoy *et al.*, 1972). A second study, however, disagreed because the serum blocking factors acted much more efficiently on immune effector cells than on target tumor cells (Barski *et al.*, 1972; Youn *et al.*, 1973). Such results would be more compatible with a blocker of either sloughed soluble tumor cell membrane antigen alone or with the antigen complexed with antibody in antigen excess. The immune response of rats to a transplantable Gross virus-induced tumor has also been studied (Herberman and Oren, 1971; de Landazuri and Herberman, 1972). Cytotoxic antibodies and cell-mediated immunity appeared within 1 week after injection of tumor cells and remained elevated for several weeks. Neither the level of cell-mediated nor humoral immunity correlated well with whether or not the tumors regressed. Good evidence exists, on the other hand, that humoral antibodies passively obtained by newborn rats are very effective for preventing Gross virus-induced tumors (Ioachim, 1970; Ioachim *et al.*, 1973). It is likely that virus neutralizing antibodies are largely responsible for this effect. It is possible, however, that the immune response for rats, an unnatural host for Gross virus, may be different from the response expected for mice with primary virus-induced tumors.

Considerably more work has been done to study the immune response to oncornavirus-induced fibrosarcomas. This system is experimentally more attractive because (a) the tumor latent period is shorter than with the leukemias and (b) by adjusting virus dose and age at inoculation, a desired number of spontaneous regressions can be made to occur. The potential disadvantage of working with fibroblastic target cells rather than lymphoid cells, which are easier to manipulate for membrane fluorescence and cytotoxicity tests, can be eliminated by using the appropriate cross-reacting oncornavirus-induced lymphoma target cell in such tests.

Significant differences exist regarding the beneficial effects of serum and cellular components on inhibiting growth of MuSV-induced tumors. Experiments with a combination chemotherapy–immunotherapy model suggested that, while therapy with regressor serum had some benefit, it was not nearly as pronounced as the beneficial effects observed when therapy was done with immune cells (Fefer, 1969, 1970). A second set of experiments showed that regressor serum was very effective for preventing the growth of transplanted Moloney sarcoma cells (G. R. Pearson *et al.*, 1973). In a third system, where mice were im-

munosuppressed with anti-lymphocyte serum to increase their risk for tumor progression, regressor serum appeared to be more effective than immune cells for inducing resistance to tumor death (Law, 1972).

Lamon *et al.*, (1972a) followed the cell-mediated cytotoxicity response of mice given MuSV injections and found that cytotoxic lymphocytes were present from 2 days after virus infection until tumors completely regressed. They found that the lymphocyte cytotoxicity response was considerably reduced during tumor progression, as others had found for virus-induced lymphomas (Barski *et al.*, 1972; Youn *et al.*, 1973). It was particularly interesting that the most important effector cell in the MuSV cell-mediated cytotoxicity response appeared to be of bone marrow rather than thymus origin (Lamon *et al.*, 1972b).

The considerable differences in results regarding the relative efficiency of cellular and humoral components to control tumor development by murine oncornaviruses could be largely due to differences between the viruses used, the different strains and species of hosts used, the types of inocula used for sensitization and challenge, and differences between the various *in vivo* and *in vitro* assays for immunity.

VII. Summary and Conclusions

Oncornaviruses of chickens, cats, and mice are morphologically very similar, although fine differences can be discerned by electron microscopy. These virus groups, defined according to species of isolation, possess virus envelope glycoproteins and virus core proteins that are biochemically similar. The proteins are antigenically distinct for each group, the only exceptions thus far being the virus core interspecies antigen (gs 3) that is common for cat and mouse viruses. The principal virus core antigens are apparently identical for all viruses within each group. The principal and most immunogenic virus envelope antigens, on the other hand, are subgroup- or type-specific. These antigens also follow the subgroup classification based on virus receptor susceptibility and interference patterns. New oncornavirus-associated cell membrane antigens are also expressed following infection and/or transformation by these viruses. The principal tumor cell membrane antigens are group-specific and antigenically distinct from virus envelope antigens.

The mode of transmission and route of infection with oncogenic oncornaviruses is apparently different for the three major groups. Avian and feline viruses appear to be transmitted horizontally, while this route seems unimportant for the murine viruses. In the case of all groups it is difficult to determine the role of endogenous chromosomally transmitted

but largely nononcogenic viruses. The type of virus transmission that occurs under natural circumstances appears to be related to the degree of genetic variation for each host species, since horizontal transmission is most important in the outbred cat and least important in inbred mouse strains. This suggests that selection of apathogenic viruses may follow inbreeding, and vertical transmission may follow the evolution of apathogenic virus strains.

Both the magnitude and nature of the immune response to oncornavirus infections depends on the infection route, since vertical infection prior to the development of the immune response should result in immune tolerance to those antigens expressed during embryonic life. Although it was previously thought that animals were tolerant to the virus core antigens, substantial evidence to the contrary has been obtained in the case of chickens and cats.

The immune response to virus core antigens probably plays little or no role in control of either virus replication or tumor growth. The immune response to virus envelope antigens can eliminate virus through neutralization. This response probably has no effect on surveillance of growing tumors, but may indirectly limit tumor growth by inhibiting cell transformation. This response may be more important when viruses of lower transforming efficiency are involved (leukemia viruses as oppossed to those causing sarcoma).

The immune response to oncornavirus-induced cell membrane antigens is probably the primary immune mechanism for controlling tumor growth. The immune response to this group of antigens is distinct from, and does not necessarily follow, the responses to either virus envelope or core antigens. The immune response directed to the cell membrane antigens can probably both block tumor outgrowth and induce regression of some established tumors. In the case of spontaneous and induced fibrosarcoma and leukemia of outbred cats, the humoral antibody response to the oncornavirus-induced cell membrane antigen is closely correlated, in an inverse sense, with tumor growth. Cats that develop high antibody titers either develop no visible tumors or develop tumors that regress, while cats that fail to develop high antibody titers die with progressing fibrosarcoma or leukemia. Both cell-mediated and humoral responses appear to contribute in a favorable sense to this activity. Although a blocking effect of the cell-mediated immune response has been observed, this is more likely to be due to circulating sloughed tumor cell membrane antigen alone and/or this antigen complexed in excess with antibody, rather than to either antibody alone or complexes with antibody in excess. This observation, along with the finding that non-T cells appear to be the important mediator of cell-mediated cyto-

toxicity in the case of murine oncornavirus-induced sarcomas suggests that B cells may play a very important role in the immune response to oncornavirus infections.

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CHAPTER 19

Prospects for Vaccines against Cancer

MAURICE R. HILLEMAN

I. Introduction

Perhaps no subject is more appropriate in a book on comparative virology than that of discussing the prospects for vaccinating against cancer in man. I say this because we know so little about the cause of cancer in man, and we know so little also about the immunology of cancer in man. Those of us who are charged with the responsibility of seeking practical application for control of cancer from the abyss of ignorance must, of necessity, give attention to those parallel developments in animal experiments that are of outstanding accomplishment and that may provide clues for technological advances that may be eventually translated to man. Our only approach for the present lies in learning what we can from comparative oncology and in establishing systems and precedents that can be applied to future developments for man. It may be worthy of note that the foundations for modern knowledge of mammalian RNA leukemia and sarcoma viruses are based on the pioneering studies carried out with chicken viruses in the chicken model system. It seems true also that the further progress with oncogenic mammalian her-

pesviruses will have its foundation in the advances that have been made with Marek's disease in chickens. The domestic fowl has provided fine models for studies of cancer and the contributions that have been made by the chicken virologists are something worth "crowing about."

II. Cancer

In its simplest concept, cancer is akin to an unruly offspring. It is a disease in which a cell or cells become disobedient to the parent host and multiply indiscriminantly. At the same time, the parent host becomes permissive and loses partial or total control.

The question is why, and it seems axiomatic that two events must occur if there is going to be clinical cancer. First, something must happen to the cells to make them different—thus, neoplastic transformation. Second, something must happen to the host to render it permissive—thus, a failure of immunologic surveillance and homeostasis. In any discussion of the immunologic control of cancer, then, one needs focus on two points alone—how does one prevent neoplastic transformation in the first place? And how does one enhance the host's capability for control of the neoplastic cell once it arises?

Specific Control

1. Viral Vaccines

a. Etiology. It is well established that a great many factors have something to do with cancer: ionizing radiation, environmental carcinogens, age of host, hormones, genetic factors, and the like. It is also well established that a great variety of tumors of animals are caused by viruses, and one needs only to point to the role of viruses in the malignant neoplasia of rodents, cats, fowl, frogs, and bovines with ancillary information obtained in studies of cancer in monkeys and man. This leads to the concept that the one indispensable element in cancer may be a virus or its genetic material, and all other factors may only be secondary. In taking this position, we are reinforced by the fact that cancer cells seem to have a new genetic input that allows them to make new and unique antigens that are present in the cells and on their surfaces. Carcinogenic chemicals and physical agents, such as ionizing radiation, do not provide such input—they only rearrange the output. If it is true that virus infection is indispensable in cancer, then the prevention of viral infection or the negation of the viral effect might permit the

breaking of an essential link in the neoplastic chain and so make the prevention of cancer possible.

This then has provided the motivation for seeking the virus or viruses that cause cancer in man. Current research in viral etiology of human cancer has been focused on the C and B type RNA viruses that are being linked with leukemia, sarcoma, and breast cancer mainly by the demonstration of viruses or viruslike particles in human neoplastic tissues and secretions. To date, however, no reliably propagable RNA virus has been recovered from human neoplasia that can be considered to be a serious candidate for an etiologic role in man; this in spite of the intense competitive effort given to it and in spite of the private and public proclamations to the contrary.

The DNA viruses have fared somewhat better, and the herpesviruses can be seriously considered as candidates for several neoplasia in man including Burkitt's lymphoma and nasopharyngeal carcinoma and possibly also cervical and prostatic neoplasia and Hodgkin's disease. As with the RNA viruses, however, proof of etiologic role remains elusive and is frustrated by the inability to carry out direct etiologic studies in the human species.

b. Spread. In judging the probability for developing vaccines against cancer, it is necessary to consider the means for spread of the virus, whether it be horizontal as in infectious disease or vertical as in transmission from mother to offspring either through transplacental infection or through integration in the germ plasm. Cancer viruses transmitted horizontally postnatally at an adequate time period after birth may be very well subject to prophylactic control by vaccine. Cancer viruses transmitted vertically afford less chance for vaccine control, primarily because infection would already have been established prenatally and because immunologic control might have been preempted in part at least by induction of immunologic tolerance. Attempts at therapeutic application of viral vaccines, either to prevent the expression of neoplasia in virus-dependent cancer or to limit its expression as clinical cancer, deserve special consideration since they involve an area in which exploratory clinical testing in man could be justified at the present time.

There is presently a theory that is fashionable, if not of significance, that is based on the observation that many if not all cells carry genetic materials called virogenes and oncogenes that code for infectious virus and for the neoplastic transformation of cells. Although normally repressed, the virogenes and oncogenes may be derepressed by a variety of means, such as carcinogenic chemicals, radiation, aging, DNA viruses, or perhaps by the assistance from related RNA helper viruses to cause cells to undergo neoplastic transformation and to produce infec-

tious transmissible virus bearing the oncogene. The theory, in effect, says that genes for RNA tumor viruses are part of the genome of animal cells. These genes have a role in (a) normal embryogenesis, (b) specifying viral genomes, and (c) acting as determinants of cancer.

If indeed, the ubiquitous oncogene were responsible for the generality of cancers, then the chance for immunologic interruption by viral vaccine would be small. But, it is a fact that infectious oncogenic RNA viruses introduced artificially by syringe and needle do cause cancer and that in some instances these viruses may correspond completely to the endogenous oncogene. Further, cat and chicken leukemia viruses can be horizontally spread. The interpretation, then, is that even if cancer can arise as a spontaneous event through activation of latent oncogenes, cognizance must be taken of the fact that cancer can also result from superinfection with an adequate dose of the corresponding infectious virus, which is already bearing the oncogene, or from horizontally spread infection with an infectious oncogenic virus. The basic question, then, is *which* kind of event—assuming both do occur in man—is *mainly* responsible for cancer as it occurs in the human species. It is probable that both may occur, and it is possible that the latent oncogene, in the practical sense, may only be the repository in nature for these viruses and the means by which their perpetuity is assured.

c. Vaccines. Experiments aimed at evaluating the virus vaccine approach to control of experimental cancer in animals have been limited to date. There are, however, sufficient examples to establish the utility of the approach to vaccination against oncogenic viruses in several kinds of circumstances in experimental cancer, and these may provide guidelines for the development of human cancer vaccines. One can cite very positive results in immunizing against the avian leukemia-sarcoma complex, against the murine leukemia-sarcoma complex, against the adenoviruses that are oncogenic for animals, and against SV40, polyoma, and myxomatosis viruses. Of special interest, immunization of mice with killed murine leukemia virus antigens in Freund's adjuvant have been shown recently to reduce significantly the incidence of tumors induced by 3-methylcholanthrene. Vaccines, in the case of RNA virus-dependent neoplasia, may even show some degree of therapeutic effect presumably by limiting the spread of the virus to new and uninfected cells within the infected host. With both RNA and DNA virus-induced tumors, vaccines may increase the recognition and response by the host to tumor cells that carry viral-specified antigens on their surface.

d. The Feline Leukemia-Sarcoma Model. The feline leukemia-sarcoma complex presents an excellent model in comparative oncology for

testing vertical versus horizontal spread of cancer and for evaluating the effectiveness of vaccines. It is the prime model for RNA oncogenic viruses that is being pursued in our laboratories. The cat might be considered an especially meaningful model for such studies, since the cat, like man, is outbred, and it is subject to many, if not most, of the same environmental carcinogens to which man is subject and which might play a role in the induction of cancer. The rates for spontaneous leukemia and sarcoma in cats are low and roughly in the same range as in man, considering the difference in life span. Most important, while cats do harbor covert indigenous leukemia viruses, horizontal transmission of the oncogenic virus does occur, and the role of the vertically transmitted genome in relation to clinical cancer as it occurs in nature is in serious doubt. Significantly, adult immunologically competent cats can be infected artificially or naturally with the virus and this can result in the induction of leukemia. With further study, it may be hoped that this model will present the means whereby live, killed, and subunit vaccines can be evaluated for prophylactic and therapeutic efficacy and whereby information can be obtained that can be applied eventually to the development of vaccines for use in man.

e. Marek's Disease Vaccine. The most outstanding example of vaccine success is the recent development of highly effective live virus vaccines against Marek's disease of chickens. Marek's disease is a lymphoproliferative neoplastic disease of chickens that causes great economic loss to the poultry industry. The disease is caused by a B type herpesvirus, and the development of the vaccine, in my judgment, is perhaps the most outstanding achievement in the virus cancer field in the last decade or two. The demonstration of an exclusively horizontal transmission of the herpesvirus that causes Marek's disease and the prevention of the disease by use of highly effective attenuated live herpesvirus vaccines present some of the best evidences against the universality of cancer caused by a ubiquitous oncogene.

The most widely used Marek's disease vaccine is a preparation of a naturally avirulent herpesvirus of turkeys that is antigenically related to Marek's disease virus but causes no tumor.

The manner in which the vaccine works is of interest as shown in Table I. The fully virulent infectious virus is released from chickens in feather follicle cells that make up chicken dander. The virulent virus infects susceptible chicks in the environment, establishes an infection that persists for the lifetime of the bird, and causes neoplasia with tumor production, deficiencies in the conversion of carbohydrate to protein, and death.

TABLE I

Progress of Marek's Virus Infection in Vaccinated and Unvaccinated Chickens

Agent	Infection	Clinical outcome
Virulent Marek's herpesvirus (in chicken dander)	Persistent lifetime infection	Death, tumors, decreased egg and meat production
Turkey herpesvirus (avirulent vaccine – parenteral)	Persistent lifetime infection	Dual viral infection but with failure to develop clinical disease
→ Virulent Marek's herpesvirus (in chicken dander)	Persistent lifetime infection	

The avirulent herpesvirus vaccine is given to chicks at 1 day of age and like the virulent virus, it also establishes a viral infection that persists for the lifetime of the bird, but it differs in that it does not cause clinical disease. Importantly, however, the avirulent virus does not prevent subsequent infection with the fully virulent Marek's virus. What the prior infection with avirulent virus does is to prevent the expression of the virulent virus as clinical neoplasia. This immunity against the development of cancer appears to reside either in some ill-defined biological antagonism between vaccine virus-infected cells and virulent virus-infected cells (e.g., interferon or intrinsic interference) or, perhaps, in the retention of a degree of immunologic function of the lymphoid reticular immune mechanisms. It is known that infection with Marek's virus does affect both the bursal and thymic lymphoid systems in chickens, with reported depression of both cellular and humoral immune responses. A complementary finding is provided by the observation in some but not all trials of Marek's vaccine that there can be reduction in deaths due to causes other than definable Marek's disease. Assuming validity of the findings, such evidence is consistent with retention of a functional immune system that can cope with a variety of life-limiting disease agents.

The conditions of presentation of the vaccine is of considerable importance in affecting its efficacy. In studies carried out by Dr. R. L. Kilgore and others of vaccine produced in our laboratories, the protective efficacy of the ordinary cell-associated vaccine runs about 80% (see Table II). The efficacy level is about the same when the more recently developed naked virion or cell-free vaccine is used in chicks hatched from dams that were not vaccinated with the turkey herpesvirus. When, however, the cell-free vaccine is employed in chicks that carry maternal antibody to the turkey herpesvirus, there is a delay in antibody produc-

TABLE II

Influence of Turkey Herpesvirus Maternal Antibody on the Efficacy of Cell-Free (Dried) Compared with Cell-Associated (Frozen) Marek's Vaccine

Kind of vaccine	Occurrence of Marek's disease			
	Group	No. positive / total	% positive	Protective efficacy (%)
Study 1[a]				
Cell-free (dried)	Vaccinated	40/1000	4	82
	Control	230/1000	23	
Study 2[b]				
Cell-free (dried)	Vaccinated	133/800	17	39
	Control	220/800	28	
Cell-associated (frozen)	Vaccinated	58/800	7	73
	Control	208/800	26	

[a] Chicks without turkey herpesvirus maternal antibody.
[b] Chicks with turkey herpesvirus maternal antibody.

tion, in vaccine-induced viremia, and a drastic reduction in protective efficacy (to 39%) (R. L. Kilgore, unpublished). It is apparent in the studies that naturally acquired Marek's virus antibody does not markedly affect the efficacy of the cell-free vaccine. It is apparent also that with cell-associated vaccine, in which virus may be spread from cell to cell by contiguous contact and without exposure to antibody, the vaccine may be fully protective in spite of the presence of homologous antibody of maternal origin.

There are at least two important lessons to be learned from Marek's vaccine, I believe. First, is the lesson that the vaccine need not prevent infection with virulent virus in order to prevent cancer. Instead, it needs only to limit the ability of the virulent virus to express itself clinically as cancer. Second, there is the alert to the need for search for avirulent counterparts of human oncogenic viruses, and it is possible that these might be bountifully distributed in nature.

f. Simian Cancer Viruses. Five oncogenic viruses of nonhuman primates have been discovered to date. Two of these are herpesviruses, viz., herpesvirus saimiri recovered from kidneys of squirrel monkeys (*Saimiri sciureus*) and herpesvirus ateles isolated from kidney culture of a black spider monkey (*Ateles geoffroyi*). Both viruses are transmitted horizontally in the parent species without causing apparent disease. In several heterologous simian species including marmosets, however, the viruses can cause lymphoma or reticulum cell tumor and leukemia in

adult animals after a short incubation period. These viruses present excellent models for studies for immunologic interruption of cancer in primate species using live, killed, or subunit viral vaccines. They are of special importance because of their possible use in elucidating the interactions among Epstein-Barr virus, Burkitt's lymphoma, and nasopharyngeal carcinoma in man.

g. Herpes Simplex. The only candidate human oncogenic viruses worthy of vaccine consideration at this time are those of herpes simplex. These viruses have been implicated in oral and genitourinary cancers of various kinds based on seroepidemiologic findings, on the demonstration of herpesvirus antigens or viral nucleic acid components in neoplastic tissue, on the occurrence in cancer patients of antibody against virus-specified nonvirion antigens, and on the induction of neoplastic transformation *in vitro* by irradiated virus.

The limitation of acute clinical disease caused by herpesviruses, once infection is established, seems to reside in mechanisms other than antibody, since herpetic disease may reoccur in spite of high levels of neutralizing antibody. Instead, immunologic control appears to reside in cell-mediated immune mechanism, based on response to antigens presented on the cell surface that are specified by the virus. Antibody may, however, be of great importance in preventing or limiting primary herpesvirus infection and should be of value if induced by vaccine. Unfortunately, killed vaccines of significance have not been adequately tested as preventives of primary infection. Instead, they have been used in attempts to cure recurrent disease. They have not been remarkably successful but they do show some evidence for being able to lessen the severity of repeated herpetic attacks and in bringing about remission in some cases. Live attenuated herpesvirus vaccines have only seen abortive trials in man, and the results have not been encouraging. The technology, now, for preparing highly purified subunit antigens and the availability of safe and effective immunologic adjuvants open the way to purposeful research investigations toward this important group of infections. Prevention of infection with prevention or limitation of the acute disease might be anticipated also to limit or prevent cancer if it be one of the longer term effects of the virus.

2. Tumor Antigens

a. Cancer Immunology. The alternative to preventing viral infections that bring about neoplastic transformation is the attempt to alter the host's ability to deal with neoplastic cells. It may be a long time before tumor immunology can bring about prevention of cancer, but it is

within our grasp to attempt improved therapy and diagnosis based on immunologic procedures.

Specific immunologic control is based on the well-known observation that neoplastic cells have abnormal antigens present that can function as weak histocompatibility antigens and may be the basis for immunologic rejection just as in homotransplantation. Cancer, in its broadest sense, may be regarded as an immunologic deficiency disease in which the potential to apprehend and destroy neoplastic cells of particular antigenic constitution is reduced or nonexistent in the first place. Credence can be given to this concept based on the observed outcome of purposeful destruction of part of the immunologic surveillance system as, e.g., by thymectomy of the neonatal animal, by treatment with antilymphocytic serum, by massive treatment with corticosteroid, or by exposure to whole body irradiation. Under such conditions, the surveillance potential is reduced and the immune system, no longer intact, becomes permissive allowing for continued proliferation of neoplastic cells in the absence of responsiveness to the feeble regulatory signals that might remain.

b. Tumor Cell Vaccines. One approach to bolstering the host's capacity to reject tumor is by use of tumor cell vaccines. These are intended to provide immunological reinforcement by getting more tumor antigens of the proper sort into the correct sites for appropriate immune responses. Favorable immune responses are most generally regarded as those that involve the cell-mediated immune response, i.e., sensitized thymus-dependent lymphocytes or T cells and perhaps macrophages to some degree.

Perhaps the greatest roadblock to the application of tumor-specific immunization is in the plurality of antigenic constitution of tumors, especially as shown experimentally with chemically induced tumors. Hence, practical application might be limited to the use of the patient's own tumor tissue to bolster his resistance to the further progression of his disease. There is, however, a commonality in tumor antigens induced by a single virus, and also to some degree in spontaneous tumor of similar type, that might permit a wider application of the principle of tumor antigen immunization.

In our own long years of investigation as well as in studies by others, the only kind of tumor cell vaccine that consistently prevents tumor, whether induced by virus or given by transplant, is a vaccine comprised of tumor cells that have not been killed but have been rendered nonreplicative by exposure to γ radiation or by treatment with iododeoxyuridine. Breakage of the cells to separate out tumor antigen from normal host cell

antigen that might be troublesome in vaccines because of the possibility for inducing autoimmune disease has nearly always destroyed the potency in our experience. There have been a few reports of successful isolation of immunizing antigens in other systems, however, and this provides the basis for continued hope that effective and safe purified tumor antigens can be derived. In our own studies, we are now giving emphasis to recovery of cell membrane polypeptides and glycoproteins that, hopefully, will be found to carry tumor cell specificity that will be separable from normal cell antigens, and that will retain immunogenic activity.

One particular kind of vaccine evolved by W. T. Shier and referred to as a chemical vaccine has attracted our attention and we have studied it extensively (M. M. Cook, A. F. Wagner, V. M. Larson, A. A. Tytell, T. Y. Shen, and M. R. Hilleman). The vaccine is based on the observation that neoplastic cells have on their cell surfaces increased amounts of receptors that render them agglutinable by plant lectins, such as wheat germ agglutinin. Shier determined that the receptor was chemically similar to di-*N*-acetylchitobiose, an aminodisaccharide that can be obtained from the exoskeletons of crustacea. He condensed di-*N*-acetylchitobiosylamine with sodium poly(L-aspartate) and coupled the whole with bovine serum albumin to render it antigenic. The complex was emulsified in Freund's complete mineral oil adjuvant. In a small series of experiments, Shier recorded a beneficial effect of the vaccine against transplant myeloma of BALB/c mice and against tumor induced in BALB/c mice by 3-methylcholanthrene.

Our efforts were directed to preparing the Shier's vaccine in the same way and to testing its efficacy in the same systems. The results shown in Table III clearly indicate a lack of significant beneficial effect. While

TABLE III

Tests with Shier's Chitobiose Vaccine

	Tumor development (No. with tumor/total)		
	Transplant challenge[a] (9 weeks)		
Material	1×10^6 cells	1×10^7 cells	3-Methycholanthrene (0.5 mg) challenge[b] (20 weeks)
Vaccine	6/15 (40%)	15/16 (93%)	32/45 (71%)
Placebo	5/17 (29%)	14/17 (82%)	35/45 (78%)

[a] Shier's regimens I, II, VI; results combined.

[b] Shier's regimens IV and V; results combined.

these results are disappointing, they in no way detract from the target of eventually evolving effective tumor-specific antigens for immunization purpose.

In immunizing against tumor, the manner of presentation to the host is of greatest importance, lest one stimulate the formation of blocking factors, such as blocking antibody or circulating tumor antibody–antigen complexes, that might bring about enhancement of tumor rather than cell-mediated immunosuppression. This is not to imply, however, that antibody responses are always adverse and that antibody plays no role in tumor immunity.

Discussion of tumor cell antigen vaccines would not be complete without mention of the need to reduce the target tumor cells to the absolute minimum if an effective immune response is to be achieved. Clearly, the system of immunologic surveillance was designed to handle the sporadic appearance of abnormal cells and could hardly be expected to be significantly effective when grams of tumor tissue are involved. Hence, tumor antigen vaccine would be expected to be most useful if given even before the appearance of the first tumor of like antigenic constitution. Failing this, such vaccine should be most effective in handling distant metastases when they are still of minimal size at the time of surgical removal of primary tumor. Such vaccines might be used after irradiation or chemotherapy has been applied to reduce the mass of residual tumor to a minimum.

c. Nonspecific Control. The attempt to prevent or treat cancer by specific stimulation of the immune system must necessarily take cognizance of the existence of nonspecific measures for immunity and the nonspecific manner in which these effector components may be stimulated to intervene in cancer. The differentiation between tumor-specific and nonspecific immunity resides in a gray area and refers primarily to the means by which the immune processes directed against tumor are initiated. The use of nonspecific measures for treating or preventing cancer is clearly feasible, and the attempt appears best directed to stimulating the accumulation of mononuclear cells that are active in delayed hypersensitivity. This includes both lymphocytes and macrophages that can be activated to act aggressively against neoplasia.

Patients with cancer usually display some degree of general immunologic impairment. One approach to restoring immune competence presently being pursued is by administration of BCG or its fractions. These act by inducing sensitivity to the mycobacterium which is so reactive that it increases the immune response against tumors as well and prevents their further development. Special use can also be made of immunologic memory in patients who may no longer be able to mount a

primary sensitivity response against a new antigen but who may be able to respond to a previous sensitivity, such as for PPD (tuberculin) or mumps antigen. Transfer factor extracted from lymphocytes sensitized to a particular tumor antigen may confer specific antigen responsiveness in the recipient who is unable to do so on his own. The recent discovery of thymosin, a factor regulating T cell activity of lymphocytes, might find eventual use for maintaining and promoting cell-mediated immunity.

III. Closing Remarks

In closing, it may be said that the immunologic control of cancer is only now seeing its serious beginnings. A concerted effort is being directed to the development of means for immunologic alteration of the host to increase his resistance to cancer either by preventing or limiting infection with oncogenic viruses or by increasing resistance to cancer on tumor-antigen or nonspecific immunostimulatory bases. Thus, the probe is being made to develop means for rational immunologic intervention in cancer where no means now exist. The odds are long but the enthusiasm runs high. Many of us who are engaged in cancer work of this nature think of achievement of targets in terms of decades and perhaps even centuries. Such is the nature of cancer and its historic development thus far.

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CHAPTER 20

Visualization of the Evolution of Viral Genomes

WACLAW SZYBALSKI AND ELIZABETH H. SZYBALSKI

I. Introduction

The evolution of viral genomes proceeds by mutation and genetic recombination. Until recently, most of our knowledge on mutation and recombination was derived from genetic analysis. We shall be concerned here mainly with the complementary approach of physical mapping by electron micrography of heteroduplex DNA, according to the formamide technique developed by Westmoreland *et al.* (1969). This

method readily permits the detection and precise localization of genetic changes that involve more than 10 to 50 nucleotide pairs, but in its simple form is not suited for detecting point mutations and rearrangments encompassing shorter sequences.

In brief, the principle of the technique is as follows. When a separated DNA strand from a particular viral strain is annealed in 50% formamide solution with a complementary strand from a genetically different mutant, the regions of sequence homology form duplex DNA, whereas the heterologous segments remain single stranded, within the defined heteroduplex molecule. The DNA is spread on the surface of distilled water in a cytochrome c matrix, according to the basic protein monolayer technique of Kleinschmidt and Zahn (1959), and finally visualized by electron microscopy and the contour lengths measured photooptically. Simple deletions, insertions, or tandem duplications appear as single-stranded "loops" emanating from a single point in the otherwise duplex DNA (Fig. 1a). Other genetic rearrangements, including substitutions and inversions, form single-stranded regions of nonhomology, referred to as "bubbles" (Fig. 1b). Unpaired regions can also be located at the ends of the DNA molecule and are denoted as "split ends" (Fig. 1c). Inversions appear as symmetric bubbles when the l_A strands are annealed with the complementary r_B strands (Fig. 1d), or as short duplexes within otherwise unpaired DNA when the like ($l_A + l_B$ or $r_A + r_B$) strands are annealed (Fig. 1e).

A very complex, largely duplex arrangement is obtained for $l_A + r_B$ heteroduplexes between noninverted translocations or identical insertions (Fig. 5 and Malamy *et al.*, 1972), but if one of them is inverted in respect to the other, the $l_A + l_B$ (or $r_A + r_B$) heteroduplex will appear as in Fig. 1e. Several other configurations are possible (Fig. 1) and some of these will be discussed. The configurations depicted in Fig. 1 are those obtained with the formamide technique of Westmoreland (1968), in which the mild denaturing action of the solvent renders the single strands visualizable in their extended form. In the independently developed "aqueous" technique of Davis and Davidson (1968) different structures are obtained due to the collapse of the single strands into "bushes."

Besides the heteroduplex technique, other physical methods have been developed for mapping the viral genome. These include (a) crosses between density labeled phages, (b) fractionation of DNA fragments followed by either transfection assay for genetic markers or hybridization with defined mRNA's, and (c) electron microscopic visualization of transcribed DNA or of transcription-translation complexes, as reviewed

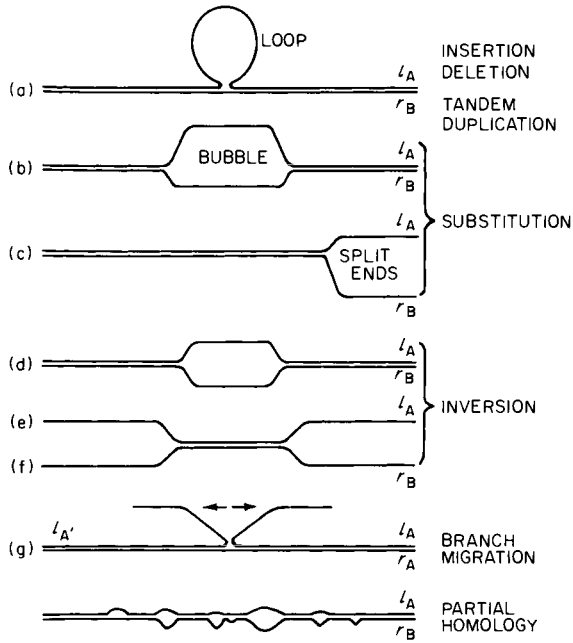

FIG. 1. Examples of heteroduplex configurations. The position of the structure is fixed in respect to the reference points on the DNA molecules, with the exception of "branch migration," where the apex of the V-like configuration can move in both directions, and the "tandem duplication" loop, which is a special case of branch migration. In "partial homology," the relative lengths and positions of the single- and double-stranded regions are variable and dependent on the conditions of annealing as controlled principally by the formamide concentration, ionic strength, and temperature. Symbols *l* and *r* denote the complementary DNA strands from the A and B strains. Modified from Davidson and Szybalski (1971).

by Davidson and Szybalski (1971). Since then, another very powerful technique has been developed which depends on precise endonucleolytic cleavage of λ DNA with restriction enzymes followed by gel electrophoresis of the fragments (Allet *et al.*, 1973; Hedgpeth *et al.*, 1972; Marks, 1973).

We have applied the heteroduplex mapping technique to the study of mutations in the *Escherichia coli* bacteriophage λ, which was subjected to various selective pressures. We found that more frequently than expected "mutations" are caused by major chromosomal rearrangements in contrast to simple point mutations. Several examples will be considered.

II. Reducing the Genome Size

A. Deletions

Phage λ^+ is composed of about equal amounts of protein and DNA. Each particle contains one double-stranded DNA molecule encapsulated in an icosahedral head. The molecular weight of the DNA is 30.8×10^6 daltons (Davidson and Szybalski, 1971). However, it is possible to pack a larger or a smaller DNA molecule into the λ head. Usually, when the DNA reaches 103% of the λ^+ length, the head becomes rather fragile, although there may be exceptions; for example, λb519b515*att*^{2}*imm*434 (Schulman and Gottesman, 1971; R. Weisberg, personal communication) is surprisingly stable even though the DNA length is 107% relative to λ^+.

The instability of λ phage particles, especially those containing a full complement of DNA, can be strongly accentuated by raising the temperature and adding chelating agents. Specifically, heating of λ^+ phage at

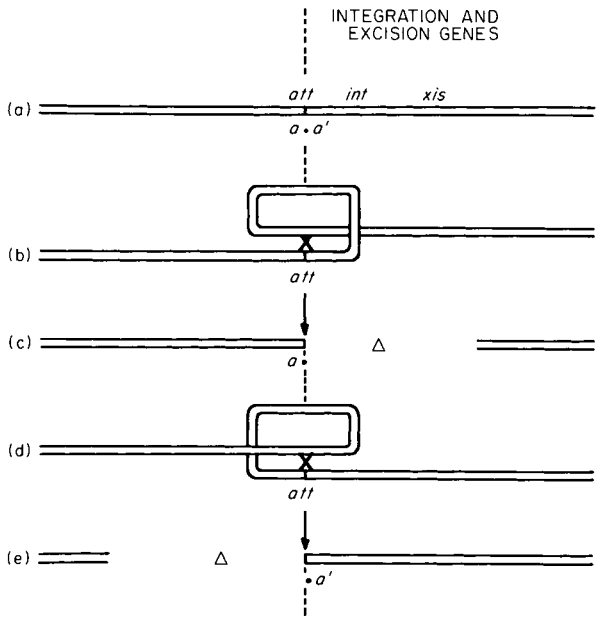

FIG. 2. Schematic representation of deletions promoted by the excision function and originating at the attachment (*att*) site. DNA to the right (b,c) or to the left (d,e) of the *att* site can be deleted. Symbol × indicates the crossover event. The circular excised DNA fragment is not shown in the figure.

37°C in 0.01 *M* EDTA inactivates over 99.9% of the plaque-forming particles. However, phage with several percent less DNA than λ^+ can survive such treatment. This technique was designed by Parkinson and Huskey (1971) for the selection of λ deletion mutants. They were able to isolate many fully viable mutants with deletions in the central region of the λ genome, since no functions obligatory for plaque formation are located there. When the phage retained a nondefective excision mechanism, controlled by genes *int* and *xis*, most of the deletions had one common end at the attachment (*att*) site located at 57.3% λ from the left DNA terminus. Apparently these deletions were promoted by the *att*-specific excision machinery through recombination between the *att* site and some site either to the left or right (Fig. 2). The excision-independent deletions could start and end anywhere in the λ genome, but in plaque-forming phages they generally did not eliminate genes indispensable to the viability of the phage. For instance, Blattner *et al.* (1974) isolated many viable λ mutants with deletions in the immunity region employing the EDTA selection technique. However, the most well-known λ deletion, b2, was isolated before the EDTA technique was developed (Kellenberger *et al.*, 1960), as well as some others (see Fiandt *et al.*, 1971).

B. Substitutions

Among phages selected for reduced genome size, one finds substitution mutants in which a fragment of λ DNA has been deleted and replaced by a shorter segment of some foreign DNA. There are two major mechanisms of substitution. The first involves illegitimate excision of the λ prophage in an induced *E. coli* lysogen. In the case depicted in Fig. 3a, λ DNA at the right prophage end (*a*) has been deleted, and a lesser amount of *E. coli* DNA adjoining the *a'* prophage end has been added. The net reduction of the λ genome would be the difference between the lengths of the λ deletion and the *E. coli* addition. An example is the replacement of most of the b2 region in λ*qin*A3 by an *E. coli* segment located between *att*λ and gene *pgl*, with a net loss of 6.6% λ units of DNA (Fiandt *et al.*, 1971). An alternative mechanism depends on recombination between λ and a related phage. As shown in Fig. 3b, one crossover has to occur between the homologous regions A and A′ and a second between B and B′. However, one of the crossover events could be the reaction between the cohesive ends or the *int*-mediated recombination. Since the distance between the A and B regions in λ is larger than the analogous segment in the lambdoid phage genome,

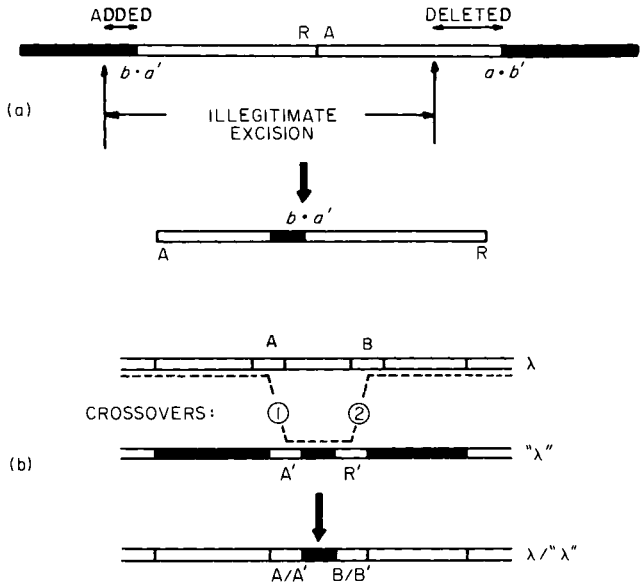

FIG. 3. Mechanisms of genetic substitution. (a) Illegitimate excision of the prophage accompanied by circularization (not shown) and opening at the R A joint. λ duplex DNA is represented by double lines and host duplex DNA is represented by solid lines. (b) Double crossover in the homology region A,A' (1) and B,B' (2) as indicated by the dashed lines. λ duplex DNA is represented by double lines and nonhomologous duplex DNA of the lambdoid ("λ") phage is represented by solid lines.

in this case the double crossover would lead to a net reduction in genome size. An example of such an exchange is the hybrid phage λ*imm*21 (21hy1 or λb5), in which the net deletion corresponds to about 5% of the λ genome (Westmoreland *et al.*, 1969). An analogous case is the crossing-in of a preexisting deletion from a λ deletion mutant.

III. Increasing the Genome Size

As already mentioned, phage λ can easily tolerate major deletions in its genome without loss of viability, as long as vital genes are not affected. However, phage with very extensive deletions form smaller plaques, probably due to impairment of packaging of the shortened DNA into the phage heads or to its injection into the host. This selective disadvantage was first utilized for the isolation of viable mutants (large plaque size) with increased DNA content by Franklin (1967). Further

investigation by Bellett *et al.* (1971) revealed that most of these isolates had undergone an internal tandem DNA duplication, which by heteroduplex analysis appears as a loop (Fig. 1a) at a variable position within the limits of the duplicated region. These results indicate that internal tandem duplication may be the most frequent event, although theoretically any genetic change which is the reverse of deletion, including insertion and substitution, could account for enlargement of the genome. For example, the p4 type of substitution (Fiandt *et al.*, 1971) would add nearly 10% to the λ genome.

The mechanism of primary duplication must be a complex process. The first event could be an illegitimate and unequal crossover between the identical phage genomes. Alternatively, it could be excision of a circular λ fragment (Fig. 2b,c) from one phage genome followed by its subsequent insertion into another intact λ genome in the same cell. The secondary events would be much more frequent, depending on normal recombination between the primary duplication regions (Bellet *et al.*, 1971). Gene duplication would appear to be an important evolutionary event and might explain the presence of repetitive DNA sequences, especially in higher organisms (Britten and Kohne, 1968).

IV. Shortening the Intergene Distance

Several types of chromosomal rearrangements have the effect of bringing two genetic units closer to each other. These include deletion of intervening material, inversion, and translocation as described below. The construction of various transducing phages carrying specific bacterial genes will serve as examples.

A. Deletions

If the distance between host gene *A* and the inserted prophage (see (Fig. 4) is too large for the construction of a transducing phage, a simple deletion of the intervening region would bring gene *A* into close proximity with the prophage (Fig. 4a,b). This was actually done by isolating *E. coli* mutants with deletions of gene *chl*D, which is located between the *gal* operon and the prophage λ attachment site (Feiss *et al.*, 1972), and then constructing nondefective *gal*-transducing λ*gal* phages. If the intervening genes have indispensable functions, they cannot be deleted but must instead be translocated in their functional form to another part of the host genome.

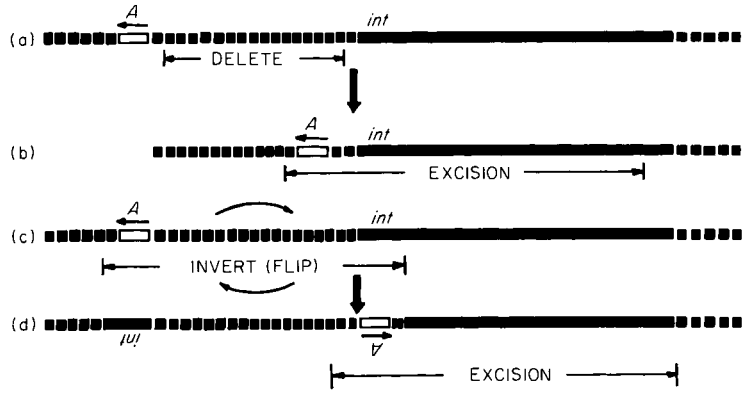

FIG. 4. Two modes of bringing host gene *A* (rectangle on the host genome represented by the heavy dashed line) close to the inserted prophage (heavy line). (a)–(b) Deletion of intervening material. (c)–(d) Inversion of a segment including intervening DNA and gene *A*. The sites of illegitimate prophage excision leading to the formation of the *A*-transducing genomes are indicated on (b) and (d). Duplex DNA is represented by single lines. For examples, see Fiandt *et al.* (1971).

B. Inversions

As depicted in Fig. 4c,d, host gene *A* can also be brought closer to the prophage by an appropriate inversion. In the case shown, the orientation of gene *A* is inverted and a small dispensable part of the prophage is detached. An example of this rearrangement is the formation of the transducing phage $\phi 80psu$III (see Fiandt *et al.*, 1971).

C. Translocations

Translocation of the *E. coli lac* gene to the vicinity of the attachment sites of phages λ and $\phi 80$ was performed by Ippen *et al.* (1971) and Beckwith and Signer (1966). Conversely, Shimada *et al.* (1972) were able to insert the prophage near to several bacterial genes.

V. Insertosomes

An insertosome, or insertion sequence IS, is a circular fragment of DNA that has a machinery and a specific site for linear insertion into the host or viral genome. Some such units, for example, episomes, have the

capacity for autonomous replication, but this is not an obligatory function of the insertosome. A still more complex unit is the lysogenic phage, such as λ, φ80, or Mu, which have the capacity for intracellular propagation and extracellular transmission.

One of the simplest insertosomes is the IS*1*, which is about 800 nucleotide pairs long and has a specific attachment site but can be inserted anywhere in the bacterial or phage chromosome (Malamy *et al.*, 1972; Fiandt *et al.*, 1972; Hirsch *et al.*, 1972). Three other classes of insertosomes, almost twice as large, IS*2*, IS*3*, and IS*4*, have also been identified. All these units carry special termination signals, which upon insertion into an operon have strong polar effects. *E. coli* apparently carries in its linear genome a considerable number of these units (Saedler and Heiss, 1973) and probably other insertosomes, which during evolution could be translocated to other sites and inactivate or modulate any genes. Insertosomes also carry a genetic recombinational function, since the region between two inserted identical IS sequences can be deleted or duplicated; it can also be inverted if the two IS sequences have opposite orientations. These are just a few examples of the many roles of the IS elements.

The formation of pretzel-like structures between identical insertosomes is depicted in Fig. 5; electron micrographs of such heteroduplexes and also heteroduplexes between inverted insertosomes (see the configuration in Fig. 1e) were published by Malamy *et al.* (1972) and Hirsch *et al.* (1972). Genetic elements that display many of the

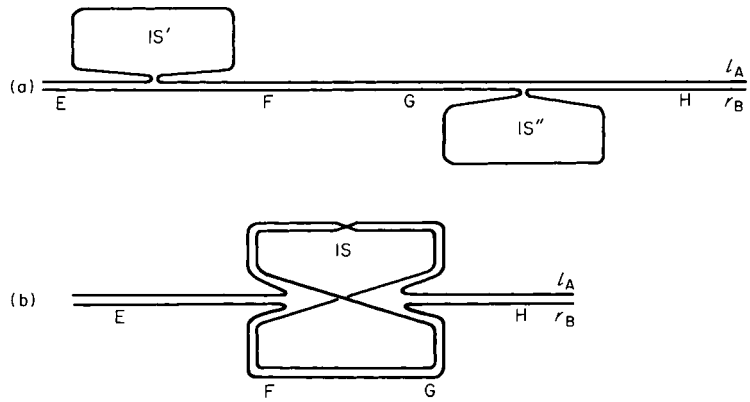

FIG. 5. Duplex formation between two identical IS sequences (insertosomes), one in phage A (*l* strand) and the other in phage B (*r* strand). The intervening F–G region has to loop out and partially unravel to relieve the tension produced by duplex formation between the IS' and IS" loops. See Malamy *et al.* (1972).

properties ascribed to insertosomes and can be translocated between the chromosomes of a higher organism were described for maize a long time ago (McClintock, 1956).

VI. Acquiring a New Function

The progress of evolution often requires a new function or a change in an existing function. A function can be acquired or modified either by addition of a new sequence, by deletion of a DNA region that contains a negative control, or by a combination of both, including inversion, translocation, and other intragenomic events.

A. Insertions

As already discussed, insertosomes of the type IS*1,2,3,* and *4* bring in a strong polar block and a capacity for deleting, duplicating, or inverting the DNA sequences located between them. Moreover, the mutational creation of a new promoter (Pereira da Silva and Jacob, 1968; Dove *et al.*, (1969) might sometimes involve an insertion. B. Allet (personal communication) concluded from the electrophoretic mobility of a λ DNA fragment containing the promoter-creating mutation *c*17 that this mutation might be an insertion of about 20 nucleotide pairs. We have not been able to detect a loop of that small size in heteroduplexes between λ and λ*c*17. On the other hand, loops of approximately 130 nucleotide length were readily detectable in heteroduplexes between two transducing phages, one of which carried a single and another a duplicated gene for the tyrosinyl tRNA (Fiandt *et al.*, 1971; Miller *et al.*, 1971).

B. Deletions

Transcriptions which originate at the p_L and p_R promoters of λ proceed only a short distance and then are blocked at the *t* termination signals (Szybalski *et al.*, 1970; Szybalski, 1971, 1972) by the action of the host *rho* factor (Roberts, 1970). However, the product of λ gene *N* permits the transcription to proceed across the *t* signals. Thus, λN^- mutants are defective, since they have lost the antitermination function of gene *N*. Court and Sato (1969) succeeded in isolating λ mutants that are able to form plaques in the absence of the *N* function. These mutations were found to be deletions (denoted *nin*) of varying size and located between the λ genes *P* and *Q* (M. Fiandt, D. Court, A. Campbell, and

W. Szybalski, as cited by Fiandt *et al.*, 1971, and unpublished). It appears unlikely that these *nin* deletions, which are 5 to 6% λ in length, could in fact be substitutions of very short, hardly detectable fragments of foreign DNA, since the length of independently isolated *nin* deletions can vary. A substitution caused by a double crossover (see Fig. 3b) should not behave as a deletion of variable length. However, it might be significant that the left termini of the *nin* deletions and the *qin* insertions (which will be discussed in the following section) appear to be the same (Fiandt *et al.*, 1971). Moreover, D. Court (personal communication) has observed recently that the λ*N*⁻*nin*5 mutant probably carries an accessory promoter mutation between genes *O* and *P*, a result which might explain the transcriptional data of Mark (1973).

C. Substitutions

Expression of the late λ functions depends on the product of gene *Q*. In the absence of *Q* product the transcription initiated at the late p_R' promoter leads to synthesis of a 198 nucleotide-long RNA terminated with the U_6A-OH sequence (Lebowitz *et al.*, 1971; Blattner and Dahlberg, 1972; Dahlberg *et al.*, 1972). The most probable role of the *Q* product is to extend this transcription into the late regions of the λ genome, in a way somewhat analogous to the antiterminating effects of the gene *N* product (Szybalski, 1972, 1974). λ*Q*⁻ mutants are defective since they cannot adequately express the late λ functions, i.e., the synthesis of the phage heads and tails and the lytic functions. However, Court and Sato (1969) and Sato and Campbell (1970) isolated *Q*-independent λ*qin* mutants that can form plaques, although derived from a phage in which gene *Q* was largely deleted. DNA heteroduplex analysis by Z. Hradecna, K. Sato, A. Campbell, and W. Szybalski (see Fiandt *et al.*, 1971) showed that in these mutants the λ DNA between genes *P* and *R* (including *R*) has been replaced by some foreign DNA. The simplest explanation is that the *qin* mutant is a product of recombination (see Fig. 3b) between a λ*Q*⁻ and a related prophage, which exhibits sequence homology with λ just to the right of genes *P* and *R* and which contains a nonhomologous but usable set of functions analogous to *Q*, p_R', *S*, and *R*. In other words the host may carry "spare parts" for the defective λ phage in the form of a "phantom" prophage.

Two additional aspects of this amazing adaptational flexibility should be stressed. (1) Since the fragment derived from the phantom prophage is much longer (about 19% λ) than the deleted λ DNA (about 10% λ) this substitution has to be compensated for by some deletions. A variety of substitutions or deletions of other regions of λ (e.g., *b*2, *a*3) or even

within the inserted foreign DNA (e.g., in λ*qin*C3 and λ*qin*Q3) have been observed (Fiandt *et al.*, 1971). Thus, genomic rearrangements needed to accommodate a new function are often quite complex. (2) An independently isolated, UV-inducted mutant, λp4 (Jacob and Wollman, 1954), was found to contain the identical substitution present in λ*qin*A3 (Fiandt *et al.*, 1971). If one accepts that the p4 or *qin*A3 "mutation" is in fact a recombinational event with a phantom prophage, it is perhaps not surprising that two identical "exotic" mutations occurred in two different laboratories 15 years apart.

VII. Hybrid Phages

The events described above involved recombination between λ and an unknown phantom prophage. This kind of recombination was actually shown to occur between phage λ and the related phages 434, 21, φ80, and others (Kaiser and Jacob, 1957; Liedke-Kulke and Kaiser, 1967; Franklin *et al.*, 1965; Szpirer *et al.*, 1969). The patterns of sequence homology between these phages were determined by heteroduplex analysis (Simon *et al.*, 1971; Fiandt *et al.*, 1971), and it was found that all the observed genetic exchanges occurred within the homology regions (Fiandt *et al.*, 1971; Westmoreland *et al.*, 1969). It was predicted that an exchange in the *OP* homology region between phages λ and φ80 should be possible (Fiandt *et al.*, 1971), and that was actually achieved by Szpirer (1972).

It should be stressed that duplex formation between homologous regions of two lambdoid phages is not proof that the sequences and the genes in those regions are identical. For instance, the *exo* genes of λ and φ80 are located in the *exo-β* homology region common to these two phages (M. Fiandt and W. Szybalski, unpublished), but the corresponding exonucleases are immunologically distinct (Szpirer *et al.*, 1969). The same appears to be the case fot the *O* functions of λ and φ80 (R. Monnat, W. Szybalski, L. Lambert, and R. Thomas, unpublished). Thus, DNA sequences that code for somewhat different proteins can still form duplexes that are stable enough to appear homologous by electron microscopic analysis.

This result points to the necessity of more refined analysis. The simplest way is to compare the stability of the duplex regions at increasing temperatures and formamide concentrations as was done by Davis and Hyman (1971). A more refined approach would be to look for changes of only one or a few nucleotides by measuring the positions of

the kinetic blocks to so-called "branch migration" by a method suggested by W. Szybalski and K. Carlson (unpublished). The ultimate level of approach would be the determination of the nucleotide sequences. Using the latter method, it was found that there are four single base changes in the homology region between λ and 434 DNA in the p_L-proximal region of gene *N* (Dahlberg and Blattner, 1973; Dahlberg *et al.*, 1974).

VIII. Plasmids

Phage λ has the capacity to persist and propagate according to three different modes. In its *lytic* form, the λ genome replicates many times, forms a large crop of phage heads and tails, and assembles about 100 complete phage particles that emerge upon cell lysis. In the *prophage* form, the λ genome becomes linearly inserted in the *E. coli* genome and propagates as an integral part of the host genome. In the *plasmid* form, the λ genome replicates as a separate unit apart from the *E. coli* chromosome, but somehow in stable symbiotic balance with the host. To propagate in the *lytic* form λ must retain all of its indispensable functions, whereas any fragment of the prophage can persist as a part of the bacterial genome, as long as the excision machinery and functions lethal to the host are repressed or inactivated. To establish a *plasmid* state, not only must the lethal functions be suppressed but the system for autonomous λ replication and its autorepressive control has to be preserved.

The replicative system of the λ plasmid is a part of the p_R-controlled, early rightward λ operon, the so-called *R1* scripton (Szybalski *et al.*, 1970; Szybalski, 1971, 1972), and includes the following eight elements (Fig. 6): the p_R promoter where the transcription of the *R1* scripton originates; that part of the o_R operator that responds to repression by the *tof* product; the s_R startpoint where mRNA synthesis begins; the repressor-like *tof* (or *cro*) product; the t_{R1} terminator, which in the absence of the *N* product reduces the transcription of elements to the right of it by a factor of about 20 (Kumar and Szybalski, 1970); the replicator site *ori*, where λ DNA replication originates; and genes *O* and *P*, the products of which are continuously required for the initiation of λ DNA replication. Moreover, it was postulated by Dove *et al.* (1969) that the rightward transcription in the vicinity of the *ori* site is required for the activation of λ DNA replication, and the experiments of Hayes and Szybalski (1973a,b) suggest that the leftward 81 nucleotide long RNA transcript in

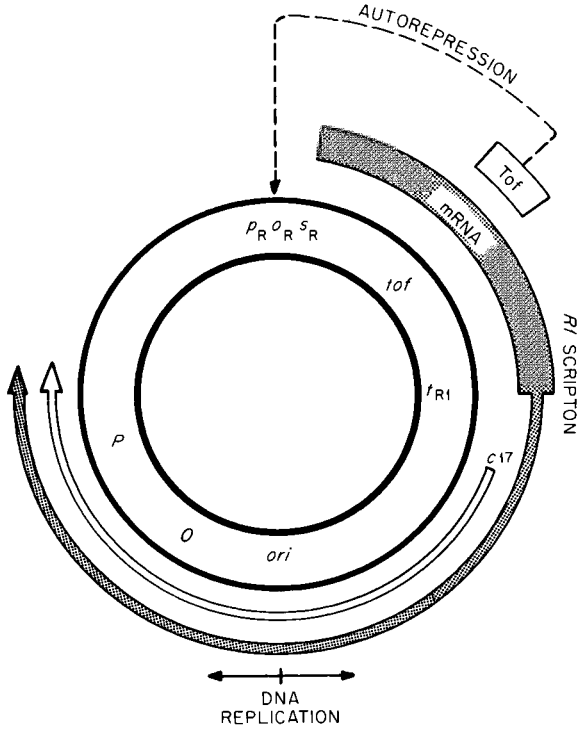

FIG. 6. Schematic representation of the DNA replication controls in the λdv plasmid. Symbols p_R, o_R, and s_R represent the promoter, operator, and startpoint for the rightward mRNA indicated by the shaded arrow. At site t_{R1} the bulk of the transcription is terminated in the absence of the *N* product of λ (Kumar and Szybalski, 1970). The autorepressive control by the Tof product of gene *tof* is indicated by a dashed arrow. DNA replication is initiated at the *ori* site and requires several factors, including the *O* and *P* products. The *oop* RNA primer, transcribed counterclockwise in the *ori* region, is not shown (Hayes and Szybalski, 1973a,b). The *c*17 mutation creates a new rightward promoter, and the *c*17-initiated transcription is symbolized by the open arrow.

the *ori* region, denoted *oop*, acts as a primer for the leftward λ DNA replication, which requires a number of host factors, including the RNA polymerase and the products of *E. coli* genes *dnaB*, *E*, and *G*.

How is the replication balance maintained with all these factors in play? The *tof* product, which acts at or near the o_R operator, seems to have an autoregulatory role, since whenever the *R1* scripton, and hence λ DNA replication, become too active the *tof* product coded by the same scripton is produced in excess and immediately depresses the expression of the $p_R o_R$-*tof-ori-O-P* operon. Thus, the *tof* product acts as

a repressor that exerts a feedback effect on the *R1* scripton, and in this manner maintains the fine balance of the plasmid replication. Elimination of any of these elements or addition of uncontrolled elements would either reduce the plasmid replication rate below that of the host, causing loss by dilution, or result in excessive synthesis of plasmids leading to the demise of the host.

If all these notions (Szybalski, 1974) are correct, the plasmid of minimum size should contain at least the $p_R o_R$-*tof-ori-O-P* segment of the λ genome, and all the elements of this segment must be functional and not bypassed by some uncontrolled function, as for instance by a new *C*17-like promotor (Fig. 6). This indeed proved true in the case of the so-called λdv1 plasmid (Matsubara and Kaiser, 1968) and several other shorter λdv plasmids isolated by D. Berg (Chow *et al.*, 1974). Moreover, the latter authors found that λdv's can exist not only in the form of single or tandem duplicated circular DNA containing one, two, three, or more identical λdv genomes in head-to-tail arrangement but also as two λdv genomes of unequal length in a head-to-head arrangement. They postulated that such unusual λdv's were formed by excision from the replicating fork. The various λdv's provide an example of the evolution of the plasmid, which one might expect to be the shortest possible unit still retaining all the functions required for replication and autoregulation, but which in fact seems to prefer the form of a multimer, a configuration with apparent selective advantage. The development of the plasmid could be thought of as evolution in reverse, the creation of the simplest possible yet still autonomous genetic unit.

IX. Conclusions

This survey of the chromosomal modifications observed in bacteriophage λ brings out the remarkable plasticity of the viral genome in response to various selective pressures. Frequent exchanges of genetic material between various phage species and between various regions of the host genome have been described. Some of these heterospecific exchanges depend on *general* recombination between small homology regions and some on *site-specific* recombination mediated by specific enzymes, as between the host and phage attachment regions in the case of lambdoid phages, or between a special site on an insertosome (or Mu-like phage) and almost any site on the chromosome. Thus, genetic changes range from point mutations to large gene rearrangements involving many thousands of nucleotide pairs. Since the genetic exchanges are rather

frequent and often are able to supply the required functions, perhaps it is not too surprising that they are rather common. After all, it should be easier to construct new genomes from preexisting "modules" rather than from scratch by the slow, haphazard process of accumulation of beneficial point mutations. When evolutionary developments must be rapid, the host genome would serve as a handy repository of "spare parts" that could be utilized by the phage to rebuild its defective genome. And, in turn, the phage could often supply the host with genes, as for instance its DNA replication genes or sites (Lindahl *et al.*, 1971). This brings up the question of which part of the phage is composed of bacterial genes and which part of the *E. coli* host consists of genes derived from various defective or repressed prophages. Perhaps this question is not quite meaningful, since host and phage appear to share most of the genes, and the particular selective pressures dictate the composition of their genomes. For instance, it is easy to adjust the selective pressures in such a way that only phages carrying large portions of bacterial genes (e.g., *gal*, *bio*, or *trp*) will survive. On the other hand, since at least 30 temperate *E. coli* phages have been identified, each about 1% of the *E. coli* genome length, and many more might be in a defective or cryptic state, one could go so far as to assume that half or even more of the *E. coli* genome is composed of prophage DNA. Thus, conceivably the *E. coli* genome may have evolved by fusion of many viral DNA's, or in turn, the coliphages could have evolved from modules belonging to the host genome. Probably there is truth in both these proposals.

What inferences could be drawn from these observations about eukaryotic genomes? Probably the genomes of higher organisms also evolved by fusion and rearrangement of genetic modules derived from various symbionts including viruses. If the *E. coli* genome contains up to 50 various prophages or their parts, then by inference the mammalian genome, almost one thousand times larger, may carry many thousands of different proviruses, most of them defective but some capable of being activated. Many would be of the DNA virus category, but some, as shown by Baltimore (1970) and Temin and Mizutani (1970), could use an RNA transcript as an intermediate form, perhaps another evolutionary trick for dispensing with a special excision mechanism.

If the eukaryotic genome is composed of large modules, carried mainly by viruses and other episomes, this would explain the mystery of why the functional genes are so sparsely distributed. Each chromomere frequently contains only one known gene, but enough DNA (on average of λ DNA size) for about 50 genes of average size. It is possible that whenever evolutionary pressures called for the addition of one functional gene, it was supplied in the form of a large module, maybe of viral

origin, with its own replicator site and a host of other irrelevant functions that might or might not still persist. A detailed genetic and physical analysis along the lines described here would be enlightening, but the experimental effort would be horrendous considering the sheer size of the eukaryotic genome. More optimistically, advances in computer technology may soon render this endeavor a practical reality. And from there on the sky is the limit. *Descriptive biology* will shift into *synthetic biology* and, using restriction enzymes, DNA ligases, and all available biochemical, synthetic, and genetic techniques, will culminate in the construction of new gene arrangements, new genes, and new controlling pathways.

Do these observations throw any light on the origin of neoplastic cells? It is well known that neoplastic transformation is often associated with major chromosomal rearrangements. Obviously, the complexity of the mammalian genome at present precludes drawing any definite correlations or conclusions. However, let us consider the λdv plasmid and how various mutations and chromosomal rearrangements can disrupt the well-balanced system of controls governing its replication, converting a plasmid from a symbiotic entity to a lethal agent. An analogy could be drawn with the neoplastic cell genome, which also has lost the capacity for controlled replication.

As discussed in Section VIII, the replicative system of the λdv plasmid consists of an operon initiated by the p_R promoter and controlled by that part of the o_R operator that interacts with the repressor-like substance Tof coded by gene *tof*, a part of the same operon (Fig. 6). Thus, the Tof product acts as an autorepressor and exerts a feedback-like control on the synthesis of several products, including *O* and *P*, required for the plasmid replication. Moreover, there is a 20-fold step-down in transcription at the t_{R1} terminator. How could these well-balanced controls be disrupted, leading to the unrestricted replication of λdv?

1. One possible mechanism would be the inactivation of the controlling genes or sites, i.e., inactivation of gene *tof* or the o_R or t_{R1} sites, which could be caused by point mutations or insertions free of termination signals.

2. Another means would be the acquisition of a new promoter function that is not subject to the repressive *tof* control and is located at any site upstream from the *ori-O-P* segment. Such a function could be acquired by mutation or by insertion of a promoter "borrowed" from some other virus or similar element. The *c*17 insertion mutation (Fig. 6) is an example of such a *tof*-independent new promoter.

Thus, there are various ways to disrupt the controls of DNA replication, involving gene inactivation or promoter acquisition, and any of these would provide tenable hypotheses to explain the neoplastic transformation leading to uncontrolled DNA replication and cell propagation. The insertion of viral genomes could account for any of these changes.

X. Summary

Employing genetic and physical methods of gene localization, especially electron micrographic mapping of heteroduplex DNA, a free flow of DNA sequences has been detected between the genomes of bacterial viruses and their hosts, creating new types of bacteriophages and modified bacterial genomes. Internal genomic rearrangements and the existence of specialized, easily translocated DNA sequences permit a purposeful evolution of genomes whenever selective pressure is applied. It would appear that new genomes can be constructed from preexisting modules in a process that is much more rapid than evolution by point mutations. One might also speculate that individual chromomeres in eukaryotic chromosomes correspond to inserted modules or whole virus-like genomes, from which the chromosome was constructed in stepwise fashion whenever evolutionary pressure called for only one particular gene function. Thus, on the average, one chromomere would code for only one relevant gene. Aside from natural evolution, many genetic rearrangements now can be engineered by the use of appropriate restriction nucleases and ligating enzymes together with chemical synthesis and genetic techniques. The era of *synthetic biology* has dawned.

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CHAPTER 21

Comparative Structure, Chemistry, and Evolution of Mycoplasmaviruses

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I. Historical Background

The mycoplasmas are a group of small prokaryotes (order Mycoplasmatales) which lack cell walls. They are the smallest known free-living

cells and, in the earlier literature, were referred to as pleuropneumonia-like organisms or PPLO. This group has been divided into two genera: the genus *Mycoplasma*, comprising those species which require sterol for growth, and the genus *Acholeplasma*, those which have no sterol requirement. The cell biology of the mycoplasmas has been recently reviewed by Smith (1971) and Maniloff and Morowitz (1972).

For a number of years there have been reports of suspected mycoplasma plasmids. These include electron microscopic observations of small DNA circles (Morowitz, 1969), satellite DNA bands in density gradient sedimentation experiments (Haller and Lynn, 1968; Dugle and Dugle, 1971; Das *et al.*, 1972), and some electron micrographs showing cytoplasmic inclusions, which have been interpreted to be "viruslike particles" (e.g., Robertson *et al.*, 1972). No infectious agent could be demonstrated in any of these studies, and, therefore, no conclusion can be drawn as to the possible viral nature of the structures. This criticism also applies to reports of "viruslike particles associated with mycoplasmalike organisms" in plants (literature reviewed by Gourret *et al.*, 1973).

The first isolation of a virus that could infect mycoplasma was reported by Gourlay (1970). This was designated MVL1 (Mycoplasmatales virus laidlawii 1). We propose to call all isolates, which are morphologically and serologically similar to MVL1, group L1 Mycoplasmatales viruses. Two other mycoplasmaviruses were later isolated, with properties different from MVL1 and from each other, and designated MVL2 (Gourlay, 1971) and MVL3 (Gourlay and Wyld, 1973). As before, we will designate similar isolates group L2 and group L3 Mycoplasmatales viruses, respectively. We use the term "mycoplasmavirus" as a common name for the Mycoplasmatales viruses.

In the last two years, about 35 additional group L1 isolates have been reported (Liss and Maniloff, 1971; Gourlay, 1972), and most mycoplasmavirus studies have concerned the group L1 viruses. Although these group L1 isolates are serologically and morphologically similar to the original MVL1 isolate, some of them have properties different from MVL1, such as host range (e.g., Section II,B), antiserum inactivation kinetics (e.g., Section II,E), UV inactivation kinetics (e.g., Section II,F), and one-step growth kinetics (e.g., Section III,A). Therefore, at the present time (and remembering that mycoplasma virology is only three years old), we think of the isolates in each group as being related in the sense that we consider T-even bacteriophages, for example, to be related (i.e., many of their gross properties appear to be similar, but there are significant measurable differences in their detailed molecular properties). From an experimental point of view, it is these differences that allow us to identify various group L1 viruses and to choose those isolates having appropriately useful properties for particular experimental designs.

It should be noted that, in spite of extensive searches in several laboratories, we know of no "cured" mycoplasma indicator host. All of the cell strains presently used to make lawns for virus growth are carrying mycoplasmaviruses; the nature of the carrier state (lysogeny or some type of pseudolysogeny) is not known. In fact, R. N. Gourlay (personal communication) has been able to obtain isolates of all three mycoplasmavirus groups from *A. laidlawii* strain M1305/68. This carrier state need not affect virology studies because the differences between the three groups and the differences between isolates within a group allow experimental confirmation that spontaneous virus release from the indicator host is not contaminating the virus being propagated. In the *A. laidlawii* strains used in our laboratory, we have found spontaneous release of carrier virus to be a negligible problem and not to be of concern for investigations of mycoplasmaviruses. This carrier virus situation is also frequently found in bacteriophage systems; one must pragmatically ignore carrier virus states, as long as they remain stable, in order to carry on with virological studies.

II. Virus Particles

A. PFU Assay

The mycoplasmaviruses are recognized by their ability to form plaques on lawns of some strains of *A. laidlawii*. Care must be taken in the interpretation of results because plaque formation is affected by the host *A. laidlawii* strain (e.g., Section II,B), the age of the cells used to make the lawn (Gourlay and Wyld, 1972; Liss and Maniloff, 1972), the water content of the agar plates (Maniloff and Liss, 1973), whether or not the incubation is under anerobic or high CO_2 incubation conditions (Maniloff and Liss, 1973), and the growth medium used (Gourlay and Wyld, 1972; Maniloff and Liss, 1973). There are also viral group differences: Group L1 viruses will form plaques on lawns that are up to 24 hours old, but group L2 viruses will only plaque on lawns less than 4 hours old (J. Maniloff and R. N. Gourlay, unpublished data). Another variable is the handling of the cells themselves. For example, in some laboratories (e.g., Gourlay's) cells are passaged only infrequently and, at each time, enough aliquots are frozen to allow some to be thawed and used for making lawns when needed; while in other laboratories (e.g., ours) cells are passaged daily, so that fresh cultures are always available for making lawns. The cells in the former procedure are probably similar to the original isolate, but the latter procedure selects for the fastest

growing cells, and so the original isolate must eventually be replaced by the fastest growing variant. Since virus plaque formation is affected by the cell growth rate, care must be taken in comparing results from two laboratories that may have started using the same indicator strain but, through adopting differing passage protocols, the cultures may now be significantly different as indicator hosts.

Group L1 plaques generally appear to have a clear center surrounded by a turbid halo. Those of L3 seem clear, while L2 plaques are slightly more turbid. L2 and L3 plaques are small (about 1 mm), while L1 plaques are much larger (several millimeters in diameter).

Plaque assays are carried out at 37°C, although all three mycoplasmavirus groups can form plaques at room temperature (22°C). Gourlay (1971) originally reported that MVL2 could not make plaques at 22°C on lawns of *A. laidlawii* M1308/68, but we have found that by changing hosts to *A. laidlawii* JA1 plaques can be observed after an overnight incubation at 22°C.

When different concentrations of group L1 (Fig. 1A) or L2 (Fig. 1B) virus are plated, it is found that the number of plaques is proportional to the first power of the relative virus concentration. This means that each plaque results from an infection by one virus and, hence, plaque-forming units (PFU) can be used to quantitate these viruses (e.g., Ellis and Delbruck, 1939). As a corollary to this conclusion, it is obvious that we have a reproducible assay system for mycoplasmaviruses, and this has allowed the development of mycoplasma virology.

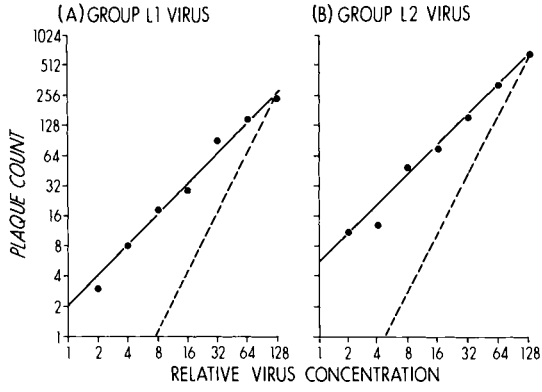

FIG. 1. Log-log plots of plaque counts from successive twofold dilutions of: (A) MVL51, a group L1 virus, and (B) MVL2, a group L2 virus. The solid lines are the theoretical curves for one-hit processes, and the dotted lines are the theoretical curves for two-hit processes. The data show that 1 PFU is produced by one virus.

B. Host Range

The ability of mycoplasmaviruses to form plaques on lawns of *Acholeplasma* and *Mycoplasma* species has been examined (Table I). Thus far, only some *A. laidlawii* strains have been shown to produce virus plaques. In a study of a number of *A. laidlawii* indicator strains, no unique pattern of biochemical or physiological properties could be found to differentiate these from other *A. laidlawii* strains (J. Fabricant, personal communication). Using a group L1 virus and *M. gallisepticum* A5969, we were able to rule out the possibility that the virus might be growing on these cells but not forming plaques by assaying for progeny virus on an *A. laidlawii* indicator (Maniloff and Liss, 1973). We also found that some group L1 virus isolates had different relative titers on

TABLE I

Mycoplasma Lawns Tested as Virus Indicators

Species	Using group L1 virus		Using group L2 virus	
	No. strains examined	No. strains producing plaques	No. strains examined	No. strains producing plaques
A. laidlawii[a,b,d]	78	23	17	6
A. granularum[b,c,d]	3	0	1	0
A. axanthum[c]	1	0	–	–
A. sp M221/69[b]	1	0	–	–
M. arthritidis[a]	1	0	–	–
M. sp. strain 14 (goat)[a]	1	0	–	–
M. gallisepticum[a]	5	0	–	–
M. sp. strain avian serotype 1[a]	4	0	–	–
M. hyorhinis[b]	1	0	–	–
M. bovirhinis[b]	1	0	–	–
M. dispar[b]	1	0	–	–
Serological group 6 (Squire)[b,c,d]	2	0	1	0
Serological group 7 (N29)[b,d]	1	0	1	0
M. mycoides var. *mycoides*[b]	7	0	–	–
M. pneumoniae[b]	1	0	–	–
M. salivarium[b,d]	1	0	1	0
M. fermentans[b]	1	0	–	–
M. hominis[b,c,d]	2	0	1	0
M. orale type 1[b,d]	1	0	1	0
M. pulmonis[c]	10	0	–	–

[a] Liss and Maniloff (1971).
[b] Gourlay and Wyld (1972).
[c] Liss (1973).
[d] Gourlay (1972).

some of the *A. laidlawii* indicator strains, and the host range, thus defined, could be a useful property for differentiating the isolates (Liss and Maniloff, 1971).

Most attempts to isolate viruses from a mycoplasma have been done by washing a lawn of the organism being examined, with either buffer or growth medium, and plating the wash on an *A. laidlawii* indicator. The two host strains most widely employed are *A. laidlawii* M1305/68 (used in Gourlay's studies) and *A. laidlawii* JA1 (used in ours); the latter is a nalidixic acid-resistant variant of *A. laidlawii* BN1. In this way, group L1 virus isolates have been obtained from 25 *A. laidlawii* strains, an *A. granularum*, an *A. axanthum*, a *Mycoplasma* sp. strain 14 (goat), a *M. hominis*, a *M. pneumoniae*, a *M. gallisepticum*, a *M. arthritidis*, and five *M. pulmonis* strains (Liss and Maniloff, 1971; Gourlay, 1972; Liss, 1973). Group L2 and L3 viruses have only been isolated from *A. laidlawii;* however, for L2, only *A. laidlawii* and *A. granularum* have been examined (Gourlay, 1972), and, for L3, only *A. laidlawii* was used (Gourlay and Wyld, 1973).

C. Electron Microscopy

The electron microscopic observations of the viral morphologies are summarized in Table II. As seen in Fig. 2A, Group L1 viruses are bullet-shaped particles, 14–16 nm wide and 70–90 nm long; this has been confirmed by observations on ten group L1 isolates (Liss and Maniloff, 1971; Gourlay, 1972; Milne *et al.*, 1972). The variation in par-

TABLE II

Properties of Mycoplasmatales Viruses

Properties	Group L1	Group L2	Group L3
Virus morphology	Naked bullet-shaped particles[a,b]	Enveloped particles[d]	Icosahedral particles with short tails[e]
Virus size	14–16 nm wide, 70–90 nm long[a,b]	80 nm mean diameter (size range: 52–125 nm)[d]	About 54 nm diameter[f]
Nucleic acid	Single-stranded DNA circle[c]	DNA[d]	Double-stranded DNA[f]
DNA molecular weight	2×10^{6} [c]	–	4 to 5×10^{6} [f]

[a] Bruce *et al.* (1972).
[b] Liss and Maniloff (1973a).
[c] Liss and Maniloff (1973b).
[d] Gourlay *et al.* (1973).
[e] Gourlay and Wyld (1973).
[f] Garwes *et al.* (1974).

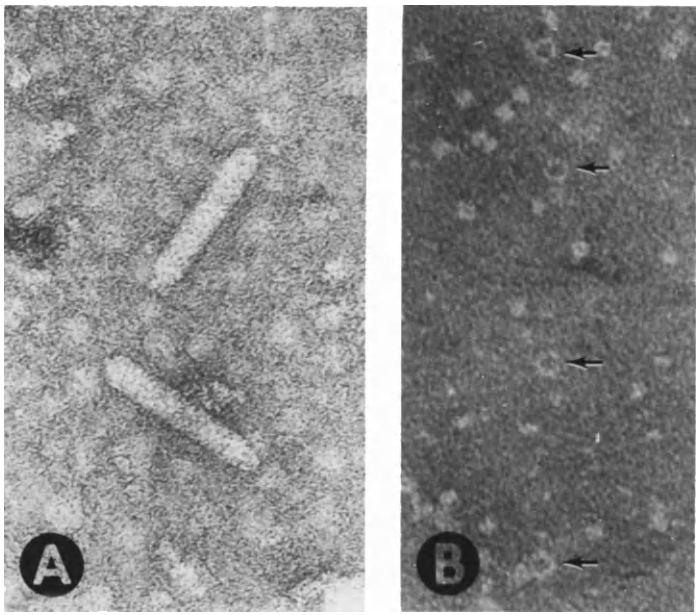

FIG. 2. Electron micrographs of negatively strained MVL51 virus particles, from Maniloff and Liss (1973). ×240,000. (A) Uranyl formate stained bullet-shaped viruses. (B) Phosphotungstate stained ring structures (arrows).

ticle length reflects two different interpretations of the micrographs. All the reports agree that one end of the virus is rounded. Bruce *et al.* (1972) report that in infectious particles the other end (the basal end that makes contact with the cell membrane) is also rounded, but that this end is frequently degraded to give 8–10 nm shorter particles, with basal ends appearing flat or with one or two short protuberances. However, Liss and Maniloff (1973a) report that the basal end is flat with some sort of short flexible protrusions, which could not be further resolved in their studies but which would add about 7 nm to the particle length. It should be noted that the ends of these thin rods have such a small radius of curvature that the drying pattern of the negative stain around any form of end structure must frequently give a hemispherical appearance. Also, since group L1 preparations usually have at least 10^{10} PFU/ml, most particles observed are probably infectious units, unless they are degraded during the negative staining.

Optical analysis of micrographs of group L1 particles have shown that the viruses have helical symmetry, and the optical transforms have a

near meridional reflection at about 4.8 nm (Bruce *et al.*, 1972; Liss and Maniloff, 1973a). The viral capsid structure units must form an hexagonal lattice (with a 9.6 nm center to center hexagon spacing) that gives rise to the helix; Bruce *et al.* (1972) have built a model of a group L1 virus from these data, with the structure units arranged in a two-start helix with 5.6 subunits per turn at a pitch of 20°.

In addition to the infectious virus particles, group L1 preparations also reveal other types of viral related structures that may eventually be useful in elucidating the mechanism of viral assembly. (1) Unpurified preparations contain 13–14 nm rings (Fig. 2B), which may be intracellular viral protein subassemblies (Liss and Maniloff, 1973a). (2) A few particles have been seen with hollow centers (Bruce *et al.*, 1972; J. Maniloff, unpublished data); these are about as wide as virus particles but are definitely shorter and may be incomplete viruses. (3) Long rods, with diameters similar to the viruses but with lengths sometimes exceeding 500 nm, have been observed (Bruce *et al.*, 1972; Milne *et al.*, 1972) and may be aberrant viral polymerization forms; we have noted that the shortest rods have both ends rounded and are about 147 nm, the length of two virus particles (Liss and Maniloff, 1973a). (4) Long hollow tubular structures have been reported (Bruce *et al.*, 1972; Milne *et al.*, 1972; Liss and Maniloff, 1973a); most widths are about 13–18 nm, but some as wide as 30 nm have been seen, and the reported lengths are up to 1 μm. Bruce *et al.* (1972) have noted a greater frequency of hollow tubes in cells infected in late log phase or in cells examined late (i.e., 4 hours) after virus infection; hence, these structures must represent some type of aberrant polymerization product of the aged infected cells. (5) Milne *et al.* (1972) showed, by thin-section electron microscopy of old (72 hour) infected cells, the presence of intracellular threads: Some formed striate masses, and others were randomly distributed but seemed to have a helical appearance. However, numerous attempts to repeat this one observation in other laboratories (e.g., J. Maniloff, unpublished data) have thus far been unsuccessful. The many possible experimental parameters (e.g., culture age and multiplicity of infection) make it impossible to evaluate these reported thread structures.

Group L2 viruses, examined by negative staining (Gourlay *et al.*, 1973), appeared as roughly spherical particles about 80 nm in diameter. No internal structure was obvious, but it is stated that an indication of a coiled structure within the envelope could be seen. The presence of an envelope was implied by the viral sensitivity to detergent and organic solvents (Gourlay, 1971) and confirmed by thin-sectioned electron microscopy showing an envelope with a "unit membrane" structure (Gourlay *et al.*, 1973).

The only group L3 isolate, MVL3, was reported to be an icosahedral particle, about 54 nm in diameter (Gourlay and Wyld, 1973). More recent studies have revealed that each particle has a short thin tail (Garwes *et al.*, 1974), giving it an appearance similar to T3 bacteriophage.

D. Chemical Composition

The fact that all three mycoplasmavirus groups are DNA viruses (see Table II) was originally demonstrated by showing that labeled thymidine, but not uridine, could be incorporated into each of the viruses (Gourlay *et al.*, 1971, 1973; Garwes *et al.*, 1974). The DNA of a group L1 virus, MVL51, was analyzed by alkaline sucrose gradient velocity sedimentation, neutral and alkaline CsCl isopycnic sedimentation, and treatment with endo- and exonucleases (Liss and Maniloff, 1973b) and shown to be a covalently linked single-stranded DNA circle of molecular weight 2×10^6 daltons. DNA from the group L3 virus, MVL3, exhibits a hypochromic shift on heating indicating that it is double-stranded and sedimentation analysis gives a molecular weight of 4 to 5×10^6 daltons (Garwes *et al.*, 1974).

The difficulty in obtaining large quantities of virus free from contaminating cell membrane material has precluded extensive studies on the number and stoichiometry of the mycoplasmavirus proteins. In addition, the group L2 viral envelope must also contain a lipid component. The envelope probably accounts for the sensitivity of group L2 (but not L1 or L3) viruses to detergent, organic solvent, and thermal inactivation (Gourlay, 1970, 1971, 1972; Gourlay and Wyld, 1972, 1973).

E. Antiserum Inactivation

The serological differentiation of the three groups of mycoplasmaviruses has been carried out by examining the ability of a specific antiserum to inhibit plaque formation (Gourlay, 1972). In this procedure, a mixture of virus and antiserum is plated and examined for plaques after a 24 hour incubation. Hence, this method allows the identification of three serologically distinct virus groups, but because the inactivation kinetics are ignored, nothing can be concluded about the degree of serological relatedness among the isolates within each group.

We have examined the inactivation kinetics of four group L1 viruses with antiserum to MVL51 (Fig. 3). There is a consistent threefold difference in inactivation rates among these isolates, indicating a degree of

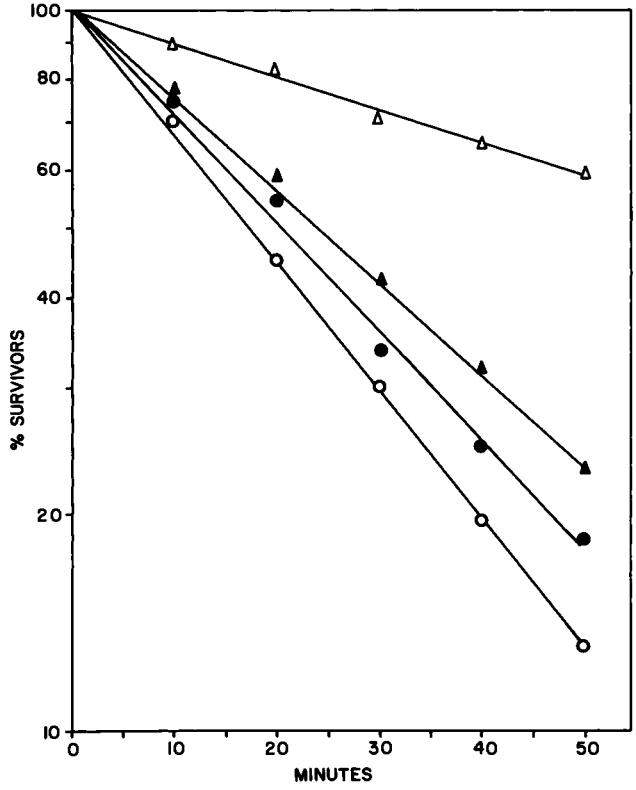

FIG. 3. Inactivation kinetics of group L1 viruses by antiserum made against MVL51: (▲) MVL1, (○) MVL51, (●) MVL52, and (△) MVG51. The ordinate is the logarithm of the surviving virus fraction after mixture of the viruses with a 1:500 dilution of a stock antiserum. The abscissa is the incubation time at 37°C, after which the mixture was assayed for PFU on lawns of *A. laidlawii* JA1.

serological heterogeneity in these group L1 viruses. This situation, where the viruses within a group are serologically related but not identical, has also been noted to occur in the T-even bacteriophage (e.g., Stent, 1963). Since the inactivation rate is a distinctive property, we have used this to identify the progeny virus produced by transfection (Liss and Maniloff, 1972).

F. UV Inactivation

All three mycoplasmavirus groups are inactivated by ultraviolet light (Gourlay, 1970, 1971; Gourlay and Wyld, 1972, 1973; Liss and Mani-

loff, 1971). In a study of the UV inactivation kinetics of three group L1 isolates (Liss and Maniloff, 1971), we found that the inactivation followed one-hit kinetics, but the three viruses each had different UV inactivation cross sections. This again indicates that not all group L1 isolates are identical. A similar result is reached in studies of the UV inactivation of the T-even phages (e.g., Stent, 1963).

III. Growth Cycle

A. One-Step Growth

In the one-step growth experiments, mycoplasma cells are mixed with viruses at a multiplicity of infection (MOI) less than 1 (so each infected cell only receives, on the average, one virus), incubated for 5 minutes to allow adsorption, diluted to eliminate possible reinfection, and samples are plated at various times to assay the number of PFU in the cultures. Figure 4 shows part of a one-step growth curve for a group L1 virus, MVL51. There is an initial, short (about 10 minutes) latent period during which there is a decrease in virus titer; this may indicate abortive viral replication in some of the infected cells. The latent period is followed by a rise period during which there is a gradual increase in the PFU titer, suggesting continuous viral production rather than a sudden release of virus by a cell lysis mechanism. After the rise period, the virus titer reaches a plateau; for MVL51 in tryptose broth, this happens at about 2 hours after infection, by which time each infected cell has released 150–200 progeny viruses (Liss and Maniloff, 1973a).

Although the general one-step growth curve pattern is the same for other group L1 isolates, the period times and the amount of progeny released per infection is different, but consistent, for the different isolates (Liss and Maniloff, 1971, 1973a).

No one-step growth data is available for group L2 and L3 viruses. Although group L2 virus grows well on lawns on solid tryptose medium, it does not adsorb well to cells in liquid medium, and this has kept us from obtaining one-step growth curves.

B. Artificial Lysis

In order to see whether group L1 virus replication involves the intracellular accumulation of mature virus, we have done premature lysis experiments (Maniloff and Liss, 1973; Liss and Maniloff, 1973a). In

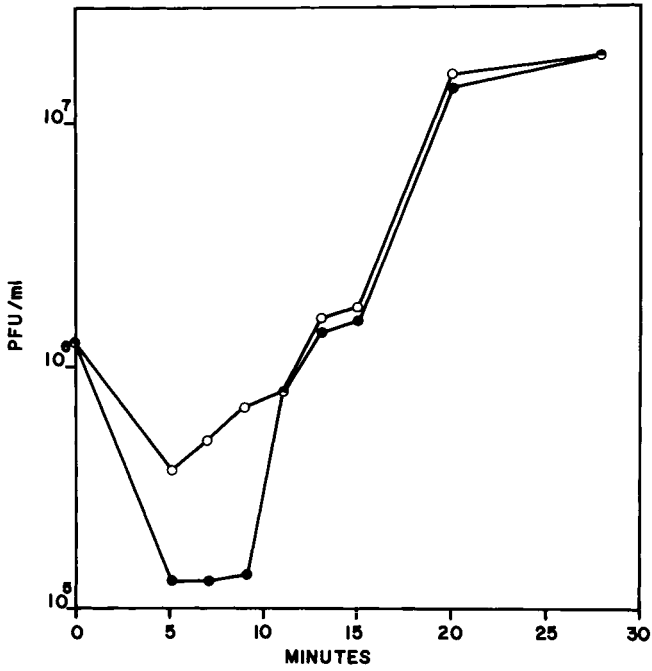

FIG. 4. Artificial lysis experiment using *A. laidlawii* JA1 infected by MVL51, from Liss and Maniloff (1973a). Half of the infected culture (MOI of 0.1) was assayed directly for PFU, giving a one-step growth curve (○), and half was lysed before the PFU assay. (●).

these, MVL51 infected cells are lysed by detergent (0.2% Triton X-100) during a one-step growth experiment and assayed for the infectivity of the intracellular contents released by the artificial lysis. It was found (Fig. 4) that, after infection, the PFU of the lysed samples was equal to the free unadsorbed virus titer during the latent period, indicating no infectious intracellular viruses. During the rise period the number of PFU in the lysed samples increased to equal the virus titer of the control unlysed samples, reflecting the release of progeny virus.

In systems where virus release is due to cell lysis, the PFU of the artificially lysed cells exceeds that of the unlysed ones up to the time of cell lysis, due to the artificial release of accumulated intracellular mature viruses (Doermann, 1952). However, for MVL51 infection (Fig. 4) the PFU titer of the lysed cells never exceeded that of the unlysed ones, showing that there is no intracellular pool of completed viruses and that new MVL51 viruses are released as they are made.

C. Single Burst Size

Although progeny group L1 viruses are not released in a burst, it is easiest to use the "burst" terminology in discussing virus yield. We have shown that both the progeny virus yield and the growth rate of infected cells vary as a function of the MOI (Maniloff and Liss, 1973; Liss and Maniloff, 1973a). Therefore, consistent experimental results and maximal virus production require a low MOI (less than about 5–10). Above this MOI, the virus yield is variable and usually less than those measured at low MOI. This probably accounts for those studies, in which the MOI was not controlled, where the burst size was reported to vary (Gourlay and Wyld, 1972).

In the one-step growth experiments, the burst size measured is an average over all of the infected mycoplasmas. For the group L1 viruses, these range from 3 for MVL1 to 150–200 for MVL51 (Liss and Maniloff, 1971, 1973a).

Using the single-burst protocol of Ellis and Delbrück (1939), the virus release from individual MVL51 infected cells was measured (Liss and Maniloff, 1973a). The average virus yield per infected cell was 88 at 60 minutes after infection (the range of values was 50–180) and was 154 at 120 minutes (range of 120–270). In agreement with the other growth experiments, these data indicate that each infected cell is releasing virus continuously at about the same rate, rather than releasing its progeny virus in a short burst.

D. Growth of Infected Cells

During the group L1 viral growth studies, we noted that at an MOI less than 5–10 infected cells produced virus without a loss of cell titer, but at a higher MOI there was a decrease in the number of viable colony-forming units (Maniloff and Liss, 1973). However, at the lower MOI the infected cells do grow slower; uninfected cells have a doubling time of 110 minutes while MVL51 infected cells have one of 160 minutes (Liss and Maniloff, 1973a).

In thin-section electron micrographs of MVL51 infected cells, no intracellular viral structures have been observed (Liss and Maniloff, 1973a). Progeny viruses were found in clusters on the cell surface; in the adjacent cytoplasmic areas, there were regions of amorphous material that may be condensations of viral structure precursors. Hence, electron microscopy confirms the absence of completed intracellular viruses and shows that virus assembly and release must take place in association with a limited number of membrane sites in infected cells.

E. Plaque Formation

The detailed studies of one group L1 virus, MVL51, and the preliminary data on other group L1 isolates have shown that these viruses produce nonlytic infections, at low MOI. Previous papers on group L1 mycoplasmaviruses have accepted a lytic mode of infection because plaque formation is generally associated with the lysis of infected cells. However, studies of the filamentous bacterial viruses have shown that plaques can be due to infected cells growing slower and making smaller colonies than uninfected ones (Hsu, 1968). The slower growth rate of infected mycoplasmas has been discussed in Section III,D, and it has been reported that MVL51 infected cells do make smaller colonies than uninfected ones (Maniloff and Liss, 1973). Therefore, group L1 virus plaque formation must be due to differential growth rate and size of infected cells, which lead to the uninfected cell lawn looking more dense and the area of infected cells appearing as a clearing or plaque. Some lysis may occur in the center of the plaque where the MOI is high.

Although group L2 and L3 plaques are clearer than group L1 plaques, no data is available on whether these represent lytic or nonlytic infections.

IV. Virus Replication

A. Adsorption

The adsorption kinetics of several group L1 viruses has been shown to be first-order (Fraser, 1973; A. Liss, unpublished data). Fraser (1973) has found little temperature dependence for the process between 30°–42°C and noted that the cation dependence indicates an ionic mechanism for adsorption. This latter conclusion is consistent with the fact that these cells have a small negative surface charge density (Carstensen *et al.*, 1971).

From the slopes of the adsorption curves, the adsorption rates for two group L1 viruses have been calculated: For MVL1 the rate is about 3×10^{-9} cm^3/minute (Fraser, 1973), and for MVL51 it is about 5.6×10^{-9} cm^3/minute (A. Liss, unpublished data). We have calculated the theoretical adsorption rate constant of these viruses from collision kinetics, using the von Schmoluchowski equation as described by Stent (1963) and approximating the rod-shaped viruses by a prolate ellipsoid (Tanford, 1961). From this calculation (see the Appendix), the theoretical rate is 5×10^{-9} cm^3/minute, in agreement with the experimental val-

ues. Hence, nearly every collision between a group L1 virus and an *A. laidlawii* cell must result in the adsorption of the virus.

We have shown that each cell can adsorb over 300 MVL1 particles, but it is not known how many of these adsorbed particles actually have their DNA penetrate the cell (Liss and Maniloff, 1971).

Nothing is known about the mechanism of penetration, nor is it known whether any of the viral coat proteins enter the cell with the DNA.

B. DNA Replication

We have investigated the intracellular replication of MVL51 (Liss and Maniloff, 1973c), by using radioisotopic labels to follow either the infecting parental DNA or the nascent progeny DNA and analyzing the extracted DNA by CsCl band sedimentation. After entering the cell, the single-stranded parental DNA was rapidly converted to a double-stranded replicative form (RF). This was then replicated to produce additional RF molecules. Later, the parental DNA was found to leave the RF pool and became associated with large molecular weight DNA; none of the labeled parental DNA could be found in released progeny viruses. Single-stranded progeny viral DNA was synthesized from the double-stranded RF and an accumulation of viral chromosome size single-stranded DNA complexed with phenol-sensitive material was observed.

C. Virus Assembly

Combining the data in Sections II,C, III, and IV,B, a model for group L1 virus assembly can be constructed. Many of these details are similar to those of other single-stranded DNA viruses (Table III).

Group L1 virus assembly must occur at a small number of sites at the inner surface of the cell membrane. The complex here may consist of both the DNA replication forms and viral proteins, some possibly in subassemblies. The single-stranded progeny DNA is synthesized on the RF and interacts with some intracellular material, probably protein, to form a phenol-sensitive complex. Ring structures have been observed which may be coat protein subassemblies and may be involved in the assembly reaction. This process must be associated with the mechanism by which progeny viruses are extruded from the cell. It may be that as a DNA region passes through the membrane it interacts with a protein ring subassembly, resulting in the extrusion of a part of the viral chromosome in its protein coat. This could be an irreversible step which would drive the extrusion reaction to completion, i.e., release of the completed virus.

TABLE III

Comparative Properties of Single-Stranded DNA Viruses

Properties	Group L1 Mycoplasmatales virus	Filamentous bacterial virus	ϕX type bacterial virus	Parvovirus
Virus symmetry	Helical[a]	Helical[c]	Icosahedral[f]	Icosahedral[h]
DNA structure,	Circular	Circular	Circular	Linear
molecular weight	2×10^{6} [b]	2×10^{6} [e]	1.7×10^{6} [f]	1.7×10^{6} [i,j]
Cell site of adsorption	Membrane[d]	Pili[c]	Cell wall[g]	Membrane[h]
Replication properties				
Conversion of parental DNA to double-stranded replicative form	Yes[c]	Yes[e]	Yes[f]	Yes[k,l]
Intracellular pool of single-stranded progeny DNA	Yes[c]	Yes[e]	No[f]	Yes[k]
Cell death	No[a]	No[c]	Yes[f]	Yes[m]

[a] Liss and Maniloff (1973a).
[b] Liss and Maniloff (1973b).
[c] Liss and Maniloff (1973c).
[d] Maniloff and Liss (1973).
[e] Marvin and Hohn (1969).
[f] Sinsheimer (1970).
[g] Brown *et al.* (1971).
[h] Hoggan (1971).
[i] Crawford *et al.* (1969).
[j] Salzman *et al.* (1971).
[k] Dobson and Helleiner (1973).
[l] Salzman and White (1973).
[m] Cytopathic effect on cell monolayers reported by Tattersall (1972).

V. Transfection

A. Properties

We have shown that DNA isolated from group L1 viruses is infectious when mixed with *A. laidlawii* cells (Liss and Maniloff, 1972), leading to production of virus progeny with the same properties as the viral source of the DNA. The infectivity was destroyed by DNase, but not by RNase, pronase, or specific antiserum to the virus (Table IV). The host mycoplasma cells are only competent for transfection during late logarithmic phase growth. The time course of the DNA–cell interaction indicates that during the first 10–15 minutes there is an attachment of the DNA to the cell, during which the transfection is sensitive to DNase, and this is followed by the entry of the DNA into the cell, after which the process is DNase-resistant. The dose–response curve for

transfection shows that an average of two molecules of DNA must interact with a cell in order to produce an infection. In addition, from the curve it can be calculated that 1.1 to 1.3 $\times$ 10^{-6} μg of DNA is required per infection; this means that 3 to 4 $\times$ 10^5 viral equivalents of DNA are needed per transfection. This efficiency is about the same as for animal virus transfection (Pagano, 1970) and at the lower end of the range of values for bacteriophage transfection (Epstein, 1971).

B. Comparison with Infection

While *A. laidlawii* cells can support group L1 viral growth throughout the logarithmic growth phase, they are only competent for transfection during the last 4–8 hours of logarithmic growth. This presumably reflects either a surface alteration or an increased extracellular nuclease activity during culture development. Table IV compares some of the growth parameters of the infection and transfection. The increased latent period and decreased progeny yield for transfection has also been observed for bacteriophage systems (Horvath, 1969). The transfection progeny have the same growth parameters as the mycoplasmavirus used as the DNA source.

We have also found (Table V) that a *Mycoplasma* species, which cannot be infected by virus, can take up the viral DNA and produce progeny virus (Maniloff and Liss, 1973).

VI. Evolutionary Considerations

At this time, no conclusions about mycoplasmavirus evolution can be stated because (1) mycoplasma virology is still young and we have insufficient data for such generalizations; (2) little is known about the origin and evolution of even those bacterial viruses which have been extensively characterized; and (3) the taxonomy and evolution of the mycoplasmas and other wall-less prokaryotes are still unclear. However, we believe it is possible to outline some considerations which may be of utility in thinking about the origin and evolution of the mycoplasmaviruses.

The first questions concern the mycoplasma host cells themselves. The cellular and molecular biology of these organisms is qualitatively the same as that of other prokaryotes; the differences are small quantitative ones due to the limited mycoplasma cell and genome size (e.g., Maniloff and Morowitz, 1972). Hence, it should not be too unexpected

TABLE IV

Infection and Transfection of JA1 Cells by MVL51[a]

	Infection	Transfection
Cells infected by	Virus particles	Viral DNA
Latent period	10 minutes	30–40 minutes
Progeny titer at 2 hours	150–200	85–100
Effect of DNase	None	Inhibition
Effect of RNase	None	None
Effect of pronase	None	None
Effect of anti-MVL51 serum	Inhibition	None

[a] From Maniloff and Liss (1973).

to find that these cells have their own viral flora. Mycoplasma taxonomy remains unsettled, and little is known about their relationship to other walled prokaryotes (Smith, 1971) or to the two other recently discovered wall-less prokaryotes: the helical spiraplasmas of plants (Davis and Worley, 1973) and the acidophilic thermophiles (Darland *et al.*, 1970). In fact, although Morowitz and Wallace (1973) have speculated about the *Mycoplasma* being the evolutionary precursor of the *Acholeplasma*, there is nothing to preclude these two genera from having originated totally independently from some primitive prokaryote.

Considerations of the origin of mycoplasmaviruses must begin with the most trivial possible explanation: Mycoplasmas are grown in media containing serum, and it has been established that a significant propor-

TABLE V

Effect of MVL51 Virus and DNA on Mycoplasmas[a]

	A. laidlawii JA1	*M. gallisepticum* A5969
MVL51 virus infection		
Virus adsorption	Yes	No
Progeny formed	Yes	No
Plaques formed	Yes	No
MVL51 DNA transfection		
DNA taken up	Yes	Yes
Virus formed	Yes	Yes[b]
Plaques formed	Yes	No

[a] From Maniloff and Liss (1973).
[b] Assayed as PFU on JA1 lawns.

tion of sera contain animal viruses and/or bacteriophages (e.g., Molander *et al.*, 1972; Merril *et al.*, 1972), so perhaps the mycoplasmaviruses originated from viral contaminants in the sera. It is probably impossible to completely eliminate this possibility, but we believe it is highly improbable that this could account for the origin of all the mycoplasmaviruses. The main argument against this is the isolation of similar mycoplasmaviruses in several laboratories both in England and the United States, where different media and serum sources were used.

It is interesting to note the morphological similarity of the mycoplasmaviruses to other viruses. Naked bullet-shaped particles such as group L1 viruses have only been reported as plant viruses (e.g., Brunt *et al.*, 1964); it is not known whether the plant viruses contain DNA or RNA. Group L2 particles resemble animal viruses because of their membrane envelopes, and Group L3 particles, with apparent head and tail structures, resemble T3 bacteriophage. Evolutionarily, it is difficult to image a cell, like the mycoplasmas, being so virologically naive as to be able to be parasitized, through media contaminants, by such diverse viruses. We must then consider other possibilities for the origin of viruses.

Table III compares some of the properties of the single-stranded DNA viruses. Each type, including the group L1 mycoplasmavirus, has distinctive properties. Interestingly, all these viruses have the same size chromosome, within experimental accuracy: 2×10^6 daltons. The group L3 virus, which has double-stranded DNA (Table I), also has the same amount of genetic information, since its DNA is 4 to 5×10^6 daltons. This may represent a lower threshold for the informational content of the DNA viruses; even this is about twice as large as the nucleic acid of the small RNA bacteriophages, which is about 1×10^6 daltons (e.g., Bradley, 1971).

The idea that the DNA bacteriophages originated as episomes and evolved in a series of steps to complete viruses has been recently reviewed by Bradley (1971). Before this scheme can be applied to the mycoplasmaviruses, more data on viral evolution will be required for the bacteriophage systems and more details on the basic biology of the mycoplasmaviruses must be accumulated.

Given the obvious fact of the origin of the mycoplasmaviruses, their survival and spread must have not been too difficult. We have shown that mycoplasma can support viral growth, and even those that are resistant to viral infection can be infected by viral DNA (Table V). The lysis of cells carrying virus in some carrier state may release infectious DNA to spread the virus to other mycoplasmas.

The mycoplasmavirus carrier state seems extremely stable, and spon-

taneous plaques are infrequent. This latter observation may be due to the fact that the viruses grow slowly (group L1 virus release takes about 2 hours) and the cells are only sensitive to infection during logarithmic growth, so once the culture ages the cells are resistant to the viruses. Parenthetically, cell densities are generally only high enough to allow a good probability of a virus colliding with a cell when the cells are in the late logarithmic growth phase. Therefore, the viral growth properties probably work against the establishment of an ongoing infection in the culture and allow the carrier state to persist.

VII. Concluding Remarks

It is three years since the isolation of the first mycoplasmavirus. We have tried to review here the outlines of mycoplasma virology that have been established in that time. The mycoplasmaviruses are ecologically distinct: Like bacteriophage they infect prokaryotic cells, but like animal viruses they must adsorb to and penetrate a lipoprotein unit membrane structure. Hence, mycoplasma virology overlaps both areas; it uses both for models and, in turn, should contribute information to both.

In view of the prevalence of the mycoplasmaviruses, we should remember that the mycoplasmas are medically significant pathogens. The future development of mycoplasma virology must allow us to examine the possible role of these viruses in determining mycoplasma pathogenicity and consider the suggestion that the role of mycoplasmas in disease may be as vectors for viruses (Atanasoff, 1972).

VIII. Appendix: Calculation of Adsorption Rate Constant

To calculate the rate of contact between a group L1 virus and a cell due to collision kinetics, we must first calculate a diffusion coefficient for the rod-shaped virus particles. The diffusion coefficient is

$$D = kT/f$$

where $k = 1.38 \times 10^{-16}$ dyne cm/deg (Boltzmann's constant), $T =$ 300°K, and $f =$ frictional coefficient (dyne second/cm). The frictional coefficient of a rod-shaped particle can be approximated by a prolate ellipsoid with semiaxes a, b, b (Tanford, 1961), where

$$\frac{b}{a} = \left(\frac{3}{2}\right)^{1/2} \frac{d}{L}$$

and d = diameter of virus = 14 nm, L = length of virus = 71 nm. With this approximation, the frictional coefficient is given by

$$\frac{f}{6\pi\eta R_0} = \frac{\alpha}{(b/a)^{2/3} \ln [a(1+\alpha)/b]}$$

where $\alpha = (1 - b^2/a^2)^{1/2}$, R_0 = radius of sphere with same volume as rod-shaped particle = $(3d^2L/16)^{1/3}$, η = viscosity of water = 10^{-2} poise. Combining these equations gives

$$D = 1.33 \times 10^{-7} \text{ cm}^3/\text{second}$$

for the virus.

The adsorption rate constant is given by (e.g., Stent, 1963)

$$k = 4\pi DR$$

where R = radius of sphere whose surface is equal to that of cell = 0.5×10^{-4} cm. Putting in the values for D and R and converting from seconds to minutes gives

$$k = 5 \times 10^{-9} \text{ cm}^3/\text{minute}$$

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CHAPTER 22

The Evolution (Evovirology) of Herpesviruses

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Whether we look at infectious diseases primarily as microbiologists concerned with understanding and circumventing the microbial aggressors or as immunologists studying the reactions of the host, we shall need above all to apply ecological and evolutionary principles.

Burnet and White (1972)

Some people are inclined to retain what separates, others what unites. We belong to the latter group.

Lwoff and Tournier (1971)

I. Introduction

The need to apply an evolutionary perspective to herpesviruses became apparent to me about two years ago when I was asked to present an overview of herpesviruses and their pathobiological aspects (Nahmias, 1972a,b). The time seemed propitious for several reasons.

There was a mounting number of reports describing the isolation of one or more distinct herpesviruses in a variety of species, spanning the vertebrate phylum from fish to man. Information had become available on the natural history in their own hosts of many herpesviruses, particularly those of medical and veterinary importance. Several common features among the various herpesviruses, such as viral persistence, had become apparent, and others, such as oncogenic potential, were becoming appreciated. Furthermore, a large variety of laboratory methods was being applied to compare relationships among many of the herpesviruses.

Over the past two years, more information has been acquired related to these various aspects of herpesviruses, providing at the same time more questions of evolutionary import as well as some approaches to their resolution. It is the purpose, then, of this report to focus on evolutionary issues concerning herpesviruses, with the realization that more questions will be raised than can be conclusively answered at this time.

II. The Problem of Classification

Herpesviruses are generally defined as large enveloped virions with an icosahedral capsid consisting of 162 capsomeres and arranged around a DNA core (Roizman, 1969). Viruses with such characteristics have been detected not only in a large number of vertebrate species from fish to man, but also more recently (Farley *et al.*, 1972; Kazama and Schornstein, 1972) in invertebrate oysters and in marine fungi (Fig. 1). As the figure also indicates, up to five different herpesviruses may be found in a single species, and several herpesviruses have been found capable of infecting, under natural conditions, species other than their natural host.

This large array of viruses has made it difficult for the Herpesvirus Study Group of the International Committee for the Nomenclature of Viruses to provide some uniform method of nomenclature. As a result, the Study Group has proposed recently (1973) that only a provisional labeling system can be considered at present, suggesting that (1) the label for each herpesvirus would be in an anglicized form; (2) each herpesvirus would be named after the taxonomic unit to which its primary natural host belongs; and (3) the herpesviruses within each group would be given arabic numbers (rather than "type").*

All classification systems, including the phanerogram, the cryptogram, and the gymnogram (Lwoff and Tournier, 1971) make use of both

* For the present discussion, however, vernacular common usage terms will be retained.

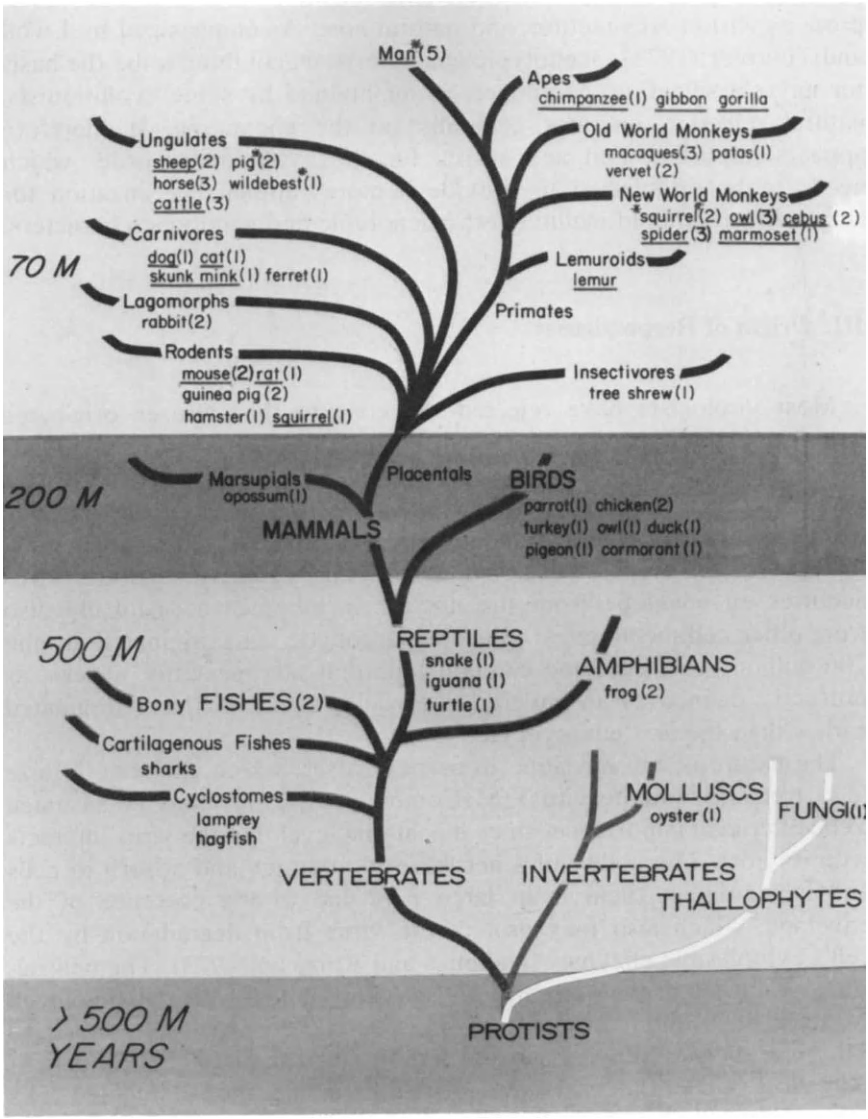

FIG. 1. Evolution of species with herpesviruses; no phylogenetic relationship among the various herpesviruses themselves is implied in this figure. Number in parentheses is the number of herpesviruses identified in each species. Species underlined are the species susceptible to herpesviruses from other species. Asterisk denotes species with herpesvirus which have been found to infect other species under natural conditions. Adapted from Nahmias (1972b).

genotypic and phenotypic characteristics of the virus, e.g., nucleic acid, proteins, virion architecture, and natural host. As emphasized by Lwoff and Tournier (1971), phenotypic characteristics continue to be the basis for any classification. Moreover, as maintained by some evolutionists, natural selection operates generally on the phenotype. It therefore appears important that any search for the evolutionary order which needs to be established to provide a more rational classification for herpesviruses should include both phenotypic and genotypic characters.

III. Origin of Herpesviruses

Most virologists have rejected the concept that viruses originated from early abiotic organic material, basing their argument primarily on the premise that all viruses require other living forms to multiply (Luria and Darnell, 1967). The current concept is that viruses were derived from genetically specific components of cells. One of the characteristic features of all herpesviruses, studied in any detail, is that the virus acquires an envelope from the nuclear membrane (occasionally also from other cell membranes). Since eukaryocytic cells originated around 700 million years ago, one can conclude that a herpesvirus, at least as presently defined as an enveloped particle, could not have originated earlier than the first eukaryocyte.

The nature of the envelope in herpesviruses, which can vary in size (e.g., tree shrew herpesvirus) (McCombs *et al.*, 1971), may be assumed to be of crucial importance, since it is at this level that the virus interacts with its host. The ability of a herpesvirus to attach and adsorb to cells and hence infect them is in large part due to the presence of the envelope, which also may protect the virus from degradation by the cell's cytoplasmic enzymes (Nahmias and Roizman, 1973). The neutralizing responses in the vertebrate host's body fluids is also at this level. Another important consideration, in several herpesviruses studied to date, e.g., herpes simplex viruses (HSV) (Roizman, 1971) or Epstein-Barr virus (EBV) (Klein, 1972a), is that the membranes of infected cells acquire biochemical and antigenic components similar to those found on the viral envelope itself. Cell membrane changes occur early after viral infection, may be produced in the absence of viral DNA synthesis, and are believed to be of importance as regards immunological responsiveness of the vertebrate host and in carcinogenesis by herpesviruses. It should be kept in mind, however, that not only is nonenveloped virus occasionally infectious, but that in case of HSV-1, infection can be

accomplished *in vitro* with deproteinized "naked" DNA (Lando and Ryhiner, 1969).

There is a large temporal gap to account for between the first clinical description of herpesvirus infection [herpes febrilis (HSV-1)] around 20 centuries ago and the first eukaryocytic cell from which an enveloped herpesvirus could have originated, approximately 700 million years ago. The presence of the various herpesvirus in today's species (Fig. 1) tells us only that a herpesvirus found in a particular species could not have occurred in its present form earlier than when that species evolved. It may be pertinent in this regard that oysters, from which a herpesvirus has recently been isolated, are believed not to have changed for 150 million years (Simpson, 1944).

An early origin of herpesviruses is also suggested by results of genotypic analyses. As noted in Table I, the guanine + cytosine content of the DNA of several vertebrate viruses ranges from 45 to 74 moles %. Such a substantial divergence in DNA base composition would imply wide evolutionary separation among herpesviruses.

The pattern of nearest-neighbor base sequences has been determined by Subak-Sharpe *et al.* (1966) for HSV-1, porcine pseudorabies virus, and equine rhinopneumonitis (type 1) viruses. The patterns differed widely not only among the three herpesviruses but also in relation to that found in mammalian cells. These workers concluded that it was unlikely that these herpesviruses originated from mammalian cells. It would be of interest, nevertheless, to compare the nearest-neighbor base sequences of herpesviruses of lower forms with invertebrate or fungal cells, now that herpesviruses have been isolated from these organisms.

IV. The Matter of Survival

> The main problem that a parasitic species has to solve if it is to survive is to manage the transfer of its offspring from one individual host to another (Burnet and White, 1972).

Another point favoring a relatively early origin for most herpesviruses, as compared to almost all other virus families, is that the herpesviruses have the greatest capacity for surviving when small numbers of susceptible hosts are available, a situation probably quite common as the host species themselves developed. This capacity to survive is conveyed to herpesviruses by their ability to persist in spite of various host defenses and hence enables them to spread to other hosts at a later time.

In evolutionary terms, this property of herpesviruses must be consid-

TABLE I

DNA Base Composition and *in Vitro* and *in Vivo* Host Range of Herpesviruses

Herpesvirus (common name)	Base composition G + C (moles %)[a]	*In vitro* tissue culture host range[b]	*In vivo* host range[b]
Porcine pseudorabies	72–74	B	B
Bovine infectious rhinotracheitis	71–72	B	L
Squirrel monkey *Herpes saimiri*	69–70	B	B
Human herpes simplex type 2	68–69	B	B
Human herpes simplex type 1	66–67	B	B
Squirrel monkey T virus	67	B	B
Tree shrew herpesvirus	66	?	?
Equine coital exanthem – type 3	66–67	L	L
Bovine mammilitis	64	B	B
Guinea pig herpesvirus	57	L	L
Human Epstein-Barr virus	56–58	L	L
Human cytomegalovirus	56–58	L	L
Murine cytomegalovirus	56–58	L	L
Equine cytomegalovirus (type 2)	56–58	L	L
Equine abortion-rhinopneumonitis (type 1)	55–57	B	L
Frog virus No. 4	54–56	L	L
Vervet monkey cytomegalovirus (SA8)	51	L	L
Chicken Marek's disease virus	47	L	L
Turkey herpesvirus	47	L	L
Chicken infectious laryngotracheitis	45–47	L	L
Frog herpesvirus (Lucké)	45–47	L	L
Feline rhinotracheitis	46	L	L
Human varicella-zoster	46	L	L

[a] Adapted from Plummer *et al.* (1969), Bachenheimer *et al.* (1972), and Ludwig (1972). The G + C content of canine herpesvirus of 33 moles% has been challenged.

[b] B, broad; L, limited.

ered as the central issue for their survival in time and, as such, would be likely to have originated very early. Most other phenomena associated with herpesviruses are also likely to be related, in some way, to this feature. Indeed, one wonders how the morphological complex of the particular geometrical configuration of the herpesvirion and its envelope relate to this major character.

Viral persistence, however, would be a dead end, unless the virus were present in a site from which it could spread to other hosts – hence, the need to examine now the various modes of spread of herpesviruses.

A. Herpesviruses—With and without Love

Although the exact mode of spread has not been well substantiated for all herpesviruses, the two major routes of viral transmission, at least for vertebrate viruses, appear to be via the air or by contact. Air dissemination could occur not only via air droplets but also via infected feather follicles (Calneck *et al.*, 1970) and possibly skin squames. In man, kissing may well be involved in the spread of HSV, cytomegalovirus (CMV), and EBV; this suggestion in the case of infectious mononucleosis has gained support with the ability to isolate EBV for long periods from the throat (Miller *et al.*, 1973).

Several of the herpesviruses have been recently demonstrated to spread primarily by a venereal route. The venereal human HSV, type 2, (Nahmias *et al.*, 1973) and the equine coital exanthem virus, type 3, (Studdert, 1973) have been found to differ in several antigenic and biological characteristics from the nonvenereal viruses in the same hosts. Preliminary studies suggest that although the venereal canine herpesvirus is antigenically similar to the nonvenereal virus, the two viruses express different biological effects (Poste, 1972). Bowling and coworkers (1969) have been unable to demonstrate any significant antigenic or biochemical differences between the venereal [infectious pustular vulvovaginitis (IPV)] and nonvenereal [infectious bovine rhinotracheitis (IBR)] bovine herpesviruses. This may be due to the adaptation of the venereal virus to respiratory spread in recent times (McKercher, 1973). The isolation of human cytomegaloviruses from the cervix (Reynolds *et al.*, 1973) and from semen (Lang and Kummer, 1972), and their common recovery in women attending venereal disease clinics (Jordan *et al.*, 1973), suggest that these viruses may also possess a venereal mode of spread. In evolutionary terms, the ability to be transmitted venereally offers another survival advantage for the virus infecting species that reproduce sexually. It should be noted that nonvenereal strains may be occasionally isolated from the genital tract, e.g., equine type 1 herpesviruses in horses (Studdert, 1973) or HSV-1 in humans (Nahamias *et al.*, 1973), suggestive of an oral-genital mode of spread.

Various herpesviruses persist in peripheral blood leukocytes (see below), and some, e.g., human CMV, can be transmitted via blood transfusions (Lang, 1972). No evidence currently exists for blood-borne virus transmission by vectors, such as insects. There is, however, evidence for the transplacental transmission of blood-borne virus in several mammalian species (reviewed by Nahmias, 1972b). In general, such transmis-

sion would have little survival value for the virus, since in most cases, the fetus would be aborted. An exception might be human CMV, which occurs in approximately 1% of newborns and kills very few of the infected infants; survivors can then serve as a long-term source of virus to other susceptible hosts, as well as demonstrating on occasion chronic neurological or other ill effects.

Transmission of human CMV via breast milk may also occur, and a similar mode of viral spread would be expected to occur occasionally when herpesviruses affect the mammary glands of other mammals, such as cattle. Although some herpesviruses, e.g., human CMV and HSV, can be isolated from the urine, there is no information regarding the possibility that urine may serve as a source of transmission of herpesviruses. Neither has the oral-fecal route of spread been documented for any of the herpesviruses.

Another mode of spread for some of the mammalian herpesviruses is from the infected maternal genital tract to the newborn around the time of delivery (Nahmias *et al.*, 1970; Reynolds *et al.*, 1973). This may be advantageous for further transmission only if the infected newborn does not die from his infection (see below). No vertical transmission via germ plasm has been documented in herpesviruses.

B. The Ecological Niche

The ecological niche for a virus is its host, in particular those host tissues and cells which the virus can infect. For a herpesvirus, the ecological niche would also include those tissues or cells in which it can persist. From an evolutionary perspective, therefore, it appears appropriate to consider what has been termed, from other viewpoints, "tropism" in both its clinicopathological and viral persistence aspects. Each of these aspects and their interrelationships will be discussed within the larger framework of the host with its nonimmune and immune influences.

1. Clinicopathological Effects of Herpesviruses

One of the most striking similarities among herpesviruses is that they generally cause either a subclinical infection or a nonlethal disease in their natural host, with important exceptions to be discussed later as a separate topic. On the other hand, there are several examples of severe, often fatal diseases when a herpesvirus is found to be at all capable of infecting another species under natural conditions (Fig. 1, species underlined). The examples include cattle killed by wildebeest virus;

gibbons, lemurs, owl monkeys, and skunks by HSV-1; sheep, cattle, cats and dogs by pseudorabies virus of pigs; and marmosets and owl monkeys by the T virus of squirrel monkeys (reviewed by Nahmias, 1972b). One interesting exception to this common theme is the nonlethal human varicella infection in chimpanzees and gorillas. Nevertheless, the observations that vervet monkey herpesvirus kills many of these animals (Hunt and Melendez, 1969) or that owl, pigeon, duck, or falcon herpesviruses kill many of these birds (Maré and Graham, 1973) would suggest that another undetermined species is the actual natural host.

The tissues affected by herpesviruses in its otherwise healthy natural host, outside infancy, are, in general, external sites from which virus can spread. These include (1) the skin, oral or genital mucous membranes, e.g., the herpesviruses of fish, turtles, cats, cattle, horses, macaque and Patas monkeys and the human HSV-1, HSV-2, and varicella-zoster (VZ) viruses; (2) the upper, and occasionally lower, respiratory tract, e.g., herpesviruses of chickens, cats, dogs, horses, cattle and VZ virus; (3) the eyes, e.g., herpesviruses of chickens, cats, dogs, pigs, and HSV-1 and VZ virus.

Internal tissues involved include the liver in case of several avian species (Maré and Graham, 1973) and the lymphoid organs, e.g., infectious mononucleosis with EB virus (Henle *et al.*, 1968). Various herpesviruses have been detected in peripheral blood leukocytes; these viruses include herpesviruses of chickens, turkeys, guinea pigs, cattle, wildebeest, pigs, cottontail rabbits, dogs, several Old World and New World monkeys, chimpanzees, and human CMV, HSV-2, and EBV (reviewed by Nahmias, 1972b). This particular affinity for lymphoid cells may be related to one of the mechanisms of persistence and, as suggested by the observations of Bryans (1969) with equine type 1 herpesvirus, could also permit the virus to escape neutralization by circulating antibodies.

There is a great deal of information available on the ability of various herpesviruses to infect cells in culture from species other than their own. Some viruses, e.g., herpes simplex viruses, have been found to have a broad *in vitro* host range, whereas others, e.g., human CMV, have a limited host range (Table I). Although all herpesviruses, except EBV, produce intranuclear inclusions, some also produce intracytoplasmic inclusions. There are many variabilities among the various herpesviruses, as well as within different strains of the same herpesvirus, in plaque size or syncytial formation. These differences can often be attributed to the cell culture used (reviewed by Nahmias and Dowdle, 1968), environmental conditions [e.g., temperature effect on iguana virus syncytial formation (Clark and Karzon, 1972)], or to genetic differences.

Indeed, syncytial formation has been used as a genetic marker for studies with temperature-sensitive mutants of HSV and is believed to represent a point mutation (Subak-Sharpe, 1973). In addition, herpesviruses have been classified by Melnick and McCombs (1966) on the basis of whether they are cell-associated or not. This differentiation appears artificial; for example, both human CMV and VZ viruses are classified as cell-associated, yet extracellular virus is found in high titers in the urine or vesicular fluid, respectively. Marek's disease virus is present in various organs of the chicken in a cell-associated form, yet infectious virus is recoverable from feather follicles (Calneck *et al.*, 1970). Iguana herpesvirus is cell-associated at warm temperatures; yet at cold temperatures, the virus is readily released extracellularly (Clark and Karzon, 1972).

A large number of observations have been made on the ability of many herpesviruses to infect foreign hosts, under natural or experimental conditions, and on the consequences of such infections. Some of the viruses have a limited *in vivo* host range, appearing to infect only its own or closely related species, e.g., human CMV, whereas others have a much broader host range, infecting widely diverse species, e.g., HSV (Table I). Experimental studies on foreign hosts have been helpful in suggesting possible pathogenetic pathways, types of persistence, or carcinogenic potential of various herpesviruses. For studies of human herpesvirus there are few other alternatives. However, it would appear that in case of nonhuman herpesviruses, results obtained with natural, or even experimental, infection of a herpesvirus in its own host would be more readily interpretable for comparative or evolutionary considerations.

2. *Viral Persistence*

Current information suggests that herpesviruses persist in the host in two types of tissue—neural and extraneural. The persistence of HSV-1, HSV-2, and equine herpesvirus in the nerve ganglions of experimentally infected animals has been well documented (Plummer *et al.*, 1970; Stevens and Cook, 1973). HSV-1 has now been repeatedly isolated from trigeminal ganglions of human cadavers (Bastian *et al.*, 1972; Stevens and Cook, 1973; Baringer and Swovland, 1973). In both the animal and human studies, infectious virus can only be demonstrated by cocultivation on susceptible cells or by growing the ganglion for several days or weeks. Whether these herpesviruses persist in nerve cells or in supporting cells of the ganglion, and whether there is back-and-forth traffic of virus in a complete or incomplete form, is still not established.

Extraneural tissues in which herpesviruses have been found to persist

include lymphoid tissues (see above) and other tissues, such as parotid glands and kidneys (e.g., human CMV).

Environmental factors play an important role in inducing reactivation of several "latent" herpesviruses. This phenomenon has been noted even in marine fungi, in which virus is only demonstrable when nutrient medium is unavailable (Kazama and Schornstein, 1972). Temperature is another important factor. The herpesvirus associated with the frog Lucké adenocarcinoma is demonstrable only at low temperatures (winter form) and remains in a latent state at warm temperatures (Granoff, 1972). In humans, it is well appreciated that hyperthermia reactivates herpes simplex virus infections. On the other hand, hyperthermia has been shown to suppress viral dissemination in mice experimentally infected with HSV (Schmidt and Rasmussen, 1960) or in pups infected with canine herpes (Carmichael *et al.*, 1970). These observations indicate that the virus–cell interaction related to persistence is separable from the virus–host interaction at the pathogenetic level.

Several other factors have been found to contribute to reactivation of herpesviruses, e.g., menstruation for HSV, corticosteroids for bovine infectious rhinotracheitis virus (Sheffy and Davies, 1972), and IUdR or BUdR and arginine depletion for EBV (see Klein, 1972b). The detailed mechanisms of viral persistence remain to be elucidated. It appears, however, that herpesviruses may persist in cells in various forms where (1) the viral genome only is detectable; (2) viral antigens are also detectable; (3) infectious virus is also demonstrable. It should also be noted that exogenous reinfection with the same virus can also occur on occasion with some animal or human herpesviruses (Nahmias and Dowdle, 1968).

3. Fatal Herpetic Disease

From an evolutionary perspective, the death of its host has no survival value for any parasite, including a virus. An attempt to explain such occurrences will now be made for herpesviruses within the context of the two aspects of the ecological niche and the nonimmune or immune influences of the host. For herpesviruses, potentially lethal consequences of infection include (a) encephalitis, (b) cancer, (c) fetal and neonatal disease, and (d) disease in the comprised host.

a. Encephalitis. There are two possible methods for virus to spread to the brain—via a hematogenous or via a neurogenic route. Hematogenous transmission to the brain of those herpesviruses that can be isolated from peripheral blood leukocytes, e.g., human CMV or EBV, is very infrequent. In case of HSV-1, such blood dissemination to the

brain appears to occur primarily in the newborn or compromised host, whereas HSV-2 infections in normal hosts generally results in a benign meningitis (Craig and Nahmias, 1973).

Several experimental and/or clinicopathological observations suggest that HSV-1 and VZ encephalitis are neurogenically transmitted (Craig and Nahmias, 1973). Such relatively infrequent outcomes of infection with these viruses may be viewed as a consequence of an aberration of their persistence in neural tissues. This may occur as a result of a primary HSV-1 infection before the virus establishes itself in a "static" relationship in neural tissue or as a consequence of virus reactivation from such an integrated state, so that the virus would now travel centrally rather than peripherally. It is also possible that herpesviruses may remain latent in parts of the brain itself, as demonstrated with B virus in rhesus monkeys (Keeble, 1960) and with HSV in experimentally infected rabbits (Stevens and Cook, 1973). The several examples of brain involvement, presumably by neurogenic spread, of a variety of vertebrate herpesviruses when they affect a foreign host may also be the result of unsuccessful attempts to establish a persistent relationship in neural tissue. Involvement of sensory ganglions of cattle, sheep, cats, or dogs by pseudorabies virus of pigs causing pruritis in these animals, which is then followed by neurogenic spread to the brain (Plummer, 1967), is compatible with this view.

b. Cancer. A variety of cancers in man and other vertebrates has now been associated with several herpesviruses (Table II). The purpose here is not to review this area, which has been the subject of several recent conferences (Biggs *et al.*, 1972; Herpesviruses and Cancer, 1972; Herpesvirus and Cervical Cancer, 1973). Rather, the question is how to explain neoplastic transformation, which has no apparent evolutionary survival advantage to herpesviruses. One likely possibility is that such an occurrence is an aberration in the integration of the viral genome in cells in which the virus would persist. For instance, in Marek's disease of chickens, the lymphomas occur in lymphoid cells, in which virus assumes a noninfectious form, rather than in the feather follicles, from which infectious virus can be recovered. The other examples of leukemias or lymphomas associated with human EBV, two New World monkey herpesviruses, and cottontail rabbit and guinea pig herpesviruses also correlate with the persistence of nonproductive virus in lymphoid cells. In case of the frog herpesvirus associated with adenocarcinoma of the kidney, there is this extraordinary effect of low temperatures in activating virus in "summer" tumors. Indeed, a variety of environmental or host factors must interplay with this virus–cell interaction (Table II). The burgeoning understanding of the role of immune factors

TABLE II

Herpesviruses Associated with Cancer (in Order of Strength of Evidence for Etiologic Association)

Strength of association	Herpesvirus	Species of origin	Type of malignancy	Associated factors
Strong	Marek's disease virus	Chicken	Lymphoma	Dose and virulence of virus; stress, genetic, age and immune factors
	H. saimiri	Squirrel monkey	Lymphoma, leukemia	Host differences suspected
	H. ateles	Spider monkey	Lymphoma	Host differences suspected
	Lucké virus	Frog	Adenocarcinoma of kidney	Temperature, immune factors (?)
	EB virus	Man	Lymphoma	Genetic factors, immune factors (?), malaria (?)
Fair to good	Herpes simplex type 2	Man	Cervical cancer	Pregnancy (?) or hormones (?)
	EB virus	Man	Nasopharyngeal carcinoma	Genetic factors, immune factors (?)
	Cottontail rabbit virus	Cottontail rabbit	Lymphoma	
	Guinea pig virus	Guinea pig	Leukemia	Association with RNA oncornavirus (?)
Weak	Herpes simplex type 2	Man	Prostatic cancer, other urogenital cancers	
	Herpes simplex type 1	Man	Lip cancer, other oral cancers, laryngeal cancer	
	Sheep virus	Sheep	Pulmonary adenomatosis, lung cancer (?)	
	Hamster virus	Hamster	Ileocecal proliferation disease, adenocarcinoma (?)	
	Canine herpesvirus	Dog	Lymphoma	
	Equine herpes type 3	Horse	Lymphoma (in hamsters)	
	Bovine herpesvirus	Cow	Lymphosarcoma, sarcomas (in hamsters)	

in this complex has recently been reviewed (Klein, 1972a). Of particular interest is the apparent ability of the turkey herpesvirus, related to Marek's disease virus, to prevent neoplastic transformation by the chicken virus without preventing infection and replication with that virus.

c. Fetus and Newborn. Many of the mammalian herpesviruses have been recognized as causing abortions (Nahmias, 1972b), a problem of particular veterinary and human import. The abortions may result from actual viral transmission to the fetus or as a consequence of disease in pregnant hosts, e.g., in cats infected with feline herpesvirus (Hoover and Griesemer, 1971). Several herpesviruses also cause lethal disease in infected infants, including the human herpesviruses (CMV, HSV-1, HSV-2, and VZ virus) and the herpesviruses of dogs, cats, pigs, cattle, and horses (reviewed by Nahmias, 1972b). Maternal antibodies to these herpesviruses convey varying degrees of protection to the fetus and newborn and are usually not fully protective.

The damaging effect of herpesviruses on the fetus and newborn may be viewed as an incidental occurrence secondary to the presence of persistent or infectious virus in the blood or genital organs of the mother. These ill effects are most likely a result of developmental and immunological immaturity of the fetus or newborn; however, the exact mechanisms remain to be elucidated. Although the advantage for herpesviruses having a host other than the mother as a second source of virus for later transmission may not be of primary survival value, some adaptation appears to have occurred in case of human cytomegaloviruses which kill very few of the infected newborns (see above).

d. The Compromised Host. There are several examples of the inducement by various stresses of profuse proliferation of herpesviruses in a variety of species. For example, in man, severe malnutrition permits a fatal disseminated disease with herpes simplex viruses. Several of the conditions in humans which are associated with severe, if not fatal, herpesvirus infections have been related to depressions in immune factors. The increasing severity of VZ virus pneumonia and of EB virus infectious mononucleosis in older individuals also suggest hormonal effects. Neither hormonal nor immune factors, however, are well understood as they relate to herpesvirus infections. The observations of persistent nonlethal herpesvirus infections in fungi and oysters, which do not possess classical immunoglobulins and delayed hypersensitivity responses, would suggest that nonspecific host factors are involved. Both nonspecific and specific immune factors appear to participate in controlling herpes simplex virus infection (see Nahmias and Roizman, 1973).

Whatever the stress might be that affects the intimate cell–virus relationship, herpesviruses would gain an important survival advantage if they were induced to proliferate extensively, increasing their transmission potential to other susceptible hosts.

V. Phylogeny of Herpesviruses

There are two evolutionary patterns for the acquisition of similar morphological characteristics among various organisms—parallel and convergent evolution. The first pattern would require all herpesviruses to have evolved from one common progenitor. Convergent evolution would demand that the same mechanisms from which herpesviruses originally evolved would repeat itself over fifty times. It is also possible that convergent evolution would have occurred only a few times, in which case there would not be fifty, but relatively few, progenitors of herpesviruses.

For any of these possible events, there are two main mechanisms to explain how a progenitor herpesvirus in an earlier species evolved into the various herpesviruses as the host species evolved themselves. One is that a herpesvirus from an older species could have infected a new species after it had already evolved, and, in so doing, the virus would now assume new characteristics. As noted earlier, however, herpesviruses generally cause severe, usually fatal infections in foreign hosts, even if the species are not too distantly related, e.g., squirrel T virus in owl monkeys, HSV in gibbons, or rhesus B virus in man. Since mild infections in foreign hosts may not be readily identifiable, the herpesvirus in such surviving hosts might in time become adapted to the new species. A well-appreciated example of attenuation of a virus on repeated passages is that of myxoma virus of rabbits (Fenner and Ratcliffe, 1965). However, to be successful, such a mechanism requires a large population of susceptible hosts and may actually be a factor in selecting the more resistant hosts in the population.

The second mechanism that could have operated in the evolution of a herpesvirus would be its concurrent evolution with its host, as the host itself was evolving into a new species. Such coevolution of host and parasite has been implicated in the evolution of animal parasites (Inglis, 1965).

Can these various possibilities be differentiated with presently available information in order to develop a phylogenetic order for herpesviruses? Can one determine, for instance, the various origins of the five human herpesviruses: EBV, CMV, VZ, HSV-1, and HSV-2? Intrahost

competition does not appear to be operative, since all five viruses can not only infect the same host at various times but also persist in its own ecological niche at the same time.

Several of the genotypic and phenotypic characters of HSV-1 and HSV-2 are so similar that the occurrence of parallel evolution is most likely for these two viruses. The genotypic similarities include closeness in guanine+cytosine content of their DNA (Table I) and in the pattern of nearest-neighbor sequences (Subak-Sharpe, 1973). Approximately 50% homology between the DNA of the two viruses has been demonstrated (Kieff *et al.*, 1972; Ludwig, 1972), and common and variable DNA base sequences have been found. Moreover, genetic recombination between temperature-sensitive mutants of HSV-1 and HSV-2 has been possible (Subak-Sharpe, 1973). The two viruses, besides possessing type-specific antigens, including viral thymidine kinase (Wildy, 1973), also share common antigens (Schneweis and Nahmias, 1971).

On the other hand, there are variable observations related to the other human herpesviruses. Thus, if relationships between the herpesviruses are considered according to their method of persistence in host tissues, the human herpesviruses might be grouped as follows: (1) HSV-1, HSV-2, VZ virus; (2) EBV; (3) CMV. Yet the G+C content of herpes simplex virus and VZ virus are quite different (Table I), whereas the G+C content of human EBV and CMV are similar. These latter two viruses are antigenically distinct and differ in several other phenotypic characteristics.

The same dilemma applies to all herpesviruses that have been studied in any detail. Various phenotypic characters, noted earlier, could be used besides mode of persistence. These include similarities or differences in mode of spread, clinicopathological manifestations, *in vivo* and *in vitro* host range, and type of inclusions produced in cells. Another important feature, not previously discussed, is the antigenic relationship found among the various herpesviruses. It would be expected that serological tests detecting cross-reactions with viral envelope antigens, e.g., by neutralization or cross-protection tests, would show closer relationships than serological tests, e.g., immunodiffusion using soluble viral proteins containing a variety of virus proteins.

Neutralization studies suggest a close relationship, not only between HSV-1 and HSV-2 but also between these human viruses, the B virus of macaques, the SA-8 virus of vervet monkeys, and the T virus of squirrel monkeys (Ueda *et al.*, 1968; Stevens *et al.*, 1968; Blue and Plummer, 1973). The stronger reactions of the human herpesviruses with the Old World monkey viruses than with the New World monkey virus are consistent with an earlier common parallel evolution. A closer rela-

tionship would be predicted among the herpesviruses of owls, pigeons, and falcons than with the duck virus or the infectious laryngotracheitis virus of chickens (Maré and Graham, 1973). It appears also that the chicken Marek's disease virus is more closely related to the turkey herpesvirus than to the other chicken herpesvirus (infectious laryngotracheitis).

Immunofluorescent, immunoprecipitin, and complement fixation tests have shown much wider cross-reactions between the various herpesviruses than neutralization assays. Such cross-reactions could suggest the existence of group antigens for herpesviruses, e.g., between HSV, human CMV and EBV, frog Lucké virus, and the chicken Marek's disease virus (Kirkwood *et al.*, 1972; Ross *et al.*, 1972) or between human CMV, EBV, and VZ virus (Hanshaw *et al.*, 1972). However, until the whole array of herpesviruses is systemically studied with a variety of serological tests under well-controlled conditions, it will not be possible to determine whether the cross-reactions that are found would be useful for phylogenetic considerations.

Studies of the genotype of several herpesviruses have included determinations of the G+C content of the DNA (Table I) and of the pattern of nearest-neighbor sequences (Subak-Sharpe *et al.*, 1966), as well as nucleic acid hybridization studies among several herpesviruses (zur Hausen *et al.*, 1970; Bachenheimer *et al.*, 1972; Granoff, 1972; Bronson *et al.*, 1972; Ludwig, 1972). The wide range of G+C content of the DNA among a large number of herpesviruses (46–72 moles %), the lack of common patterns in nearest-neighbor sequences of three herpesviruses, and the lack of nucleic acid homology among various pairs of eight herpesviruses do not shed light on the evolutionary relatedness among herpesviruses. *A priori,* one would expect to find common genes for some of the phenotypic characteristics shared by all herpesviruses. The fact that, except for HSV-1 and HSV-2, genotypic similarities among the herpesviruses studied have not been detected at present may reflect the lack of sensitivity of the various techniques employed. The studies with herpes simplex virus (Kieff *et al.*, 1972), demonstrating common and variable sequences in their DNA, suggest that the common genes among far-related herpesviruses would be small in number, as compared to the variable genes that would be segregated as evolution progressed. More precise information on genetic relatedness awaits resolution of methods to measure specific nucleotide sequences of the DNA of herpesviruses. Although such studies are currently being attempted with other viruses, the size of the herpesvirus DNA (around 100×10^6 daltons) will make such endeavors particularly difficult.

Certain correlations between G+C content of the DNA of several

herpesviruses and their *in vivo* and *in vitro* host range have suggested some form of grouping (Nahmias, 1972b). Another form of grouping, according to mode of persistence, has also been suggested here. However, the overall picture of the phylogeny of herpesviruses awaits further developments. At the accelerated pace with which herpesviruses from a wide variety of species are currently being studied, resolution of some of these evolutionary issues may not be too far in the future.

VI. Envoi

The evolutionary perspective with its unique four-dimensional time-encompassing view of the universe has been applied at various levels—atomic, molecular, macromolecular, genetic, cellular, multicellular, neural, mental, and social (Rolfing and Oparin, 1972). Although interdependence among a variety of scientific disciplines has proved necessary to provide the larger canvas, certain general principles have guided the study of evolutionary questions at any of these levels. At the level of viruses, such evolutionary principles can also offer a different perspective than heretofore used in the study of viruses. I have attempted in this presentation to employ the evolutionary perspective in the overview of herpesviruses from fungi to man. It appears, as evidenced by the various chapters in this volume, that the study of the evolution of viruses, which I have termed evovirology, is indeed gaining recognition as a specialized field of study in virology.

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CHAPTER 23

Evolution of Orthomyxoviruses

ROBERT G. WEBSTER AND ALLAN GRANOFF

. . . it began with a roughness of the jaws, small cough, then a strong fever, with a pain of the head, back and legs; some felt as though they were over the breast and had a weight at the stomach; all of which continued to the third day at the farthest; there the fever went off with a sweat or bleeding at the nose. In some few, it turned to pleurisy, or fatal peri pneumony.

Description of the 1557 influenza epidemic
by Short as quoted by Thompson (1852).

I. Introduction

Influenza continues to be one of the major epidemic diseases of man and, is in fact, his only remaining pandemic disease. This is largely because influenza virus is an evolving pathogen and continues to un-

dergo antigenic variation. The severity of pandemics also varies; in 1918–1919 at least 20 million people infected with influenza virus died of influenza or its complications. The concern of those that work in this field is that future pandemic strains may have the virulence of the 1918 strain.

Influenza viruses are divided into types A, B, and C on the basis of their antigenically distinct ribonucleoprotein antigens. Influenza A viruses cause the major pandemics in man, and various subtypes are found in animals and birds. Influenza B viruses cause less severe epidemics in man and have never been isolated from animals or birds; influenza C causes rare infections only in man. This chapter deals with the evolution of influenza A viruses, particularly with regard to the origin of pandemic strains.

II. The Virus

Influenza virus structure will be briefly discussed only in relationship to those parts of virus anatomy that have dominant roles in its evolution.

A. The Virus Genome

The genome of influenza viruses is segmented, single-stranded RNA which exists in five to seven pieces (21 S to 8 S) (Duesberg, 1968; Pons and Hirst, 1968; Choppin and Pons, 1970; Skehel, 1971). The amount of adenosine tetraphosphates (pppAp) on the 5′ terminal of influenza RNA (Young and Content, 1971) and the presence of nonphosphorylated 3′ termini in the different size classes of RNA (Lewandowski *et al.*, 1971) strongly indicate that the different pieces of genomic RNA result from replication and assortment into the virion and not by endonuclease cleavage of a single polynucleotide some time after maturation at the plasma membrane. The total molecular weight of the pieces of RNA is approximately 4 to 5×10^6 (Compans *et al.*, 1970; Skehel, 1971) and is compatible with the amount of RNA in the virion.

The RNA codes for at least six structural polypeptides and one nonstructural polypeptide. Quantitatively, these polypeptides are in good agreement with the coding capacity of genomic RNA. The virus contains lipid from the host cell and carbohydrate (host antigen) covalently attached to the hemagglutinin and neuraminidase subunits. Detailed information on the polypeptides of influenza virus can be found elsewhere (Kilbourne *et al.*, 1972). This chapter will deal only with the hemagglutinin and neuraminidase subunits—the antigens involved in the evolution of antigenic variants. A schematic representation of the virion components is shown in Fig. 1.

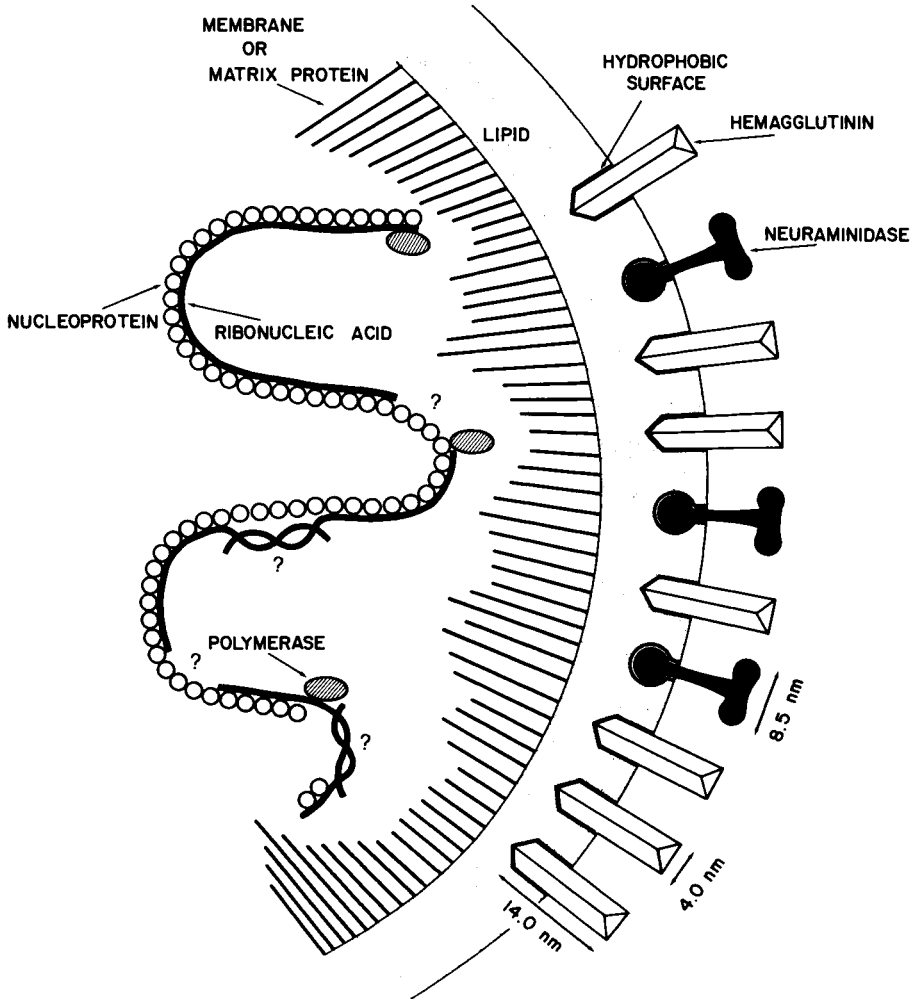

FIG. 1. Diagrammatic representation of a portion of an influenza virion. The hemagglutinin subunit is triangular in cross section and the neuraminidase subunit is "cuff-link"-shaped. The ratio of hemagglutinin to neuraminidase spikes varies from strain to strain and may be as high as 2:1 for some viruses (Webster *et al.*, 1968). The membrane or matrix protein lies beneath the lipid envelope but the arrangement of this layer is not known. The orientation of the nucleoprotein–RNA complex in the virion has not been fully elucidated; these complexes occur in segments when extracted from the virion, but may be linked within the virion by labile bonds. RNA polymerase activity has been associated with the core of the virion but its exact location in the core is unknown.

B. The Virus Hemagglutinin Subunit

The hemagglutinin (HA) is the major surface antigen of the virus and is responsible for the attachment of influenza virus to cell receptors and for the induction of neutralizing antibodies. The HA subunit of influenza virus is a rod-shaped structure about 14 nm long and 4 nm wide with a molecular weight of approximately 150,000 (Laver and Valentine, 1969). The hemagglutinin subunit is a glycoprotein (Compans *et al.*, 1970; Schulze, 1970; Skehel and Schild, 1971) composed of two heavy glycopolypeptide chains [of about 60,000 MW (HA1)] containing the antigenic determinant (Brand and Skehel, 1972) and two light glycopolypeptide chains [of about 30,000 MW (HA2)]. The light chains are joined by —S—S— bond(s) to the two heavy chains to form a dimer (Laver, 1971). The precursor of the two chains of the HA is a polypeptide of molecular weight of about 80,000 which may or may not be cleaved to give two smaller polypeptides depending on the host cell and virus strain used (Lazarowitz *et al.*, 1971, 1973).

C. The Virus Neuraminidase Subunit

The enzyme neuraminidase is the second virus-coded surface subunit on the virion; it hydrolyzes terminal *N*-acetylneuraminic acid (sialic acid) from the specific glycoprotein on the cell surface with which the hemagglutinin attaches, causing the virus to elute. The neuraminidase also induces the production of antibodies, but inhibition of enzyme activity has little effect on the infectivity of the virus, although it prevents the spread of virus to other cells (Schulman *et al.*, 1968).

The neuraminidase subunit of influenza viruses is a "cuff-link"-shaped structure with a cap about 9 nm long and 5 nm wide to which a 10 nm long fiber is centrally attached. The fiber has a diffuse knob about 4 nm in diameter at its end (Laver and Valentine, 1969). The neuraminidase is a polymer composed of four identical molecules of glycoprotein, molecular weight 60,000 (Lazdins *et al.*, 1972; Wrigley *et al.*, 1973).

III. Antigenic Variation of Influenza Viruses

Two kinds of antigenic variation occur in influenza A viruses; major antigenic shift and minor antigenic drift, both involving changes in the hemagglutinin and neuraminidase antigens on the surface of the virus.

A. Antigenic Shift

The major antigenic shifts in the human influenza viruses occurred in 1934 (H0N1), 1947 (H1N1), 1957 (H2N2), and 1968 (H3N2) (Table I). The changes in 1947 and 1968 involved a major antigenic change in the hemagglutinin only, while in 1957 both the hemagglutinin and neuraminidase were different from the preceding viruses. Table I also lists examples of influenza viruses from lower mammals and birds. The follow-

TABLE I

Hemagglutinin and Neuraminidase Composition of Mammalian and Avian Influenza A Viruses[a]

Year isolated	Composition	Prototype strain
1934	H0N1	PR8
1947	H1N1	FM1
1957	H2N2	Asian
1968	H3N2	Hong Kong
1930	Hsw1N1	Swine
1956	Heq1 Neq1	Equine 1
1963	Heq2 Neq2	Equine 2
1949	Hav2 N1[b]	Chicken/Germany
1956	Hav2 Nav1	Duck/England
1965	Hav6 N2	Turkey/Massachusetts

[a] The designation of influenza strains is according to the revised system of nomenclature for influenza viruses (World Health Organization Report, 1971). It consists of a strain designation [antigenic type, host of origin (except isolates from man), geographic origin, strain number, year of isolation] and a description of the hemagglutinin (H) and neuraminidase (N) antigens. The strain designation is followed by the antigenic description and includes in parentheses an index describing the antigenic character of the hemagglutinin subtype (e.g., human H0, H1, H2, H3; equine Heq1, Heq2) and an index describing the antigenic character of the neuraminidase subtype (e.g., human N1, N2; equine Neq1, Neq2). The host designation of H and N antigens is based on the origin of the virus; it does not imply phylogenetic or evolutionary relationships between viruses containing common H or N designation. For simplicity the following abbreviations are used: A/FPV/Dutch/27 (Hav1 Neq1), FPV; A/turkey/Wisconsin/66 (Hav6 N2), T/Wis; A/turkey/Ontario/7732/66 (Hav5 N?), T/Ont; A/swine/Taiwan/70 (H3N2), HK; A/swine/Wisconsin/1/67 (Hsw1 N1), Sw; A/NWS/33 (HO)-FPV/Dutch/27 (Neq1), $NWS_{(H)}$-$FPV_{(N)}$); A/FPV/Dutch/27 (Hav1)-Singapore/1/57 (N2), $FPV_{(H)}$-$Sing/57_{(N)}$; A/turkey/Wisconsin/66 (Hav6)-Swine/Wisconsin/67 (N1), $T/Wis_{(H)}$-$Sw_{(N)}$; A/swine/Wisconsin/1/67 (Hsw1)-equine/Prague/1/56 (Neq1), $Sw_{(H)}Eq1_{(N)}$.

[b] There are eight avian H subtypes, three of which are shown.

ing points can be made: (1) Different subtypes of influenza A do not coexist in the human population, whereas in the equine or avian populations more than one subtype can be present at the same time. (2) Similar, if not identical, neuraminidase antigens are shared between some of the influenza viruses of man, lower mammals, and birds.

B. Antigenic Drift

Antigenic drift involves gradual changes in the surface antigens of influenza virus and is thought to result from the selection by an immune population of mutant virus particles with altered antigenic determinants. These mutants, therefore, possess a growth advantage in the presence of antibody (Francis and Maassab, 1965). Antigenic drift occurs in the interpandemic periods. A recent example of antigenic drift of the Hong Kong strain (H3N2) which first appeared in 1968 is the 1972 London variant (A/England/42/72) that caused epidemics of moderate severity.

In the laboratory it has been possible to obtain influenza viruses showing antigenic drift by passing virus in the presence of antibodies (Archetti and Horsfall, 1950; Hamre *et al.*, 1958). An example of laboratory-induced antigenic drift is shown in Table II, where after seven passages in homologous antiserum the variant virus was approximately 30-fold less reactive with parental antibody than with antibody to the variant. Similarly, after 19 passages in parental antiserum the variant virus was approximately 36-fold less reactive with homologous antibody and 13-fold less reactive with the variant of the seventh passage. Peptide maps of the isolated hemagglutinin subunits of these variants (Laver and

TABLE II

Laboratory-Induced Antigenic Drift[a]

Virus	Number of passages in presence of antiserum	HI titer of antisera to viruses passed		
		None	7×	19×
H0N1	None	25,000	4,700	370
	7	1,600	47,000	800
	19	560	1,500	20,000

[a] The A/Bel/42 (H0N1) influenza virus was serially passed with low avidity antiserum in embryonated chicken eggs. Antisera used in the above tests were prepared in rabbits to wild-type virus and to virus obtained from the seventh and nineteenth passages. (Modified from Laver and Webster, 1968.)

Webster, 1968) showed differences in one or two peptides suggesting minor changes in the amino acid sequence of the hemagglutinin subunits. The significance of this finding will be dealt with later.

IV. Possible Mechanisms for Evolution of Pandemic Influenza A Viruses

The HA and neuraminidase components of the influenza virus are the antigens mainly involved in providing immunity to infection. At the same time they are the antigens involved in antigenic variation. The mechanism(s) of antigenic variation leading to the evolution of a new pandemic strain of human influenza virus is not known, but several hypotheses can be proposed. Table III lists three possible mechanisms which we will discuss and evidence for or against each one will be given.

A. Mutation of Existing Human Influenza Virus Strains

The most recent influenza pandemic was caused by a virus that arose in July 1968, apparently in southeastern China. This virus [A/Hong Kong/68 (H3N2)] possessed neuraminidase subunits that were similar to those of the Asian viruses (H2N2). On the other hand, the hemagglutinin subunits of the Hong Kong virus were found to be completely different immunologically from those of the Asian strains. Immunodiffusion tests with antisera prepared to the isolated hemagglutinin subunits of Hong Kong and Asian influenza viruses and tested against each virus showed lines of precipitation that were specific only for the homologous virus (Fig. 2).

Laver and Webster (1972) have tried to answer the question: "Where did the hemagglutinin subunit (H3) of Hong Kong influenza A virus come from?" A single mutation in an Asian (H2N2) influenza virus might have caused the polypeptide chains of the hemagglutinin subunits to refold in such a way as to expose completely new antigenic deter-

TABLE III

Possible Mechanisms for Evolution of Human Influenza A Viruses

1. Mutation of existing human influenza virus strains
2. Direct transmission from other animal sources (e.g., pigs, horses, birds)
3. Recombination between human and other mammalian or avian species

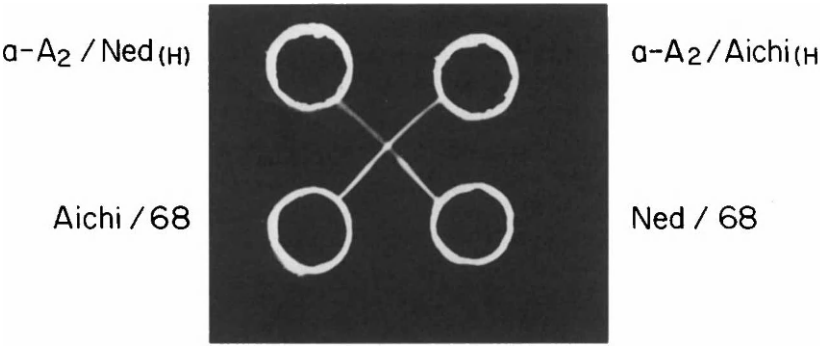

FIG. 2. Immunodiffusion tests with antisera to the isolated hemagglutinin subunits of Asian (H2N2) and Hong Kong (H3N2) influenza viruses. (From Webster and Laver, 1972). a-A_2/$Ned_{(H)}$, hyperimmune rabbit antiserum to isolated hemagglutinin subunits from A/Netherlands/84/68 (H2N2); a-A_2/$Aichi_{(H)}$, hyperimmune rabbit antiserum to isolated hemagglutinin subunits from A/Aichi/2/68 (H3N2); Ned/68, A/Netherlands/84/68 (H2N2) influenza virus treated with 1% SDS; Aichi/68, A/Aichi/2/68 (H3N2) influenza virus treated with 1% SDS.

minants. They thought that if the hemagglutinin subunits of Hong Kong influenza virus were derived by mutation in this fashion from the earlier Asian viruses, then the amino acid sequence of the polypeptides of the H2 and H3 subunits would be similar. On the other hand, if the H3 hemagglutinin subunit of the Hong Kong virus came from an influenza virus of a lower mammal or from an avian source, then the amino acid sequences of their polypeptide chains probably would be vastly different from those of the Asian viruses. To test this, hemagglutinin subunits were isolated from three strains of Asian influenza virus obtained from different parts of the world in 1968 before the occurrence of the Hong Kong influenza pandemic and from three strains of Hong Kong influenza virus isolated in different parts of the world in 1968, 1970, and 1971. The hemagglutinin subunits were isolated from recombinant (antigenic hybrid) viruses by electrophoresis on cellulose acetate strips after disruption of the virus particles with cold SDS. The subunits isolated in this way were pure insofar as they contained none of the other structural proteins of the virus (Laver, 1971; Webster and Laver, 1972). They did, however, contain a host-specific antigen, which is carbohydrate in nature and covalently attached to the protein of the subunits (Laver and Webster, 1966).

The hemagglutinin subunits were dissociated by treatment with guanidine hydrochloride and dithiothreitol, and the light and heavy polypep-

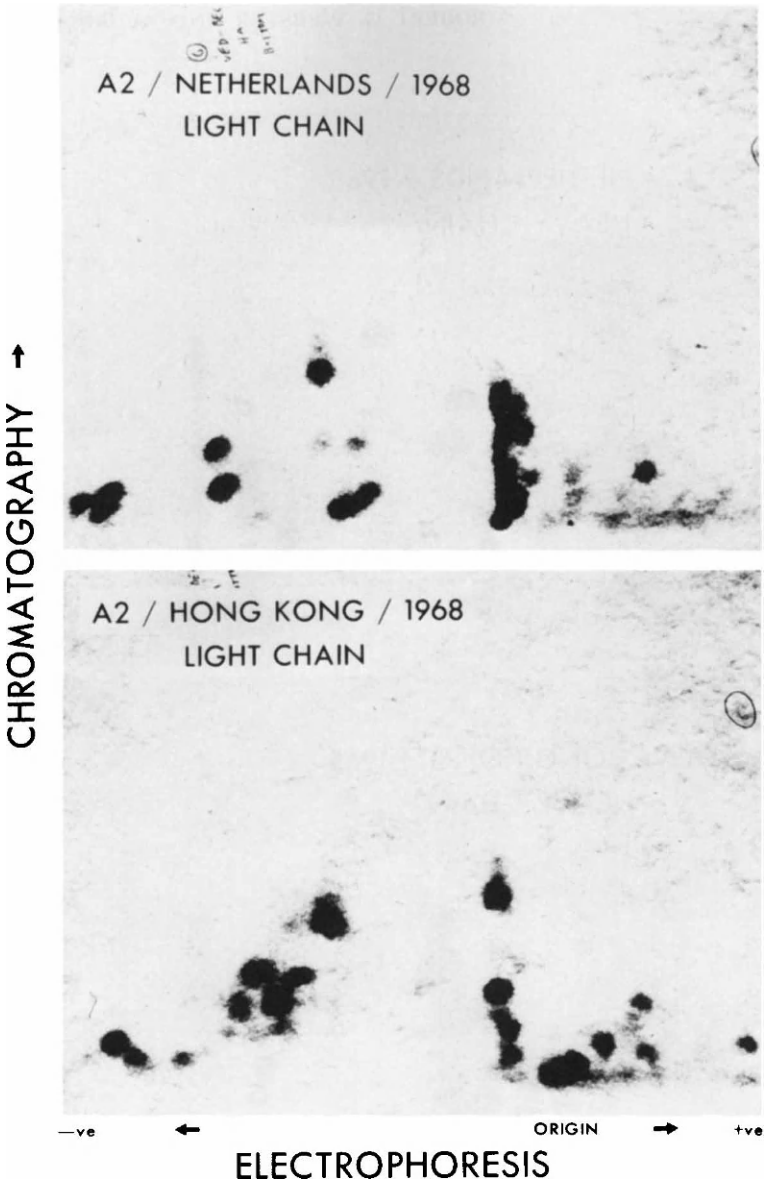

FIG. 3. Maps of the tryptic peptides (soluble at pH 6.5) from the light polypeptide chains (HA2) of the hemagglutinin subunits of A/Netherlands/68 (H2N2) influenza virus isolated before the occurrence of the Hong Kong virus influenza pandemic and of the light chains of A/Hong Kong/68 (H3N2) influenza virus. Electrophoresis at pH 6.5 was followed by ascending chromatography in pyridine–isoamyl alcohol–water (35:35:30). The circle drawn in the top right hand corner of each map shows the position of the phenol red marker added to the tryptic peptides before mapping. (Peptide maps by Dr. Graeme Laver.)

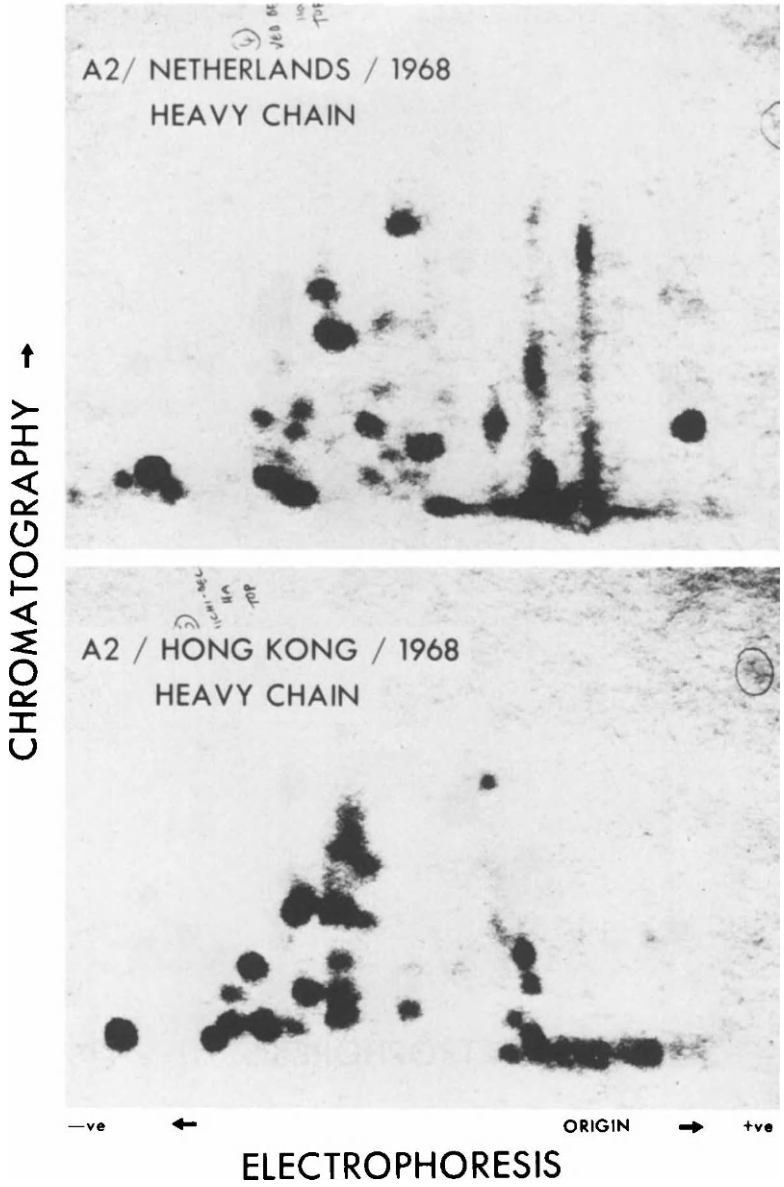

FIG. 4. Maps of the tryptic peptides from the heavy polypeptide chains (HA1) of the hemagglutinin subunits of A/Netherlands/68 (H2N2) and A/Hong Kong/68 (H3N2) influenza viruses. (Peptide maps by Dr. Graeme Laver.)

tide chains were separated by centrifugation (Laver, 1971). Each of the isolated polypeptide chains was then digested with trypsin, and the tryptic peptides were mapped (Laver, 1969).

The maps showed that the polypeptide chains from the hemagglutinin subunits of the influenza viruses isolated in 1968 prior to the appearance of the Hong Kong strain differed greatly in amino acid sequence from the polypeptide chains of the Hong Kong strains. Striking differences occurred in both light and heavy chains (Figs. 3 and 4). On the other hand, the maps of the light chains from three Hong Kong strains were similar, and the maps of the heavy chains were almost identical, the exception being one or two peptides only (Laver and Webster, 1972). The maps from three Asian 1968 strains differed in a number of peptides (both in the light and heavy chains), but none bore any resemblance to the maps of the Hong Kong strains, corroborating the immunodiffusion results.

These results indicate that the hemagglutinin subunit of Hong Kong influenza virus was not derived by mutation from a preexisting human Asian strain.

B. Direct Transmission from Other Animal Sources

It could be argued that the pandemic Asian strain of influenza virus (H2N2) of 1957 originated by direct transmission to man from another animal species. The Asian influenza virus showed major changes in both the hemagglutinin and neuraminidase antigens from the preceding H1N1 influenza viruses. Direct transmission from another animal source seems less likely to offer an explanation for the origin of the Hong Kong influenza virus (H3N2); in this case, only the hemagglutinin antigen was different from the preceding viruses, while the neuraminidase was the same as on the preceding human strains. A more convincing case can be made for the origin of Hong Kong influenza virus invoking recombination between a human Asian strain possessing N2 neuraminidase and an influenza virus from horses or ducks having an H3 type hemagglutinin (see Section V).

Serological evidence indicates that the agent responsible for the 1918 pandemic of influenza in man was caused by swine virus, or at least by a virus that was antigenically very similar to swine influenza viruses (Davenport *et al.*, 1953), but it is likely that the virus was transmitted from man to pigs rather than in the other direction (Laidlaw, 1935; Shope, 1936). There is also serological evidence suggesting that the 1899–1900 epidemic of influenza in man was caused by a virus related to equine-2 influenza virus (Minuse *et al.*, 1965; Masurel and Mulder, 1966).

Equine-2 influenza virus (Heq2 Neq2) will cause infection in humans and A/Hong Kong/68 (H3N2) virus will cause infection in Chincoteague ponies (Kasel *et al.*, 1965; Kasel and Couch, 1969). Serological evidence from lower mammals and birds (Kaplan and Payne, 1959; Dasen and Laver, 1970) suggest that influenza A viruses of man either infect these hosts or share antigenic determinants with influenza A viruses that do infect these hosts.

The absence of strict host barriers for mammalian influenza viruses is evident with Hong Kong influenza virus. This virus has been isolated from pigs (Kundin, 1970) [A/swine/Taiwan/1/70 (H3N2)], and antibodies to the virus have been detected in pigs from many countries in the world (Kundin and Easterday, 1972). Thus, it would appear that a human influenza virus was again (*vis-à-vis*, 1918) transmitted from man to pigs, but the reverse cannot be ruled out. Hong Kong influenza virus has also been reported to infect baboons (Kalter *et al.*, 1969) and gibbons (Johnsen *et al.*, 1971) and can be naturally transmitted from man to cats (Paniker and Nair, 1970). The isolation of Hong Kong influenza virus from chickens (Zhezmer *et al.*, 1973) is the first demonstration of natural transmission of a human influenza virus to an avian species. In the case of Hong Kong influenza virus it is evident that this virus can be transmitted directly from man to other hosts, but there is no convincing evidence for transmission of influenza viruses from lower mammals and birds to man to explain the emergence of a pandemic strain.

C. Recombination between Human and Other Mammalian or Avian Species

Recombination between animal viruses was first shown to occur with influenza viruses by Burnet and co-workers (Burnet and Lind, 1949, 1951). Up to 50% recombination has been shown for some influenza virus markers, while no recombination can be demonstrated for other markers (Simpson and Hirst, 1961; Simpson, 1964; Tumova and Pereira, 1965). This unusual genetic behavior of influenza virus led Burnet (1956) and Hirst (1962) to suggest that this virus contains several pieces of RNA, and this is now well established (see above). The unusually high frequency of recombination with certain markers and the low or virtual absence of recombination with others can be explained in terms of a segmented genome (Hirst, 1962; Simpson and Hirst, 1968). Thus, genetic interactions of influenza virus may be of two types: (1) random assortment of entire segments of RNA giving high frequency

recombination and (2) intragenic recombination between homologous segments of RNA giving low frequencies of recombination.

Antigenic hybrids (recombinants) of many mammalian and avian influenza A viruses have been isolated after mixed infection of chick embryos or tissue cultures with different influenza A viruses (Tumova and Pereira, 1965; Kilbourne and Schulman, 1965; Kilbourne *et al.*, 1967; Kilbourne, 1968; Easterday *et al.*, 1969). These studies have been reviewed by Kilbourne *et al.* (1967) and by Webster and Laver (1971). It is now evident that recombinant influenza A viruses with the desired surface antigens (Webster, 1970), growth potential (Kilbourne *et al.*, 1972), or other biological characteristics (McCahon and Schild, 1971) can be made "to order." Thus, "new" influenza viruses can be produced in the laboratory.

In 1970 Kilbourne pointed out that recombination between different strains of influenza A viruses had not ". . . yet been demonstrated in the intact animal—even under experimental conditions." Over the past several years experiments in our laboratory have been designed to answer the question of whether or not recombination could occur *in vivo* especially under conditions of natural transmission. The following is an account of the results of these experiments.

1. Recombination in Vivo following Inoculation of a Mixture of Two Different Influenza Viruses

a. Recombinants Isolated from Nonimmune Pigs and Turkeys. Initially two systems were used (Webster *et al.*, 1971). In the first, only one of the parental viruses multiplied in the host animal, and, in the second, both parental viruses multiplied (Table IV, Expts. 1 and 2). The general scheme was to inoculate animals with large doses of a mixture of parental viruses, and on the third day, when at least one of the viruses had multiplied, the animals were killed and homogenates of lung were tested directly in the allantois-on-shell system for the presence of recombinant viruses (Webster, 1970). This system selects for antigenic hybrids since parental viruses are inhibited by specific antisera.

In the first experiment (Table IV) pigs were inoculated with a mixture of swine influenza virus (Sw) (A/swine/ Wisconsin/1/67 [Hsw1N1]) and fowl plague virus (FPV) (A/fowl plague virus/Dutch/27 [Hav1Neq1]); the latter virus does not yield infectious virus after inoculation of pigs. Lung suspensions collected 3 days later contained parental viruses, and the recombinant viruses possessing swine influenza virus hemagglutinin and FPV neuraminidase $Sw_{(H)}$-$FPV_{(N)}$ (A/Hsw1-Neq1) and $FPV_{(H)}$-$Sw_{(N)}$ (A/Hav1-N1).

TABLE IV

In Vivo Production of "New" Influenza Viruses[a]

Expt. No.	Host	Parental viruses inoculated	Method of inoculation or infection	Viruses isolated from lung suspensions
1	Pig (nonimmune)	Artificial mixture of swine (Sw) + fowl plague (F) (Sw_H-Sw_N + F_H-F_N) Sw multiples. F does not	Intranasally	Parental plus recombinants Sw_H-F_N; F_H-Sw_N
2	Turkey (nonimmune)	Artificial mixture of turkey/Wis (T) + fowl plague (T_H-T_N + F_H-F_N), both viruses multiply	Intratracheally	Parental plus recombinants T_H-F_N: F_H-T_N
3	Chicken (immunized against T_H-F_N)	Lung suspension from turkey of Expt. 2 containing: turkey/Wis. fowl plague and recombinants F_H-T_N and T_H-F_N	Intratracheally	Recombinant F_H-T_N

4	Turkey (nonimmune)	Turkey/Ont. turkey/Wis (Each virus has antigenically distinct H and N)	Two birds each infected intratracheally with T/Ont or T/Wis introduced into flock of 16 sentinel birds	From sentinel birds: parental plus recombinant T/Ont_H-T/Wis_N
5	Turkey (nonimmune)	Turkey/Wis, fowl plague	(a) Two birds each infected intratracheally with a parental virus introduced into flock of 26 sentinel birds	From sentinel birds: parental virus + recombinant F_H-T_N
			(b) At 10th day, 12 nonimmune chickens and 12 chickens immunized against F_N were introduced[a]	Parental fowl plague virus only
6	Pig (nonimmune)	Hong Kong swine	Two pigs each infected intranasally with HK or Sw were introduced into a room with 4 sentinel pigs	Parental + recombinants Sw_H-HK_N; HK_H-Sw_N

[a] See text and Table I for details.

In the second experiment (Table IV), where both viruses replicated, turkeys were infected with FPV and turkey influenza virus (T) (A/turkey/Massachusetts/3740/65 [Hav6N2]), and antigenic hybrids possessing $T_{(H)}$-$FPV_{(N)}$ (A/Hav6-Neq1) and $FPV_{(H)}$-$T_{(N)}$ (A/Hav1-N2) were isolated.

There are two possible objections to the interpretation that the recombination described above occurred *in vivo*. The first is that the recombination might have occurred in the tissue culture system used for selection of viruses, and the second is a question of whether the antigenic hybrids were genetically stable and not merely phenotypically mixed particles.

The first objection can be ruled out because the selection of antigenic hybrid viruses was done directly in very high concentrations of antibodies that would have neutralized the parental viruses. To provide more rigorous evidence that the antigenic hybrid viruses did not arise in the isolation procedures outside the infected hosts, it was necessary to plate the mixed yield from the lung suspension for plaque isolation and to characterize the virus stocks grown from plaque isolates. Twenty-five percent of the plaques isolated from the lung suspension of turkeys mixedly infected with FPV plus turkey influenza virus were antigenic hybrids containing FPV hemagglutinin and turkey influenza virus neuraminidase. Inoculation of host animals with cloned hybrid viruses and their reisolation from inoculated animals provided evidence for their genetic stability.

b. Recombinants Isolated from Immune Chickens. The preceding experiments provided formal evidence that recombination of influenza A viruses could occur *in vivo*, but they did not answer the question of whether selection could also occur *in vivo*, analogous to what might occur in nature during emergence of a "new" influenza virus. To answer this question the lung suspensions from turkeys mixedly infected 3 days earlier with FPV and turkey influenza virus (Table IV, Expt. 2) were inoculated into groups of chickens immune to the hemagglutinin of one parental virus and also to the neuraminidase of the other parental virus (Webster and Campbell, 1972) (Table IV, Expt. 3). Thus, groups of seven chickens were vaccinated so that they were immune to parental FPV (group A), $FPV_{(H)}$ plus $T_{(N)}$ (group B), or $T_{(H)}$ plus $FPV_{(N)}$ (group C). Groups of chickens were not immunized with turkey influenza virus, since preliminary studies showed that this virus does not replicate in chickens or induce a detectable antibody response after intratracheal administration of virus.

Chickens vaccinated against FPV (group A) and against the antigenic

hybrid possessing $FPV_{(H)}$-$A_2/57_{(N)}$ (A/Hav1-N2) (group B) remained normal during the course of the experiment, and viruses were not recovered from tracheal swabs or from lung suspensions. Chickens immunized against $T_{(H)}$ and $FPV_{(N)}$ either died of a fulminating infection or remained apparently normal. Lung samples collected from the birds yielded pure cultures of a recombinant influenza virus possessing $FPV_{(H)}$-$T_{(N)}$ (A/Hav1-N2), which killed embryonated chicken eggs. Lung suspensions from the birds in group C that remained apparently normal (two out of seven) yielded pure cultures of parental FPV. The infectivity titers of the lung suspensions from the apparently normal birds were at least 100-fold lower than in the birds infected with recombinant viruses, suggesting that the immune status of the birds resulted in a modified or inapparent infection.

These studies demonstrate that recombinants of different influenza A viruses can be selected *in vivo*, where the immune status acts as a selection pressure and thus mimics what might occur in nature during the emergence of a "new" influenza virus.

2. *Recombination in Vivo under Simulated Conditions of Natural Transmission*

The above studies (Section IV,C,1,a) showed that two different strains of influenza A viruses could recombine *in vivo* when they were simultaneously introduced into the same animal artificially. The simultaneous inoculation of animals with large doses of two different influenza A viruses, however, constitutes an artificial system that probably would not occur in nature. To obviate this problem and to demonstrate recombination under conditions of transmission more closely resembling natural infection, the following experiments were undertaken.

a. Recombination between Two Turkey Influenza Strains in Turkeys. Two antigenically distinct turkey influenza viruses were simultaneously allowed to spread in a flock of susceptible birds in the following way (Table IV, Expt. 4). Two young turkeys were infected by the intratracheal route with T/Ont influenza virus (A/turkey/Ontario/7732/66 [Hav5 N?]),* and others were similarly infected with T/Wis influenza viruses (A/turkey/ Wisconsin/66 [Hav6N2]). The infected birds were put into a flock of sentinel turkeys. Tracheal and lung samples were collected daily and tested for the presence of parental and recombinant (antigenic hybrid) viruses by the immune sera-allantois-on-shell system,

* The neuraminidase of this strain has not been identified but is different from T/Wis/66.

by limit dilution in the allantois-on-shell, and by plaque isolation directly from plated samples.

The results of these studies (Webster *et al.*, 1973) demonstrated three points: (1) One influenza virus can replicate in the upper respiratory tract of turkeys, while an antigenically distinct influenza A virus can replicate in the lower respiratory tract. (2) Recombination can occur between influenza viruses under conditions simulating natural transmission. In these experiments the recovery of recombinant viruses was low and of a single variety ($T/Ont_{(H)}$-$T/Wis_{(N)}$. All isolates had the virulence of the parental T/Ont influenza virus. (3) Under conditions of mixed infection there was a sparing effect: The highly virulent T/Ont influenza virus did not cause death of the contact birds during the course of the experiment.

b. Recombination between a Turkey and a Chicken Influenza Virus in Turkeys. If a "new" strain of influenza virus were to arise in nature by recombination as described above and become an epidemic strain, it would be necessary for the "new" virus to have some selective advantage. This selective advantage could lie in possession of antigens to which the population at large was not immune, but in addition the virus would have to possess the capacity to transmit to susceptible hosts. Both of these factors were investigated in an experiment on sequential transmission of two influenza viruses in a flock of turkeys (Webster *et al.*, 1973) (Table IV, Expt. 5).

Two turkeys infected with T/Wis influenza virus were introduced into a flock of 26 contact birds, and 2 days later two turkeys infected with FPV were also put into the flock (Table IV, Expt. 5a). By the fourth day after initial exposure to the infected birds, 35% of the contacts had T/Wis influenza virus in their tracheas. Similarly, by the fourth day after exposure to FPV (sixth day after initial exposure to T/Wis), the first doubly infected bird was detected. The incidence of dually infected birds increased with time, and, by the tenth day, 71% of the contact birds were doubly infected with influenza viruses; one-half of these birds had antigenic hybrids possessing $FPV_{(H)}$-$T/Wis_{(N)}$ in their tracheas.

On the tenth day after initial exposure to T/Wis influenza virus, when 50% of the contact turkeys had recombinant influenza viruses ($FPV_{(H)}$-$T/Wis_{(N)}$) present in their tracheas, additional birds were introduced into the flock (Table IV, Expt. 5b). There were two groups of birds: (1) 12 fully susceptible turkeys and (2) 12 chickens vaccinated with a recombinant possessing $NWS_{(H)}$-$FPV_{(N)}$, so that they had high levels of antibodies to $FPV_{(N)}$ and antibody to an irrelevant hemagglutinin. Tracheal samples were collected from birds beginning on the second day after introduction to the flock. Parental FPV was isolated from the tracheal

swabs and from lung samples, but no recombinant viruses were isolated from any of the samples from either group of birds. This experiment demonstrates that even though recombinant influenza viruses can arise with a high frequency under natural conditions of transmission *in vivo*, these viruses do not necessarily have the properties that would permit them to readily spread and initiate a pandemic.

c. Recombination between Hong Kong and Swine Influenza Viruses in Pigs. To determine if recombination can occur *in vivo* between influ-

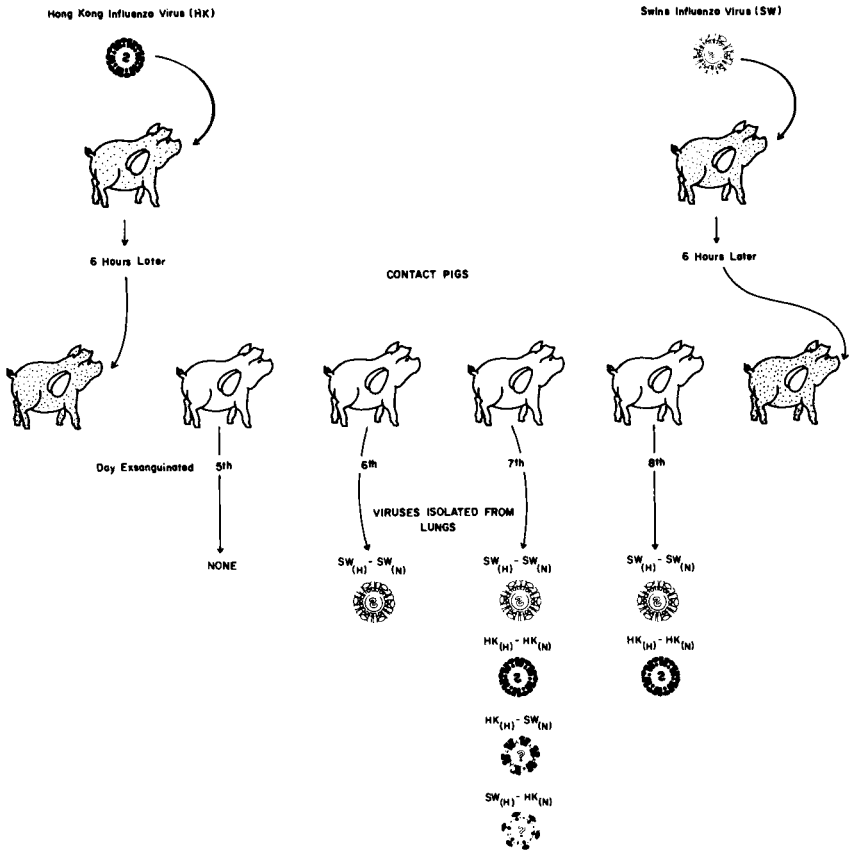

FIG. 5. Recombination between Hong Kong and Sw influenza viruses under conditions of natural transmission. One pig was infected with HK influenza virus, and a second pig was infected with Sw influenza virus. Six hours later the infected animals were put into a room with four contact pigs. Beginning on the fifth day after introduction of the infected animals, one pig was exsanguinated each day and lung suspensions were examined for recombinant viruses (from Webster *et al.*, 1973).

enza viruses from man and from lower mammals, the following experiment was done. Two antigenically distinct influenza A viruses that will replicate in pigs were used; these were A/swine/Wisconsin/67 (HswlN1) (Sw) and a strain of Hong Kong influenza virus isolated from pigs – A/swine/Taiwan/70 (H3N2) (HK) that is antigenically identical to strains of human origin. One pig was infected with Sw influenza virus and another with HK influenza virus; 6 hours later both animals were introduced into a herd of four susceptible pigs (Fig. 5 and Table IV, Expt. 6) (Webster *et al.*, 1973).

Lung suspensions from the contact pigs were passaged directly in the allantois-on-shell system in the presence of specific antibodies to the hemagglutinin of one parental virus and to the neuraminidase of the other. Figure 5 and Table IV (Expt. 6) shows that both of the parental viruses were detected in the lungs of the animal killed on the seventh day after exposure to the pigs infected with parental viruses. Recombinant viruses possessing $HK_{(H)}$-$Sw_{(N)}$ and others possessing $Sw_{(H)}$-$HK_{(N)}$ were also isolated on the seventh day after exposure.

The recombinant viruses were isolated at limit dilution in the allantois-on-shell system and then reinoculated into pigs. Both types of recombinants caused mild respiratory infections in pigs and were genetically stable; the same viruses were reisolated from the pigs. The antigenic hybrid possessing $HK_{(H)}$-$Sw_{(N)}$ was naturally transmitted from infected to control pigs.

These studies demonstrate that recombination between influenza viruses from man and animals can occur *in vivo* under conditions simulating natural transmission.

V. Evidence for the Progenitors of the Hong Kong Strain of Human Influenza Virus

The above studies provide evidence of a mechanism for the formation of "new" influenza viruses, such as the Hong Kong strain, but give no idea of the parents. The following experiments provide information on the possible lineage of Hong Kong influenza virus.

Previous hemagglutination inhibition tests showed that two of the known mammalian and avian influenza viruses, A/equine/Miami/1/63 (Heq2 Neq2) and A/duck/Ukraine/1/63 (Hav7 Neq2), were related immunologically to Hong Kong influenza virus (Coleman *et al.*, 1968; Tumova and Easterday, 1969). However, the cross-reactions found were very small and could have been mediated through the neuraminidases via steric hinderance. The hemagglutinin subunits from equine,

TABLE V

Cross-Reactions in Hemagglutination-Inhibition Tests between the Hemagglutinin Subunits of Hong Kong, Equine 2, and Duck/Ukraine Influenza Viruses[a]

Hyperimmune antisera to isolated HA subunits	HI titers with the following HA or virus antigens				
	Hong Kong (H3)	Equine 2 (Heq2)	Duck/Ukraine (Hav7)	A2/Asian (H2N2)	Duck/Penn. (Hav6N?)
Hong Kong	38,000	3,600	3,600	<20	<20
Equine 2	3,400	13,800	2,600	<20	<20
Duck/Ukraine	11,800	1,500	20,400	<20	<20

[a] The hemagglutinin subunits (HA) were isolated by electrophoresis of SDS-disrupted virions. Hyperimmune antisera were prepared in rabbits, and hemagglutination-inhibition (HI) titers represent the reciprocals of the serum dilution causing 50% inhibition of four hemagglutinating doses of antigen. A2/Asian and Duck/Penn influenza viruses were used as controls. (Modified from Laver and Webster, 1973.)

duck, and Hong Kong influenza viruses were therefore isolated and tested for immunological and biochemical relatedness (Laver and Webster, 1973).

Hyperimmune antisera raised against the isolated hemagglutinin subunits of duck/Ukraine, equine 2, and Hong Kong influenza viruses were tested in hemagglutination-inhibition and immunodiffusion tests with each of the viruses. The results of these tests showed strong serological cross-reactions between the isolated hemagglutinin subunits of the three viruses. In hemagglutination-inhibition tests using the isolated subunits (Table V), a 10-fold difference was found between Hong Kong and the other two viruses with antiserum to Hong Kong. The cross-reactions were asymmetrical. Antiserum to duck/Ukraine showed a 2-fold difference between Hong Kong and duck/Ukraine hemagglutinin subunits, while the reciprocal reaction showed a 10-fold difference.

Maps of the tryptic peptides from the light chains of the hemagglutinin subunits of the equine 2, Hong Kong, and duck strains of influenza virus were almost identical. In contrast, maps of the heavy polypeptide chains of the hemagglutinin subunits of each virus showed a number of changes indicating that the heavy chains of the hemagglutinin subunits from the three viruses differed considerably in amino acid sequence. The similarities of the HA light chains of each virus suggest a common progenitor of the HA. The differences in the heavy chains can best be explained by antigenic drift from the progenitor. This idea is in keeping with the heavy chain containing the antigenic determinant (Brand and Skehel, 1972).

VI. Conclusions

We have attempted in this chapter to present evidence to account for the evolution of pandemic influenza viruses. In the laboratory, recombination among influenza viruses from man, lower mammals, and birds can occur *in vivo* after natural transmission from individually infected hosts and give rise to "new" influenza viruses. There is no reason to believe the same does not occur in nature. The experimental data (Laver and Webster, 1972) strongly indicate that the hemagglutinin subunit of Hong Kong influenza virus could not have arisen by mutation from a preexisting human influenza virus. It is more likely that genetic recombination between an animal or avian influenza virus can explain its appearance, a view which is strengthened by the transmission experiments we have described. The evidence for animal and avian influenza viruses having hemagglutinin subunits closely related antigenically to those of Hong Kong influenza (Laver and Webster, 1973) further supports this hypothesis as do data from the peptide maps of the light and heavy polypeptide chains of human, equine, and avian strains (Section V). The light chains of these strains were virtually identical, in contrast to peptide maps of the light chains from human H2N2 and H0N1 influenza viruses, which were quite different (Laver and Webster, 1973).

It is not known whether the light polypeptide (HA2) of the hemagglutinin subunits constitutes any part of the antigenic area. These findings do not imply that the immunological relationships between equine, duck, and Hong Kong viruses are due to similarities in light chains. They are, however, very difficult to explain if Hong Kong influenza virus arose by mutation from a preexisting human influenza virus. The only acceptable alternative seems to be that it arose by genetic recombination from an animal or avian influenza virus having hemagglutinin subunits related to those of equine 2 and duck/Ukraine viruses.

The segmented nature of the influenza virus genome probably accounts for the high frequency of recombination between different influenza A viruses and for the above findings of efficient recombination *in vivo* between different influenza viruses. Biochemical analysis of gene products of each of the segmented genomic RNA's of influenza viruses isolated from man, lower mammals, and birds should help to elucidate the origin of new strains.

In toto the evidence presented strongly favors a recombinational event in the evolution of new pandemic strains of influenza virus.

There is little information available on the number of different influenza viruses in lower mammals and avian species or on the extent of genetic variation in these viruses. The eventual control of the last great

plague of man may well depend on ecological information on this virus in lower species and information on the available antigens contributing to major antigenic shift.

Since the topic of this treatise is viruses, evolution, and cancer, perhaps it is worth noting that RNA tumor viruses may also have a segmented genome (Vogt, 1971). As with influenza virus, high frequency recombination with members of this virus group has been demonstrated. Although human RNA tumor viruses (C type) have yet to be uncovered, indirect evidence suggests their presence in certain human tumors (e.g., Spiegelman *et al.*, 1973). The possibility that recombination between a putative human RNA tumor virus and an avian or animal RNA tumor virus might occur, based on the considerations for evolution of orthomyxoviruses presented in this chapter, should not be considered lightly. Experimental design and the resulting data obtained with influenza viruses may well be applied one day to the study of the evolution of human cancer viruses.

ACKNOWLEDGMENTS

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CHAPTER 24

Reovirus: Early Events (in the Infected Cell) and Structure of the Double-Stranded RNA Genome

STEWART MILLWARD AND ANGUS F. GRAHAM

I. Introduction

The diplornaviruses are a group of viruses whose genetic material is composed of double-stranded RNA (dsRNA). Membership in this group has expanded rapidly in recent years to the point where there are now a large number of representatives that have been isolated from a broad spectrum of mammals, insects, plants, and molds (for a comparative

review of the dsRNA viruses, see Wood, 1973). The first dsRNA virus from bacteria has been reported recently with the isolation and characterization of the $\phi 6$ bacteriophage from *Pseudomonas phaseolicola* (Semancik *et al.*, 1972; Vidaver *et al.*, 1973). The $\phi 6$ bacteriophage is unique among this group of viruses in that its single-capsid core is encapsidated within a hostlike membrane and therefore can be classified as a separate group among the diplornaviruses. The genome of $\phi 6$ bacteriophage, like that of all the diplornaviruses characterized to date, exists in the form of discreet segments of double helical RNA.

A second group of dsRNA viruses is the single-capsid mycoviruses, whose morphology resembles that of the core or inner capsid of the dsRNA viruses isolated from higher plants and animals. However, the genome segments of the mycoviruses may be packaged in separate particles (Wood and Bozarth, 1972).

The third and most thoroughly studied group of dsRNA viruses are those with a double-capsid morphology. The dsRNA segments of these viruses are packaged together in an inner capsid structure. Representatives among this group include the reoviruses (serotypes 1, 2, and 3) which are extremely widespread among the vertebrates; wound tumor virus and cytoplasmic polyhedrosis viruses, which are widespread among plants and insects, respectively. Rice dwarf virus, blue tongue virus of sheep, African horse-sickness virus, and Colorado tick fever virus are other arthropod-borne viruses that share this double-capsid morphology and dsRNA genome.

The host range specificity usually associated with morphologically similar viruses is not in evidence among the double-capsid group of diplornaviruses. Since insects not only transmit most of these viruses but also act as primary and intermediate hosts, the possibility exists that these viruses have evolved to their plant and animal hosts from a central pool existing among the arthropods. A central role of the insects as a common host through which different evolutionary lines might arise has been suggested by Wood (1973).

The ubiquitous nature of the dsRNA viruses makes them a particularly useful model for studying the evolution of virus–host relationships. A case can be made for the hypothesis that once a virus has entered a cell, it must meet certain basic requirements of that particular host. For example, the "early" messenger RNA's (mRNA) synthesized in cells infected with reovirus must be compatible with the translational machinery of the host cell to give rise to virus-specific proteins. These "early" viral proteins might modify the host or in some other way aid establishment of the viral infection. However, once established, the "late" events rapidly predominate, leading to replication of the viral genome and, in the

case of reovirus, to lysis of the host cell. The "late" events might be as variable as the diversity of nature permits.

Reovirus is the most thoroughly studied member of the double-capsid group of dsRNA viruses. Its genome is composed of ten segments of dsRNA (Shatkin *et al.*, 1968; Watanabe *et al.*, 1968) packaged together within the inner capsid or core. When reovirus enters the host cell, the virions are sequestered within lysozomes and ultimately migrate to a region peripheral to the nucleus (Silverstein and Dales, 1968). The outermost capsomeres are removed, presumably by lysozomal hydrolases, thus activating the latent, virion-associated RNA polymerase (transcriptase) (Borsa and Graham, 1968; Shatkin and Sipe, 1968; Silverstein *et al.*, 1972). The dsRNA and protein of these subviral particles are conserved throughout the course of infection (Silverstein *et al.*, 1970).

II. Regulation of Transcription

A. Size of the Viral Transcript

It has been known for some time from both *in vivo* and *in vitro* studies that the virion transcriptase transcribes the viral genome as though there were discontinuities along the length of the dsRNA. When virus-induced mRNA is isolated from infected cells and hybridized with denatured genome RNA, the hybrids migrate at the same rates as the native dsRNA segments. This is illustrated in Fig. 1, where mRNA isolated from infected cells late in the replicative cycle was hybridized with genome dsRNA and analyzed by polyacrylamide gel electrophoresis (Millward and Nonoyama, 1970). The ten mRNA hybrids (open circles) and the ten genome hybrids (closed circles) coelectrophorese. From this observation one can measure the extent of the genome transcribed during infection. Since the distance each segment migrates in the gel is proportional to the logarithm of its molecular weight (Shatkin *et al.*, 1968; Millward and Nonoyama, 1970), we can calculate that 0.35 mm (one gel fraction) corresponds to 15,000 daltons or approximately 46 nucleotides per segment. This means that on the average at least 99% of the total genome is transcribed in infected cells.

B. Frequency of Transcription

Using the same technique we can also measure the relative frequency of transcription of each segment throughout the course of infection.

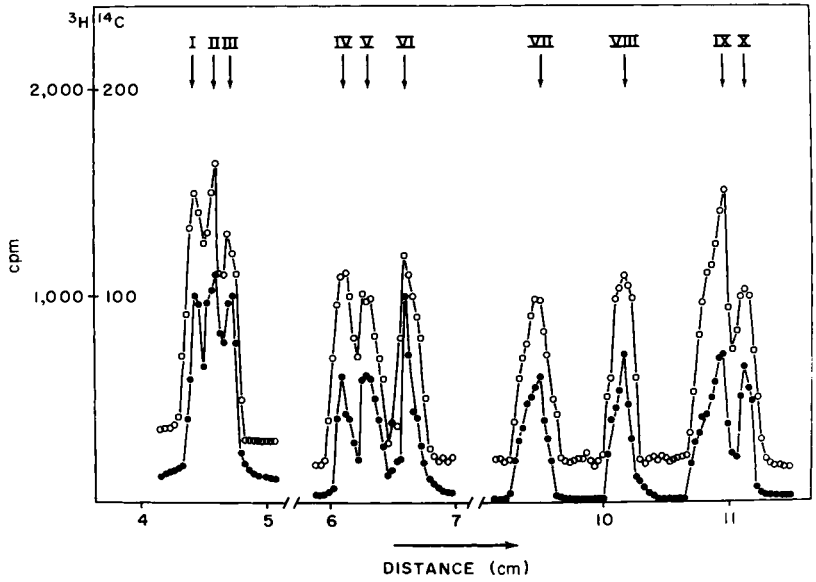

FIG. 1. Electrophoretic analysis on polyacrylamide gel of the hybrids formed between viral dsRNA and "late" mRNA. Closed circles, ^{14}C-labeled dsRNA; open circles, ^3H-labeled mRNA hybridized with dsRNA. (From Graham and Millward, 1971.)

Thus, it was shown that some control is exerted over transcription of the various genomic segments in infected cells (Watanabe *et al.*, 1968). To show this, suspension cultures of L cells were infected with reovirus in the presence of actinomycin D to suppress the synthesis of cellular RNA (Kudo and Graham, 1965; Shatkin, 1965). Infected cells were exposed to [^3H]uridine at early, intermediate, and late times after infection and the virus-specific mRNA was isolated. This ^3H-labeled mRNA was hybridized with an excess of ^{14}C-labeled dsRNA obtained from purified virus and the hybrids analyzed by electrophoresis in polyacrylamide gels (Millward and Nonoyama, 1970). Under the conditions used, the efficiency of hybridization decreases with increasing length of the dsRNA segments and also from one experiment to another. However, since the dsRNA is in large excess, the ^{14}C label provides an internal standard by means of which the hybridization results can be quantitated. Thus the ratios of $^3H/^{14}C$ for each peak in a given gel are a measure of the relative frequency of transcription of the various segments of the genome.

When infected cells were labeled with [^3H]uridine between 10 and 11 hours postinfection and the mRNA isolated hybridized with [^{14}C]dsRNA and analyzed, the pattern shown in Fig. 1 was obtained

TABLE I

Ratios of $^3H/^{14}C$ in Hybrids Formed between Viral dsRNA and Virus-Induced mRNA Labeled under Various Conditions

Component[a]	$^3H/^{14}C$ for mRNA labeled between[b]		
	0–8 hours with cycloheximide	4–5 hours	10–11 hours
I	2.1	4.2	11
II	–	3.0	10
III	–	2.1	10
IV	–	4.2	14
V	–	3.7	11
VI	*10*	*10*	*10*
VII	–	6.9	11
VIII	–	8.4	12
IX	2.2	13	14
X	2.2	15	12

[a] Refers to the components separated by gel electrophoresis in Figs 1, 2, and 3.
[b] Figures within each column normalized to a value of 10 for segment VI.

(Millward and Nonoyama, 1970). The $^3H/^{14}C$ ratio calculated for each peak is shown in the last column of Table I. There is no marked difference in the ratio from one peak to another, and similar results were obtained whether the labeling period was 30 minutes or 2 hours. It is concluded that late in the infectious cycle all ten segments of the genome are transcribed at approximately the same frequency.

Synthesis of progeny dsRNA in this system commences about 4 hours postinfection and is proceeding at its maximum rate by about 7 hours postinfection. If one adds an inhibitor of protein synthesis, such as puromycin or cycloheximide, the synthesis of dsRNA is blocked while synthesis of ssRNA continued (Kudo and Graham, 1965; Watanabe *et al.*, 1967). Thus, cycloheximide added at the time of infection blocks the synthesis of protein and of progeny dsRNA, but does not block uncoating of the virus in the cell (Silverstein *et al.*, 1970) nor does it completely block transcription of the viral genome. As shown in Fig. 2, four of the ten segments, namely, segments number I, VI, IX, and X, are transcribed when cycloheximide was present from the beginning of infection. These segments are by definition associated with "early" viral functions. It is still far from clear why segment VI was transcribed at a frequency 5-fold greater than the other three "early" segments (Table I). To get around the possibility that these results were due to some unknown side effect of cycloheximide, the transcription pattern at 4 to 5

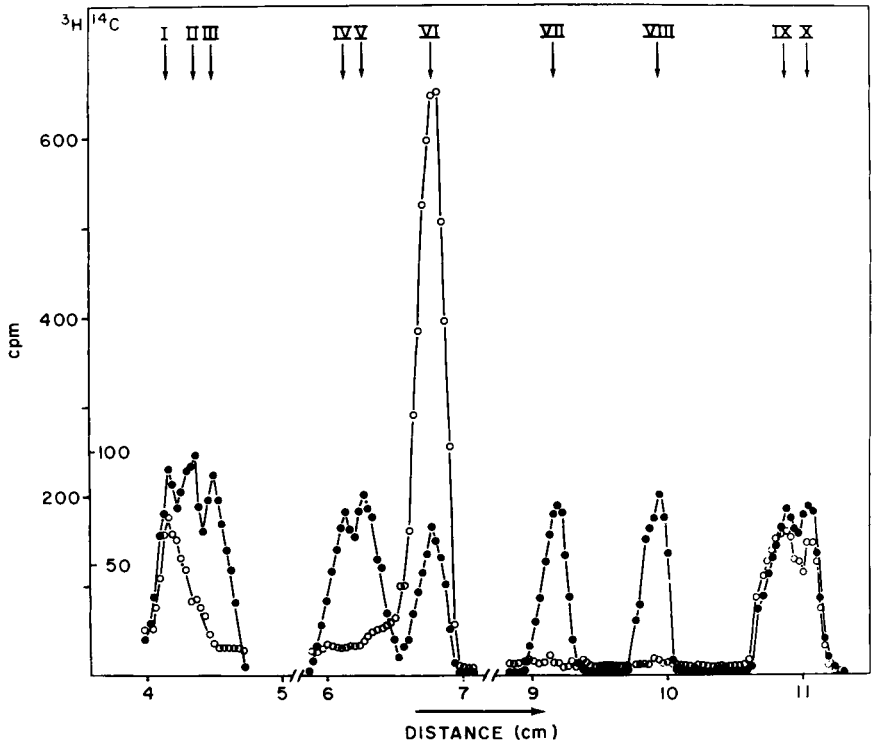

FIG. 2. Electrophoretic analysis on polyacrylamide gel of hybrids formed between viral dsRNA and "early" mRNA. Cycloheximide was added at the time of infection to inhibit protein and progeny dsRNA synthesis. Closed circles, ^{14}C-labeled dsRNA; open circles, ^3H-labeled mRNA hybridized with dsRNA. (From Graham and Millward, 1971.)

hours postinfection was examined without using cycloheximide. The pattern obtained is shown in Fig. 3 and the ratios of $^3H/^{14}C$ are summarized in Table I. Between 4 and 5 hours postinfection, all the later segments are being transcribed but not yet at their maximum frequencies. We conclude that Fig. 3 represents a transition between "early" and "late" transcription and strengthens the argument that "early" viral functions are associated with genome segments I, VI, IX, and X.

What controls the switch over from "early" to "late" transcription *in vivo* is still not understood. No one has yet been able to obtain this "early" transcription pattern *in vitro*. Virions, which have been uncoated *in vivo* in the presence of cycloheximide and reisolated, transcribe all ten segments *in vitro* (Silverstein *et al.*, 1970). In addition, seven of a possible ten recombination groups of conditional lethal (temperature sensi-

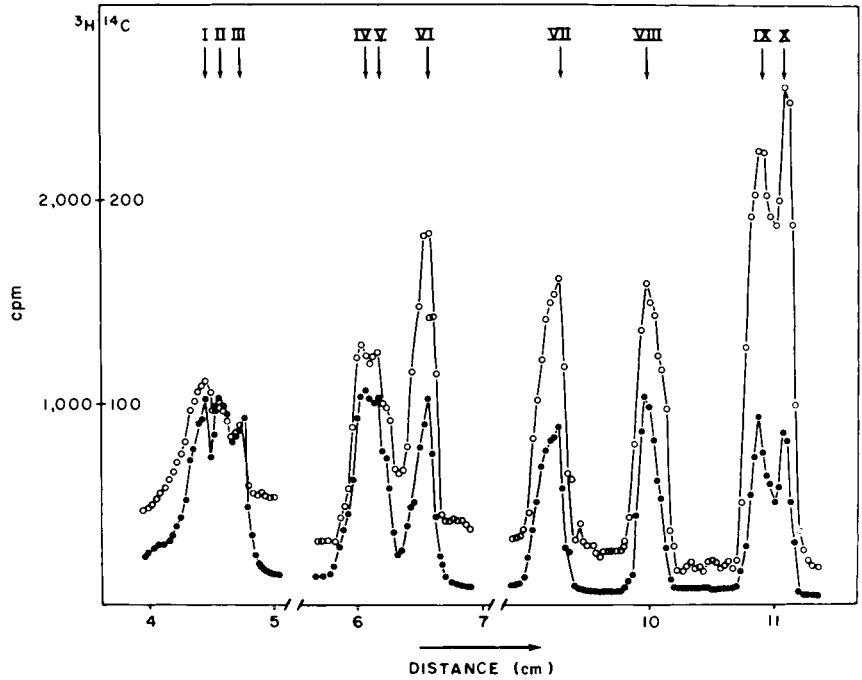

FIG. 3. Electrophoretic analysis on polyacrylamide gel of hybrids formed between viral dsRNA and mRNA synthesized 4 to 5 hours postinfection without cycloheximide. Closed circles, ^{14}C-labeled dsRNA; open circles, ^{3}H-labeled mRNA hybridized with dsRNA. (M. Nonoyama and A. F. Graham, unpublished data.)

tive) mutants of reovirus transcribe all ten segments at the nonpermissive temperature in spite of the fact that three of these groups are dsRNA^{-} (Ito and Joklik, 1972; Cross and Fields, 1972). The simplest explanation at the present time is that the host cell contains a repressor that prevents transcription of the "late" segments. This repressor might be inactivated by one of the early viral functions, thus derepressing transcription of the six "late" segments.

In summary, all ten segments of the reovirus genome are transcribed at approximately equal frequencies at late times during the course of infection. At intermediate times, certain of the segments are transcribed at a greater frequency than others. When cycloheximide is used at the time of infection to block protein and progeny dsRNA synthesis only 4 of the 10 segments are transcribed, and these correspond to the presumptive "early" functions that are responsible for establishing the viral infection. It is on these "early" viral functions that we now wish to focus our attention.

III. "Early" Proteins Synthesized in Infected Cells

A. Detection of the "Early" Polypeptides

Since cycloheximide has the effect of locking the system into the "early" transcription pattern, we can use this observation to advantage by allowing cells infected with reovirus, in the presence of cycloheximide, to accumulate "early" mRNA. Following the period of accumulation, cycloheximide can be removed and its inhibitory effect reversed (Watanabe *et al.*, 1967). The experimental design was as follows: Cells were treated with actinomycin D for 6 hours prior to infection. This pretreatment was necessary to depress host protein synthesis, since reovirus does not inhibit host protein synthesis until later in the infectious cycle (Kudo and Graham, 1965; Zweerink and Joklik, 1970). Cells were then infected with reovirus at relatively high multiplicities and incubated in the presence of cycloheximide for 18 hours at 31°C. The drug was removed and the cells resuspended in fresh medium and incubated at 31°C. Proteins synthesized after reversal of the cycloheximide block were labeled *in vivo* with [^{3}H]leucine, then cytoplasmic extracts of these cells were analyzed by electrophoresis in polyacrylamide gels. Virus-specific proteins present in the cytoplasm of cells infected in the absence of cycloheximide were labeled with [^{14}C]leucine and analyzed in parallel gels to determine the position of the viral specific proteins, which are easily identified at late times in infection (Zweerink *et al.*, 1971). Nine viral protein bands are normally seen under these conditions and are represented by the arrows along the top and bottom of Fig. 4. The λ, μ, and σ proteins correspond to the large, medium, and small size class genome segments, respectively. The pattern in the top panel of Fig. 4 illustrates the polypeptides obtained when cells infected with reovirus and incubated in the presence of cycloheximide were labeled with [^{3}H]leucine for 50 minutes after removal of the drug. Since the host background is still rather high, particularly in the σ region, a cell culture was similarly treated except no virus was added. This culture was labeled with [^{14}C]leucine and analyzed in a parallel gel as shown in the middle panel of Fig. 4. In order to emphasize the viral specific proteins over the host background, the ^{3}H/^{14}C ratios throughout the gel were determined. As shown in the bottom panel of Fig. 4 there are four major polypeptides discernible by this technique. The largest polypeptide co-electrophoreses with λ_2, a structural component of the inner capsid. We know from data presented earlier that the only large segment transcribed under these conditions is L1 and therefore λ_2 must be the gene product

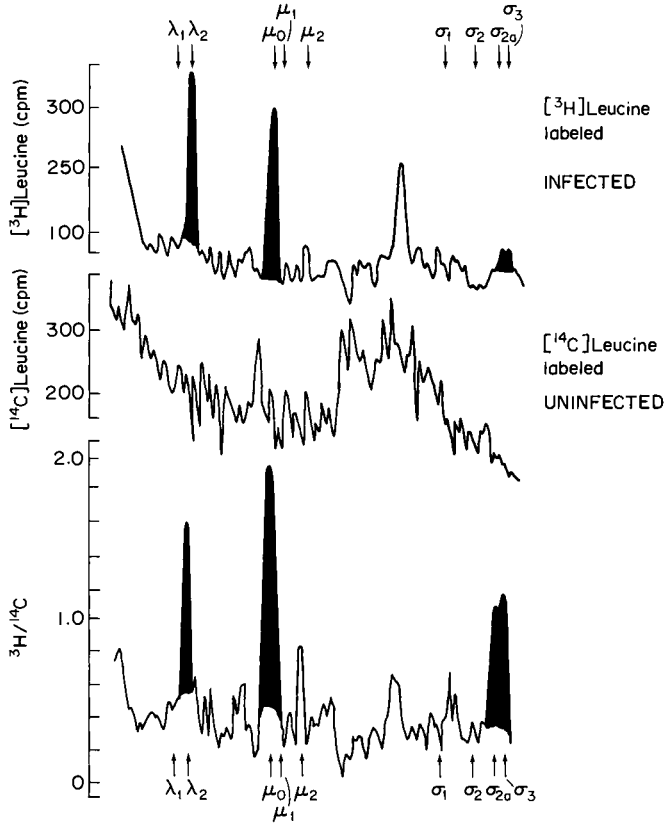

FIG. 4. Electrophoretic analysis of polypeptides in SDS-polyacrylamide gels. Top panel: ^{3}H-labeled polypeptides synthesized during the first 50 minutes after removal of cycloheximide. Middle panel: [^{14}C]leucine labeled polypeptides from cells treated as for the top panel except no virus was added. Bottom panel: $^{3}H/^{14}C$ ratios calculated from the top and middle panels to emphasize virus-specific polypeptides over the host background. The arrows indicate the position of the nine viral polypeptides normally found late in infection. Direction of migration is from left to right. (D. Van Alstyne and A. F. Graham, unpublished data.)

of the largest dsRNA segment. The second polypeptide band migrates in the μ region and coelectrophoreses with the nonstructural polypeptide μ_0. Thus it would appear that the smallest of the three medium size dsRNA segments gives rise to the largest of the medium-size polypeptide class. This apparent anomaly could be due to glycosylation of the primary gene product, or it could also happen if the other μ polypeptides were cleaved during some type of processing. The remaining two

"early" polypeptides migrate in the σ region and coelectrophorese with σ_{2a} a nonstructural protein, and with σ_3, a component of the outer capsid layer. These correspond to segments IX and X, to which we have previously assigned "early" functions.

Although the correlation between the four "early" segments and the four polypeptides detected by this technique is a good one, it does not constitute proof that these polypeptides were translated from mRNA's synthesized during the period of incubation with cycloheximide.

B. Effect of Cordycepin on "Early" Polypeptide Synthesis

A more direct test was obtained by adding 3′-deoxyadenosine (cordycepin) to the infected cell culture to prevent new RNA synthesis after removal of the cycloheximide block. Cordycepin has been shown to be an effective inhibitor of reovirus-specific mRNA and dsRNA under conditions in which protein synthesis is only slightly affected (R. J. Berckmans and A. F. Graham, unpublished data). The bottom panel of Fig. 5 shows the polypeptides synthesized in the presence of cordycepin. These results are qualitatively the same as those obtained over the same labeling period (50 minutes) in the absence of cordycepin (Fig. 4, bottom panel) and suggests that viral mRNA's made in the presence of cycloheximide are functional. The polypeptides synthesized in the presence and absence of cordycepin between 4 and 5 hours after removal of cycloheximide are shown in the middle and upper panels respectively of Fig. 5. It is clear from the middle panel that the polypeptides synthesized in the presence of cordycepin between 4 to 5 hours are essentially unchanged from those made during the first 50 minutes. On the other hand, the pattern obtained between 4 to 5 hours in the absence of cordycepin is beginning to show a transition, particularly in the μ region, to the pattern normally found late in infection. Thus it would appear that in the presence of cordycepin, the cells are "locked in" to the "early" pattern. The transition from "early" to "late" pattern is more evident in infected cells 8 to 10 hours after release from cycloheximide. As shown in Fig. 6, all the polypeptides normally found late in infection are present by this time. This transition from "early" to "late" polypeptide pattern is reminiscent of the transition that occurs between "early" and "late" mRNA synthesis (Graham and Millward, 1971; Millward and Nonoyama, 1970).

In summary, four virus specific polypeptides are made from the four "early" mRNA's synthesized in the presence of cycloheximide. Two of the "early" polypeptides (μ_0 and σ_{2a}) are nonstructural components, and two (λ_2 and σ_3) are structural components. One of these "early" poly-

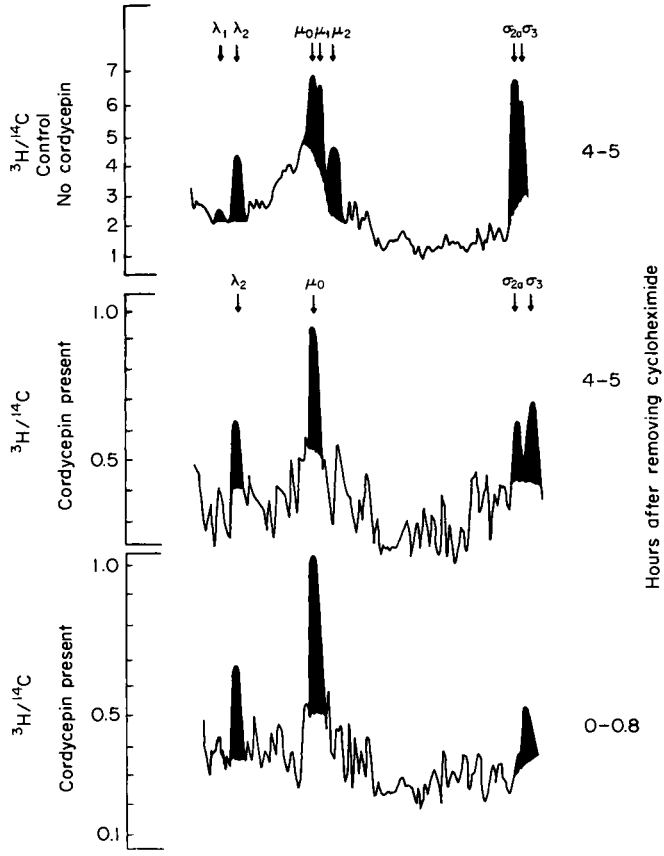

FIG. 5. Electrophoretic analysis of polypeptides synthesized in the presence and absence of cordycepin. Top panel: polypeptides synthesized between 4 and 5 hours after removal of cycloheximide and without cordycepin present. Middle panel: analogous to top panel except cordycepin was added to block new RNA synthesis after removal of cycloheximide. Bottom panel: viral polypeptides synthesized during first 50 minutes after removal of cycloheximide and in the presence of cordycepin. Direction of migration is from left to right. (D. Van Alstyne and A. F. Graham, unpublished data.)

peptides may be involved in derepressing the "late" genomic segments. Although their role in establishing the viral infection is presently unknown, the fact that they can be synthesized in the absence of the "late" polypeptides provides us with the experimental means for isolation and characterization of these "early" functions. These data permit the assignment of λ_2 to dsRNA segment I, μ_0 to segment VI, and σ_{2a} and σ_3 to segments IX and X, respectively. In addition, these data also show that mRNA made in the presence of cycloheximide is functional.

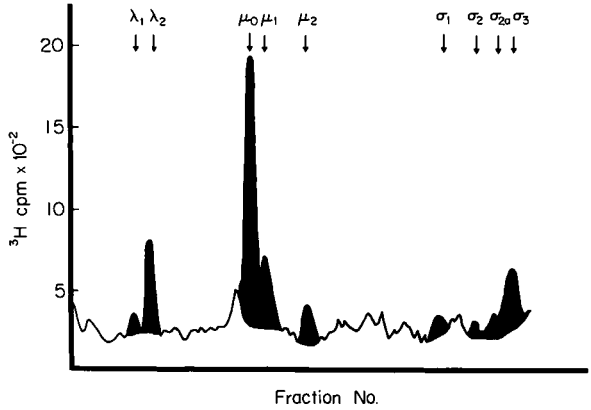

FIG. 6. Electrophoretic analysis of the virus-specific polypeptides synthesized between 8 and 10 hours after removal of cycloheximide. The arrows indicate the position in the gel of the nine viral polypeptides normally found late in infection. Direction of migration is from left to right. (D. Van Alstyne and A. F. Graham, unpublished data.)

IV. Structure of the Viral Genome

A. General Considerations

A question that naturally arises from these studies is what controls the switch over from the "early" to "late" transcription pattern? Putting the question in another way, are there structural differences between the "early" and "late" genome segments which might account for the selective binding of a host repressor? We have approached this problem by investigating the conformation of the nucleotides at the ends of the duplex segments. Before describing these experiments it is important to realize that the replication of this virus is asynchronous (Schonberg *et al.*, 1971). That is, the first step in the replication of reovirus is equivalent to a transcriptional event that gives rise to single-stranded RNA (ssRNA) copies of the ten segments. These ssRNA's have the same polarity as mRNA from polyribosomes and will therefore be referred to as plus strands. Isotope labeling experiments indicate that these plus strands accumulate relatively early during infection and then serve as a template pool for the synthesis of minus strands during progeny virus formation somewhat later in the infectious cycle (Schonberg *et al.*, 1971; Acs *et al.*, 1971; Sakuma and Watanabe, 1971). Because of this asynchrony, the precursor plus strands and ultimately the dsRNA segments themselves must be maintained in a specific alignment. Most

likely this occurs at the time of synthesis, and if we assume a viral protein is involved in linking the strands, then a probable candidate would be the large "early" protein λ_2, which we know to be a structural component of the inner capsid.

Whatever is involved in linking the segments *in situ* must depend on the conformation of the nucleotides at the ends of the segments. A "perfect duplex" model has been suggested for the genomic segments of reovirus (Banerjee and Grece, 1971). In this model both strands of the duplex segments are hydrogen bonded throughout their length as shown here for one end of a segment:

$$5'\text{ppGpUpUpPup} \cdots \qquad (\text{Pu} = \text{G or A})$$

$$3' \cdots \text{OdAdAdPy} \qquad (\text{Py} = \text{C or U})$$

This model was based on knowledge that (1) all 5′ termini contain the sequence ppGpPyp (Py = pyrimidine) (Banerjee and Shatkin, 1971) and (2) the 3′ termini contain the common sequence —ApApC (Banerjee and Grece, 1971). However, this model was not consistent with our earlier results, which suggested that the 5′ ends of the segments consisted of single-stranded tails composed of purines (Millward and Nonoyama, 1970).

B. Release of Nucleotides from dsRNA with T_1 Ribonuclease

In those experiments we had treated purified dsRNA with T_1 ribonuclease under conditions where this nuclease is highly specific for guanylic acid residues in a non-hydrogen-bonded configuration. Upon removal of the nuclease, the dsRNA was labeled by incubating with polynucleotide phosphokinase and [γ-^{32}P]ATP (Richardson, 1965). This enzyme transfers the γ-phosphate of ATP to the newly exposed 5′-OH group in the dsRNA (Millward and Nonoyama, 1970). Untreated dsRNA or dsRNA treated with pancreatic ribonuclease cannot be labeled with ^{32}P under these conditions. If the reovirus segments were perfect duplexes, we could explain our results only if T_1 ribonuclease was releasing the 5′ terminal ppGp's, simply because they were situated at the ends of the duplex segments. This might happen if there was a localized destabilization of the hydrogen bonds between the terminal G—C residues. To test this possibility we examined the release of nucleotides from virion dsRNA. To do this, uniformly labeled [^{32}P]dsRNA was incubated with T_1 ribonuclease in high salt and then the mixture was layered on a sucrose density gradient and centrifuged. Figure 7 (open circles) illustrates one such separation in which only the

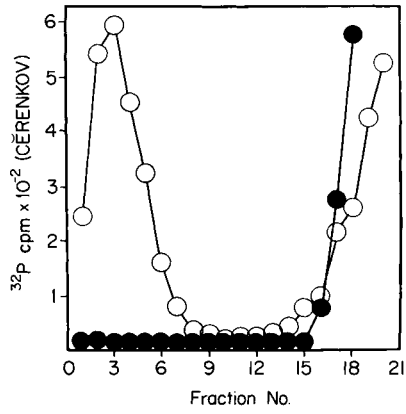

FIG. 7. Separation of nucleotides from dsRNA after release by T_1 ribonuclease. Uniformly labeled [^{32}P]dsRNA was layered over each of two sucrose density gradients. Just prior to centrifugation both preparations were incubated at 37°C for 30 minutes and then were made 1% with SDS. One (open circles) was incubated with T_1 ribonuclease, while the control (closed circles) was treated identically but without enzyme. Fractions were removed successively from the top and assayed for radioactivity. Only the upper two-thirds of each gradient is shown in this figure to emphasize the cpm released by this enzyme. Sedimentation is from left to right. (J. E. Shaw and S. Millward, unpublished data.)

upper two-thirds of the sucrose gradient is shown to emphasize the separation between the dsRNA (fractions 15 and greater) and material released as a result of T_1 ribonuclease digestion (fractions 1 to 8). An equivalent amount of ^{32}P-labeled dsRNA treated in an identical manner, except no T_1 ribonuclease was present, failed to release any detectable radioactivity to the upper regions of the gradient (Fig. 7, closed circles). This control sample also reflects the purity of the dsRNA.

Kinetic analysis of the release of these nucleotides showed an initial fast reaction at 37°C indicative of a single-stranded region in the duplex segments (Fig. 8, open circles). Lowering the temperature to 0°C (Fig. 8, hatched circle) or adding $MgCl_2$ to 10 m*M* (data not shown) did not alter this release. Both these conditions are known to stabilize helix formation and limit hydrolysis by T_1 ribonuclease. These results show that T_1 ribonuclease was releasing a specific number of labeled nucleotides from the dsRNA, which means that there must be a region in the genome segments which is not in a hydrogen-bonded duplex structure.

C. Analysis of the Nucleotides Released

When the nucleotides released by T_1 ribonuclease were fractionated according to chain length, four major peaks of radioactivity were found

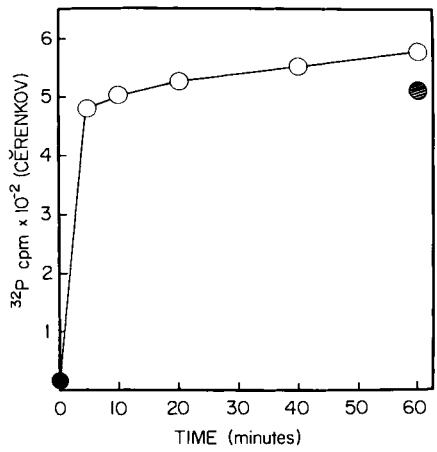

FIG. 8. Kinetics of release of nucleotides from viral dsRNA. An incubation mixture containing uniformly labeled [^{32}P]dsRNA was digested with T_1 ribonuclease. At the times indicated aliquots were removed (open circles), made 1% with SDS, and chilled to 0°C. A control mixture was incubated at 37°C without enzyme (closed circle). Another sample was kept at 0°C for 60 minutes in the presence of enzyme (hatched circle) then each mixture was layered on a sucrose gradient and analyzed for radioactivity released as described in Fig. 7. (J. E. Shaw and S. Millward, unpublished data.)

(Fig. 9). These corresponded to the elution position of the absorbance markers Xp_1 to Xp_4, and their structures are summarized in Table II. Numerical analysis of the data presented in Fig. 9 is also summarized in Table II and shows that the twenty 5′ terminal ppGp's were indeed released. However, for every 20 ppGp's (equal to one genome equivalent) released there were 39 additional phosphate equivalents released during hydrolysis with T_1 ribonuclease. Structural analysis showed they existed as 4 Gp, 4 XpGp, and 9 $(Xp)_2Gp$. Radioactivity eluting in positions of $(Xp)_3Gp$ (the elution position of ppGp, Fig. 9) and greater corresponded to one or less oligonucleotide per genome and were not analyzed.

D. Site of Release

The question now arises concerning where along the length of the segments the nucleotides other than ppGp are being derived. Three types of experiments were performed to answer this question. The first was designed to determine if nucleotides had been released from internal regions of the segments; the second was designed to test for loss of nucleotides from the 3′ termini; and the third was to examine the possi-

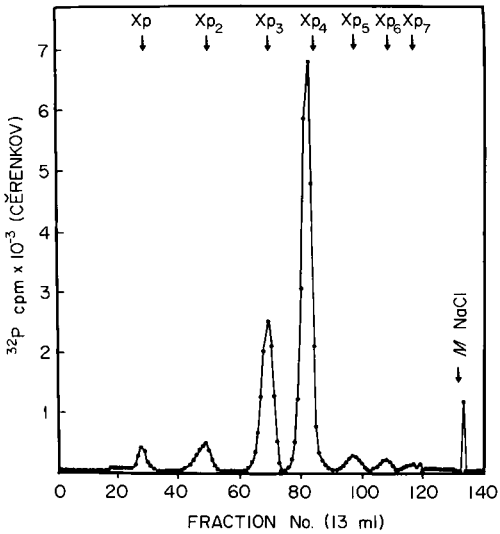

FIG. 9. Sephadex A-25 chromatography of the T_1 released material. Material released by T_1 ribonuclease such as that shown in Fig. 7 was pooled (fractions 1–8) and the SDS removed by four *n*-butanol extractions. A T_1 digest of wheat germ RNA was added as optical density marker; the sample was added to a column of Sephadex A-25 in the presence of 7 *M* urea and eluted with a salt gradient. The absorbance profile is not shown, but the elution position of the markers are indicated by the arrows. (J. E. Shaw and S. Millward, unpublished data.)

TABLE II

Structural Analysis of Nucleotides Released from dsRNA by T_1 Ribonuclease

Chain length[a]	cpm[b]	Mole fraction per genome[c]	Structure[d]
Gp	1636	4	Gp
(Xp) Gp	3079	4	2 ApGp, 1CpGp, 1 UpGp
$(Xp)_2Gp$	11313	9	1 (ApCp) Gp, 2 (CpUp) Gp, 6 ApUpGp
$(Xp)_3Gp$ + ppGp[b]	26,536	< 1	NA
$(Xp)_4Gp$	1736	< 1	NA
$(Xp)_5Gp$	1121	< 1	NA
$(Xp)_6Gp$	873	< 1	NA
ppGp[b]	24,970	20	ppGp

[a] Chain lengths and cpm calculated from data presented in Fig. 9.

[b] The tetranucleotide region of Fig. 9 was composed of 24,970 cpm ppGp and 1620 cpm $(Xp)_3Gp$.

[c] The cpm under each isostich was divided by the number of constituent phosphates to obtain cpm/P equivalent. Letting cpm/P equivalent ppGp = 20 (since ppGp was released quantitatively), the mole fraction per genome for each isostich was determined.

[d] NA, not analyzed.

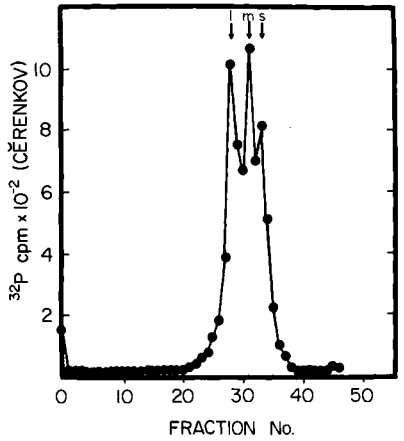

FIG. 10. Sedimentation analysis of denatured dsRNA previously treated with T_1 ribonuclease. Uniformly labeled [^{32}P]dsRNA was treated with T_1 ribonuclease, made 1% with SDS, and then separated from the released materials as described in Fig. 7. The dsRNA was precipitated from sucrose, phenol extracted, and then denatured with 90% DMSO and reprecipitated with ethanol. The [^{32}P]ssRNA was then sedimented through a linear sucrose density gradient. Just prior to centrifugation the sample was heated to 60°C for 3 minutes to disrupt aggregates. Reovirus ssRNA prepared *in vitro* was included as marker (l, m, s represent the large, medium, and small size class of ssRNA). Sedimentation is from right to left. (J. E. Shaw and S. Millward, unpublished data.)

bility that nucleotides other than ppGp were removed from the 5′ termini.

In the first experiment [^{32}P]dsRNA which had been treated with T_1 ribonuclease was recovered from a sucrose gradient, such as the one shown in Fig. 7 (fractions 15 and greater). The dsRNA was denatured, mixed with ssRNA synthesized *in vitro* from reovirus cores, and then subjected to a second sucrose gradient analysis. If nucleotides had been released from internal regions of the segments, a portion of the ^{32}P radioactivity should sediment heterogeneously. The fact that the ^{32}P radioactivity exactly cosedimented with the ssRNA marker (Fig. 10) means that T_1 ribonuclease must be releasing nucleotides from regions at or near to the ends of the dsRNA segments.

To determine if nucleotides were released from the 3′ ends by T_1 ribonuclease the 3′ termini were labeled specifically by oxidizing the dsRNA with periodate then reducing with tritiated borohydride (Millward and Graham, 1970). Analysis of the [3′-^{3}H]dsRNA showed that all twenty 3′ termini were represented. Samples of the [3′-^{3}H]dsRNA were incubated separately with pancreatic and T_1 ribonucleases then analyzed by velocity sedimentation in sucrose. The results of these analyses, which are summarized in Table III, show that neither enzyme treat-

TABLE III

Distribution of [3′-^{3}H]Label among the Large (L), Medium (M), and Small (S) Classes of Reovirus dsRNA[a]

Nuclease treatment	L	M	S
Control	1.2	1.3	1.0
Pancreatic	1.1	1.3	1.0
T_1	1.0	1.2	1.0

[a] [3′-^{3}H]dsRNA was purified, mixed with A_{260} quantities of cold dsRNA, and then divided into three samples. One was incubated with 100 U/ml T_1 ribonuclease, another with 0.3 μg/ml pancreatic ribonuclease, and the third was not treated with enzyme. After 30 minutes at 37° C, SDS was added to 1% and each sample was separated into large (L), medium (M), and small (S) size classes by velocity sedimentation on sucrose. The absorbance profile and radioactivity were coincident for each of the three samples. The TCA precipitable cpm in each class were summed and divided by the representative number of 3′ termini (L = 6, M = 6, S = 8). The cpm was normalized to S in each gradient and the ratio of each class determined as shown above.

ment released tritium from the dsRNA segments. The [3′-^{3}H]dsRNA was recovered from these gradients, denatured, and then subjected to a second velocity sedimentation in sucrose. Still the ^{3}H label sedimented into the gradient with the bulk of the RNA. Therefore, not only are the 3′ termini resistant, but treatment of dsRNA with either of these nucleases does not introduce nicks or gaps in regions near to the 3′ termini.

Since we knew that T_1 ribonuclease quantitatively releases the 5′ terminal ppGp's, we had to use a different approach to determine if nucleotides other than ppGp were also released from the 5′ ends. The experimental approach was based on a report that the base penultimate to all twenty 5′ termini is a pyrimidine (Banerjee and Shatkin, 1971). Thus, if ppGp was the only nucleotide released from the 5′ end, polynucleotide kinase should introduce ^{32}P label on the penultimate pyrimidine. The test of this prediction involved taking dsRNA that had been treated with T_1 ribonuclease and labeling the newly exposed 5′-OH with ^{32}P in the polynucleotide kinase reaction. When the product was denatured and analyzed on a sucrose gradient the ^{32}P label cosedimented with marker ssRNA, indicating that the label had been introduced at the ends of covalently intact strands. Base analysis of the product showed that essentially all the label chromatographed as the purine ^{32}pAp (Fig. 11) rather than a pyrimidine. This means that T_1 ribonuclease was releasing more than just the terminal nucleotide from the 5′ ends of the segments and supports the contention that all nucleotides released by this enzyme are derived in line to the terminal ppGp's. It should be emphasized at this point that although nucleotides were released from all twenty 5′

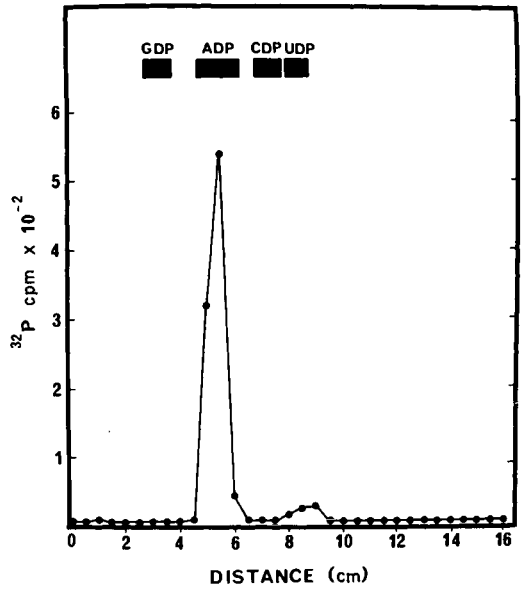

FIG. 11. Identification of the nucleotide exposed following hydrolysis of dsRNA with T_1 ribonuclease. dsRNA was hydrolyzed with T_1 ribonuclease in high salt, separated from the released material as described in Fig. 7, and then labeled with ^{32}P in the polynucleotide kinase reaction. The [5′-^{32}P]dsRNA was hydrolyzed in alkali, and an aliquot mixed with authentic 3′-5′-ribonucleoside diphosphates and chromatographed on PEI cellulose using 0.5 *M* KH_2PO_4, pH 3.4. The PEI sheet was cut into 1 cm fractions, and the radioactivity in each fraction was determined. (J. E. Shaw and S. Millward, unpublished data.)

ends, we do not know if the same number of nucleotides were lost from each strand of the segments.

Structural analysis of the nucleotides released (Table II) can be interpreted to mean that the base sequences penultimate to the 5′ terminal ppGp's are heterogeneous and are in a form, unlike the 3′ termini, which permits their removal by a nuclease highly specific for ssRNA. Structural analysis also shows that pyrimidines as well as purines are present in the oligonucleotides released by T_1 ribonuclease (Table II). Yet, in an earlier report we showed that treatment of dsRNA with pancreatic ribonuclease did not expose new 5′-OH groups as assayed in the polynucleotide kinase reaction (Millward and Nonoyama, 1970). The simplest explanation for this apparent contradiction might be that the pyrimidines were in a hydrogen-bonded form and therefore resistant to hydrolysis by pancreatic ribonuclease. This requires that the 5′ ends of the duplex segments be looped in a hairpin conformation. However, such a conformation seems unlikely because the number of nucleotides released is insufficient to form stable loops.

E. Conformational Inequality between the Ends of the Segments

It is also possible that the pyrimidines referred to above are susceptible to hydrolysis by pancreatic ribonuclease but that the dsRNA is not converted by this enzyme to an acceptable substrate for polynucleotide kinase. This possibility is supported by the following observation; when dsRNA was treated first with T_1 ribonuclease and the new 5′-OH groups labeled in the polynucleotide kinase reaction, label was incorporated only on the 5′ end of the minus strands (S. Millward and J. E. Shaw, unpublished data). This conformational inequality could be explained if one end was a perfect duplex while the other end has a single-strand tail. Such a structure would result if the replicase enzyme did not copy the plus strand template right to the end during synthesis of dsRNA. We are presently exploring this possibility in greater detail.

F. Possible Significance of the AUG's Released

Among the oligonucleotides released by T_1 ribonuclease were six ApUpGp's per genome (Table II). However, we do not know if these were released from six different segments or not. Nevertheless, it seems reasonable to suppose that since the sequence —GAUG— is accessible to the active site of T_1 ribonuclease, this sequence might also be available for the formation of an initiation complex. The reported inhibitory effect on initiation of *in vitro* protein synthesis by dsRNA from reovirus is consistent with this hypothesis (Ehrenfeld and Hunt, 1971). Extending this line of speculation even further, let us suppose that the six AUG's are released from the six "late" segments of the genome. If the ends of these segments were exposed in the uncoated subviral particle, they might interact with a host initiation factor resulting in repression of their transcription. If this was the case, a function of the early proteins would be to displace this factor, thereby permitting complete expression of the genome. While these remarks are strictly speculative some of the questions they raise can be answered experimentally.

G. Organization of the Genome Segments as Revealed by Electron Microscopy

A major problem in trying to design experiments to answer some of the questions raised here results from our almost complete lack of knowledge of how the genome is organized *in situ*. The only chemical approach reported to date was that of Millward and Graham (1970),

who showed that the genome had as many free 3′ ends *in situ* as purified dsRNA extracted from virions. This result was interpreted to mean that the genome existed in a segmented form within the intact virus. Studies using the electron microscope to examine the release of RNA from reovirus have also been reported (Granboulan and Niveleau, 1967; Vasquez and Kleinschmidt, 1968). Preparations of reovirus were spread on a subphase of 4 *M* urea at 4°C, and after various periods of time the protein film was sampled for electron microscopy. Under these conditions there was a gradual decomposition of the virus particles which ultimately (in 12 minutes) lead to a complete dispersal of the genome segments. One of the intermediate stages of decomposition was called a "spider" form (Plate II of Vasquez and Kleinschmidt, 1968), which consisted of filaments radiating outward from a central, electron-dense body. We have obtained similar structures by a completely different approach (G. Mark and S. Millward, unpublished data). The experimental design was based on a report that methylene blue can photochemically cross-link protein to free guanylic acid residues. Thus, if a protein was involved in linking one segment to another, it might be possible to stabilize such a linkage by cross-linking the single-stranded tails

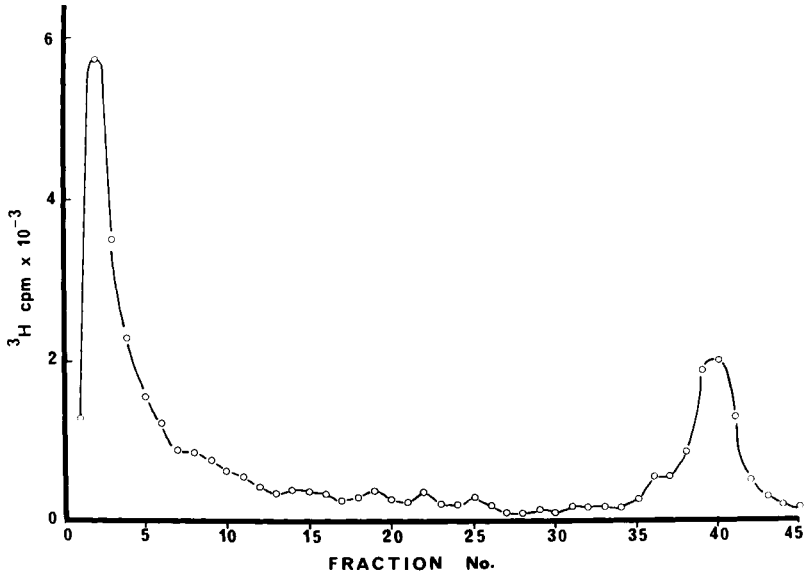

FIG. 12. Velocity sedimentation analyses of dsRNA photoreacted *in situ* with methylene blue. Prior to sedimentation the subviral particles were heated at 80°C in SDS. Sedimentation is from right to left. (G. Mark and S. Millward, unpublished data.)

containing guanylic acid with the linker protein. To test this possibility, the outer capsid layer of the virus was removed by incubation with chymotrypsin and the core structures purified by isopycnic centrifugation in CsCl. The purified core particles were photoreacted with methylene blue and then dissociated to the maximum extent by heating at 80°C in 6% sodium dodecyl sulfate (SDS). The mixture was layered on a sucrose gradient and centrifuged under conditions in which the genome RNA of fully dissociated virus remains near the top of the gradient. As shown in Fig. 12 over 60% of the dsRNA of the virions that had been photoreacted with methylene blue sedimented to the cushion at the bottom of the tube. No RNA sedimented to this position in

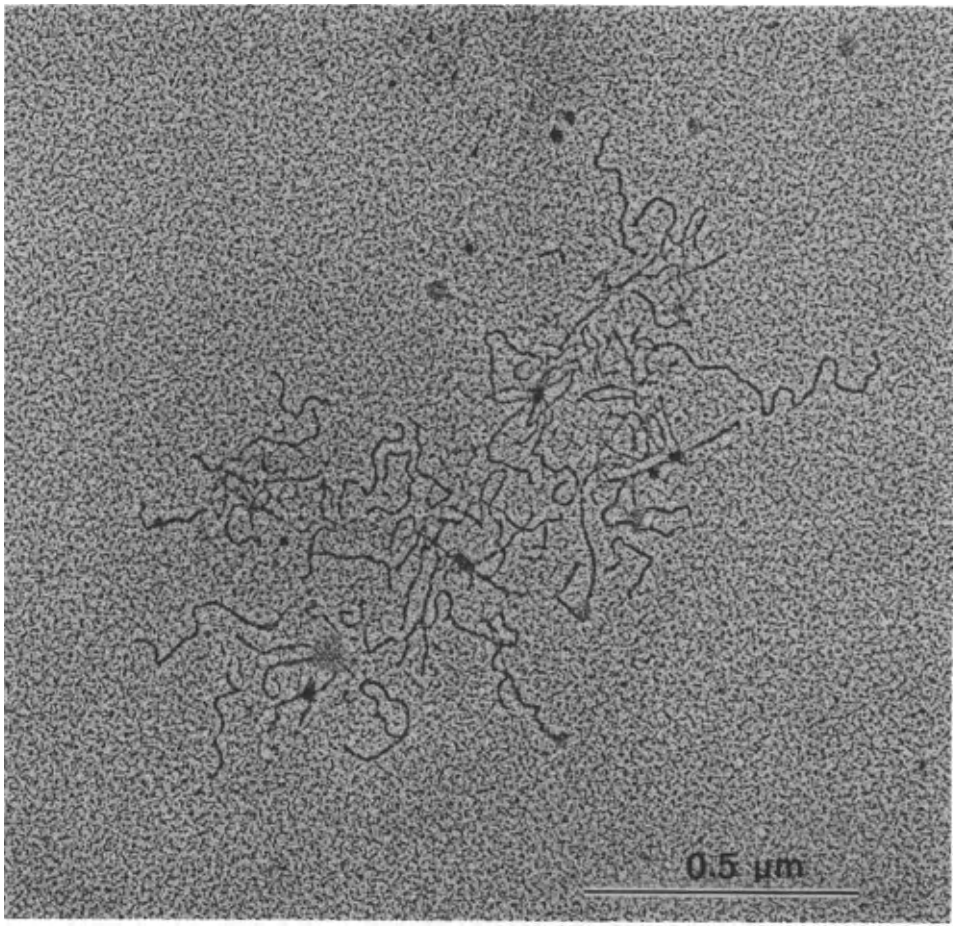

FIG. 13. Electron micrograph of the rapidly sedimenting RNA from Fig. 12. The sample was spread by the aqueous method and rotory shadowed with uranium oxide. (G. Mark and S. Millward, unpublished data.)

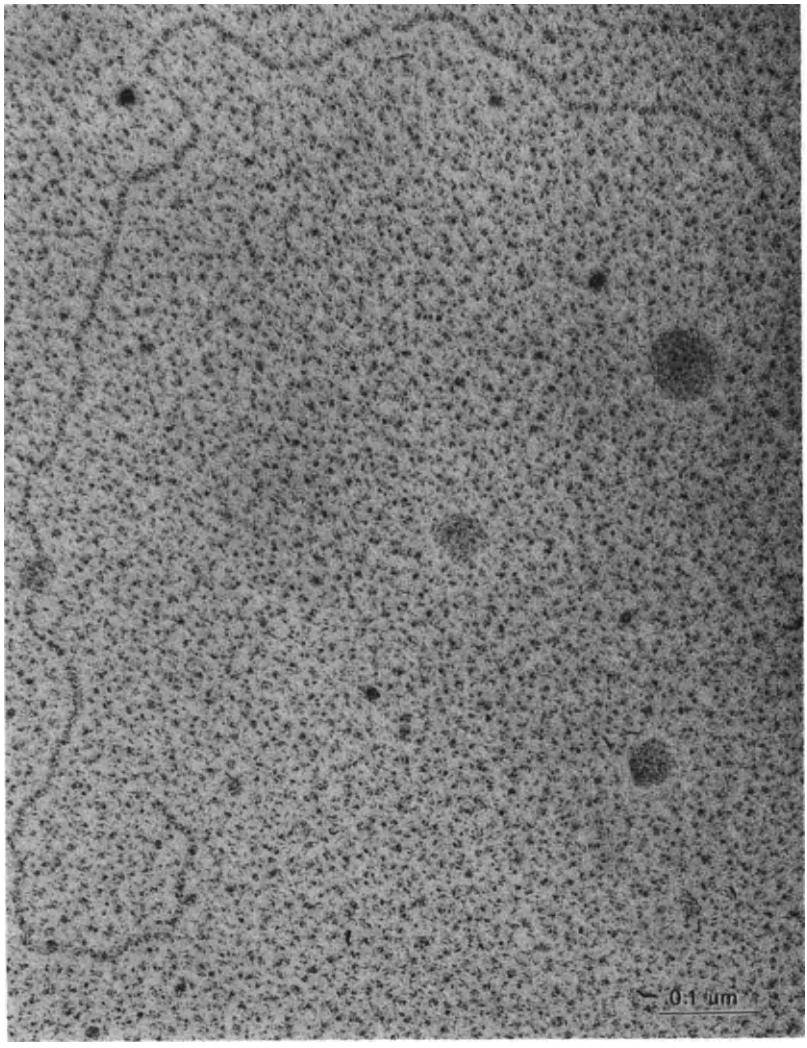

FIG. 14. Electron micrograph of a filament released during spreading of the rapidly sedimenting RNA fraction from Fig. 12.

the control sample. Examination of the rapidly sedimenting RNA in the electron microscope revealed structures that were indistinguishable from the "spider" forms obtained from gently lysed virions (Fig. 13). Filaments radiating out from an electron-dense central body are evident. A number of single filaments, such as that shown in Fig. 14, were also present. These presumably broke free from the large spider structures either during spreading or during storage of the sample. A number of fil-

aments, such as the one shown in Fig. 14, have an electron-dense spherical structure either at the end or situated along the length of the segment. The contour length of the lower arm measured 0.57 nm, while the upper arm measured 1.11 nm. These measurements correspond to the contour length of the medium and large size segments classes, respectively. This raises the possibility that the spherical structures seen in these electron micrographs contain the linker proteins. If this turns out to be true, analysis of a large number of such micrographs should reveal the sequence of the segments in terms of the large, medium, and small size classes. It may also be possible by this technique to isolate and characterize the protein(s) involved in keeping the segments in correct alignment.

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CHAPTER 25

Physiological Properties of Vesicular Stomatitis Virus and Some Related Rhabdoviruses

LUDVIK PREVEC

I. Introduction

The mammalian rhabdoviruses, by virtue of their structural and physiological characteristics, provide an extremely interesting and experimentally amenable group for biological investigation. Members of this virus group are notable for their wide host range in animals and man, for their transmission and apparent growth in arthropod vectors, for their sensitivity to inhibition by interferon, and for the production of characteristic defective-interfering particles. At the molecular and biochemical level this virus group provides a laboratory system in which many of the fundamental questions concerning the biosynthesis, regulation, and function of nucleic acids, proteins, and lipid membranes may be experimentally approached.

Many of the physical and immunological characteristics of this group of bullet-shaped viruses and a large number of other similar vertebrate and nonvertebrate viruses have been described in a review of Howatson (1970). In this chapter we shall consider only the particular characteristics of those viruses that may be loosely defined as comprising the vesicular stomatitis (VSV) group. These include the virus strains Indiana, Brazil, Argentina, Cocal, New Jersey, Piry, and Chandipura. Experiments by Cartwright and Brown (1972), which compare the complement-fixing activity and the neutralization of infectivity of both whole virus particles and internal ribonucleoprotein, suggest that the strains Cocal and Argentina are most closely related, these in turn showing common antigens with the Brazil and Indiana strains. The New Jersey strain shared some complement-fixing antigens with these four virus strains, while the strains Piry and Chandipura appeared to be antigenically unrelated to each other or to any of the other members of the vesicular stomatitis group.

II. Structural Features of the VSV Group

A. Infectious Particles

The infectious particles of the VSV group, including Piry and Chandipura, are virtually indistinguishable on electron microscopic observation (Cartwright and Brown, 1972). These bullet-shaped, or B particles, are cylinders that are rounded at one end and flat at the other, approximately 170 nm long and 70 nm in diameter. Each particle consists of a ribonucleoprotein core helically wound around a central axial hollow and enclosed in an envelope consisting of a lipid bilayer and associated structural proteins. Spike-like structures projecting from the lipid surface can easily be seen in negatively stained preparations (Fig. 1).

Complete dissociation of purified infectious particles using sodium dodecyl sulfate (SDS), 6 *M* urea, and mercaptoethanol followed by analysis on neutral SDS-polyacrylamide gels distinguishes five protein species within the virion. Following the nomenclature of Wagner *et al.* (1972b) the proteins of the Indiana serotype designated L, G, N, NS, and M have molecular weights of 190,000, 69,000, 50,000, 45,000 and 29,000, respectively (Kang, 1971).

Proteins G and M are solubilized after treatment of the virions with the ionic detergent sodium deoxycholate (Kang and Prevec, 1969). Protein G, a glycoprotein (Burge and Huang, 1970; Wagner *et al.*, 1970), can be removed from the virion by treatment with trypsin, a procedure

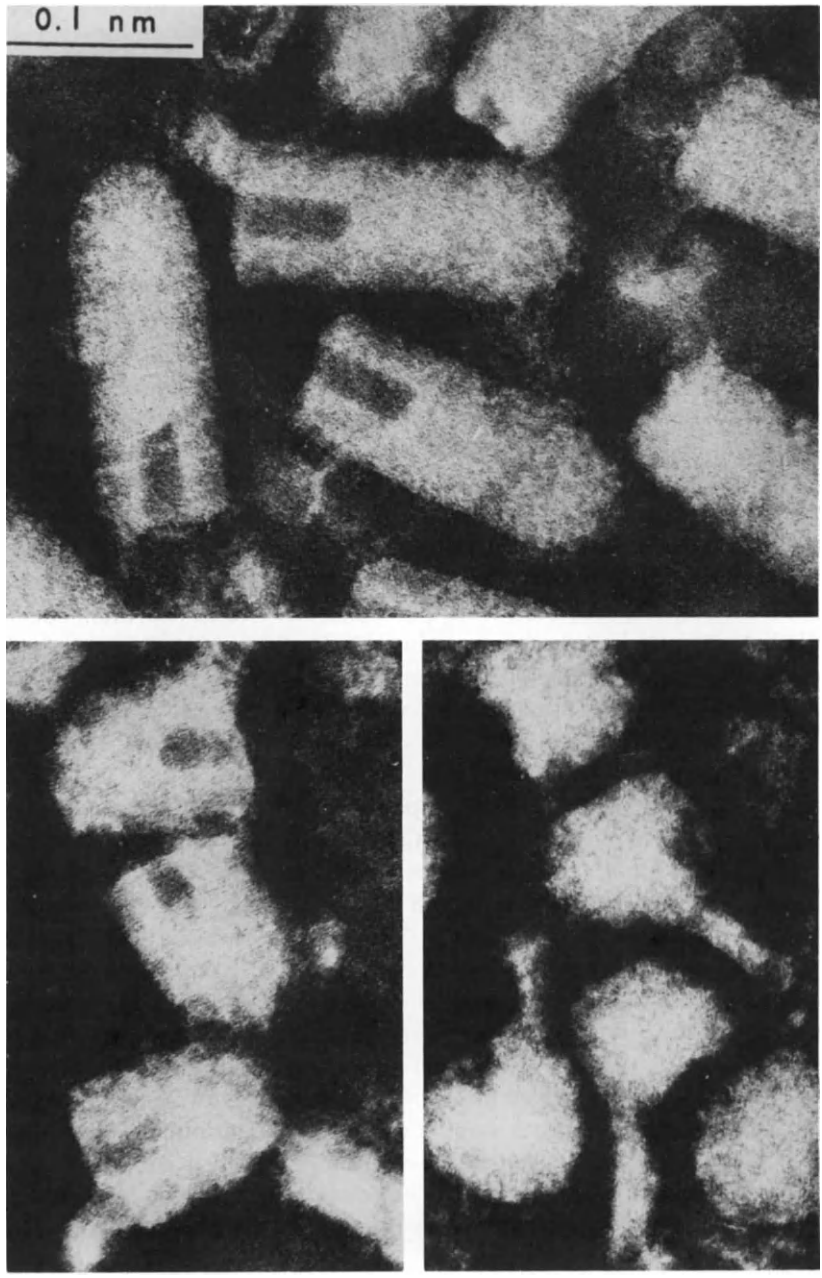

FIG. 1. Electron photomicrographs of infectious "B" (upper half), defective LT (lower left), and ST (lower right) particles of the Indiana serotype. Particles were grown in L cells in suspension culture and purified by rate zonal centrifugation on sucrose gradients as described by Prevec and Kang (1970). The particles were negatively stained with 1% phosphotungstic acid.

which removes the surface projections of the virus (Cartwright *et al.*, 1969). This protein constitutes the antigen against which whole virus neutralizing antibody is directed (Kelley *et al.*, 1972).

Since the protein M is unaffected by trypsin treatment of the virion and remains associated with the nucleoprotein core after treatment of the virus with nonionic detergents, such as NP40 (Cartwright *et al.*, 1970), it is concluded that M underlies the lipid membrane.

The ribonucleoprotein component released from the virion by deoxycholate is a ribbonlike, helically coiled structure consisting of repeated units of N protein arranged along a single-stranded RNA molecule with a molecular weight of approximately 4×10^6 (Nakai and Howatson, 1968). Isolation of the nucleoprotein free of proteins G and M, using either nonionic detergents together with high ionic strength or two phase polyethylene glycol–dextran, both treatments preserving the inherent polymerase activity present in the virion (Baltimore *et al.*, 1970), shows that proteins L and NS are also associated with this component (Bishop and Roy, 1972; Emerson and Wagner, 1972; Szilágyi and Uryvayev, 1973). Bishop and Roy (1972) further observed two new protein species, called A and B, in this fraction, but it remains to be shown whether these are novel proteins or structural modifications of one of the five major virus proteins. Treatment of virions with Tween–ether yields an infective ribonucleoprotein core still having associated M protein (Cartwright *et al.*, 1970).

The protein NS is the only phosphoprotein normally present in VSV of the Indiana serotype (Sokol and Clark, 1973), although all of the virus proteins are capable of being phosphorylated *in vitro* by a virion-associated phosphokinase (Strand and August, 1971).

Analysis of the proteins of other VSV strains shows a similar assemblage of proteins in the virions. Comparative studies of the electrophoretic mobility of these proteins on continuous neutral SDS polyacrylamide gels show some differences in the corresponding proteins (Wagner *et al.*, 1969; Wunner and Pringle, 1972). A recent thorough comparison of proteins from VSV of the Indiana, New Jersey, Piry, and Chandipura strains on both continuous and discontinuous buffer SDS-polyacrylamide gels reveals a number of minor differences in the smaller structural proteins of these strains, but does not allow any simple correlation of protein pattern and strain relatedness (Obijeski *et al.*, 1973).

B. Defective Particles

In addition to infectious B particles, replication of VSV virus in appropriate cells can produce defective particles (for review, see Huang

and Baltimore, 1970). Repeated clonal isolation of infectious virus by plaque picking followed by repeated high multiplicity infection of the appropriate cell line will generally lead to a virus stock containing both defective and infectious particles (Stampfer *et al.*, 1971). Except for their overall length and hence their molecular weight and sedimentation coefficient, the defective particles are morphologically and structurally similar to the infectious B particle. The designation "T particles" (Cooper and Bellett, 1959) is in common use for the defective particles of VSV.

A number of T particles of distinct lengths have been derived from virus of the Indiana serotype of VSV. The most common particle produced by laboratory strains has a length approximately one-third that of the B particle and an RNA genome with a molecular weight of approximately 1×10^6 (Huang *et al.*, 1966). Recent experiments by Lesnaw *et al.* (1973) show that there are significant differences in the electrophoretic mobilities of RNA genomes extracted from these standard T particles derived from different laboratories. This result suggests the need for caution in generalizations concerning the biochemical nature of T particles.

In 1970, Petric and Prevec described a larger or LT defective particle produced by an isolate of the Indiana serotype selected for increased temperature stability. This LT particle is approximately one-half the length of the B particle and contains an RNA genome with a molecular weight of approximately 2×10^6. As seen in Fig. 2 the structural proteins present in the LT particles as well as those in the standard or ST particles are qualitatively and quantitatively similar to those of the parental Indiana B particles.

In our laboratory we have examined T particles produced by five independent plaque isolates of both heat-resistant and wild-type virus of the Indiana serotype. In all cases the heat-resistant strain gave LT-like particles, while the wild-type gave ST-like particles as determined by electron microscopic examination. This result suggests that to some extent the size of defective particle produced is genetically determined by the parental virus. In this regard Reichmann *et al.* (1971) isolated T particles of different sizes by repeated passage of virus stocks originally selected after mutagenesis for conditional lethality at 38.5°C. As there was no apparent correlation between the size of the defective particle produced and the complementation group of the mutant giving rise to it, it is possible that the range of particles produced by these lines is a reflection of mutagenic action on the site(s) governing defective particle formation.

Defective particles arising from VSV of the New Jersey serotype ap-

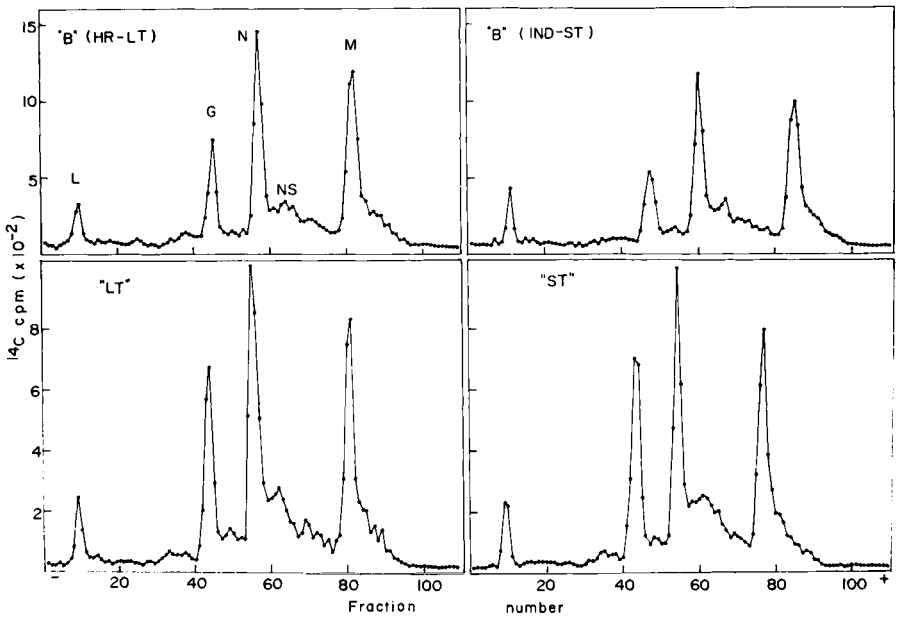

FIG. 2. Polyacrylamide gel analysis of the proteins obtained from purified B and T particles. LT and ST particles together with the corresponding parental strain of Indiana B particles (HR-LT and IND-ST, respectively) grown in L cells in the presence of ^{14}C-labeled amino acids were purified on sucrose gradients; disrupted with urea, SDS, and mercaptoethanol; and analyzed on polyacrylamide gels as described by Kang and Prevec (1971). The proteins are lettered following the convention of Wagner *et al.* (1972b). Figure from Kang (1971).

pear to be somewhat less homogeneous in size than those described above (Hackett *et al.*, 1967; Perrault and Holland, 1972a). Whether this is an inherent feature of a genetically identical virus population of this strain or an indication of difficulty in maintaining a genetically homogeneous population of B particles remains to be determined.

Crick and Brown (1973) have isolated T particles of Cocal, Piry, and Chandipura viruses.

III. Some Intracellular Events in the Replication of VSV

The synthesis of virus-specific RNA following infection of cells with VSV has been examined in a number of laboratories (Stampfer *et al.*, 1969; Mudd and Summers, 1970b; Schincariol and Howatson, 1970,

1972; Huang *et al.*, 1970; Wild, 1971). While complete interpretation of many of the studies still remains difficult, particularly due to the complication of coincident defective particle replication, a number of very significant facts have emerged. Associated with polyribosomes of infected cells via an EDTA-labile linkage (Huang *et al.*, 1970) is a class of single-stranded RNA molecules that are complementary in base sequence to the infectious virus genome (Schaffer *et al.*, 1968; Schincariol and Howatson, 1972) and have associated polyadenylic acid segments (Ehrenfeld and Summers, 1972; Soria and Huang, 1973). This group of RNA molecules can be separated by rate zonal centrifugation on sucrose into two major size classes—a homogeneous 30 S class with a molecular weight of approximately 2×10^6 and a more heterogeneous class sedimenting from 10 S to 16 S (Stampfer *et al.*, 1969; Petric and Prevec, 1970; Wild, 1971; Schincariol and Howatson, 1970). This latter RNA class can be further fractionated on polyacrylamide gels into three general size classes corresponding to molecular weights of approximately 7.5×10^5, 6×10^5, and 3.5×10^5 (Mudd and Summers, 1970b; Wild, 1971; Schincariol and Howatson, 1972). While some unexplained heterogeneity of electrophoretic mobilities still exists within each of these RNA species, they are of the proper size to contain the genetic information for the proteins G, N or NS, and M, respectively. Evidence that the 28 S RNA may serve as a messenger for the synthesis of L protein comes from the results of Stampfer and Baltimore (1973). Work by Huang and Manders (1972) shows that the complete set of mRNA species is synthesized by the virion-associated transcriptase after entry of the viral ribonucleoprotein within the infected cell.

Intracellular virus-specific protein synthesis following infection has also been examined by a number of laboratories (Mudd and Summers, 1970a; Wagner *et al.*, 1970; Kang and Prevec, 1971). While there is general agreement that the five proteins found associated with the virion form the bulk of the intracellular virus-specific protein synthesis, the possibility of other minor species is raised by Mudd and Summers (1970a).

The combined molecular weights of the five major virus proteins require a coding capacity equivalent to RNA with a molecular weight of approximately 3.8×10^6, which is in agreement with the value obtained by Nakai and Howatson (1968) from length measurements of the B particle ribonucleoprotein. Thus even allowing for reasonable error in estimates of the B particle genome size, it is doubtful if more than one additional protein of some 30,000 molecular weight could be specified by the viral genome. Aside from the obvious difficulty in totally suppressing nonviral, cellular protein synthesis, the minor species might represent

viral proteins that have yet to undergo minor structural modifications that may alter their electrophoretic mobility. Nonphosphorylated precursors to protein NS and nonglycosylated or partially glycosylated precursors to protein G (Kang and Prevec, 1971) are potential candidates.

Protein NS, which is present in low molar amount in the virion (Bishop and Roy, 1972), is found in the soluble fraction of the cell cytoplasm (Wagner *et al.*, 1972a) and constitutes a large proportion of the total intracellular virus-specific protein. Kang and Prevec (1971) showed that the rate of NS synthesis relative to other virus-specific proteins was higher at early stages of infection, suggesting that NS may have an important intracellular role in virus development.

The proteins G and M rapidly associate with large membrane structures of the cell, presumably the sites of eventual viral maturation and release (Cohen *et al.*, 1971; David, 1973). Kinetics of protein synthesis and release in Indiana virus infection suggest that one of these membrane proteins may be rate limiting in virus development (Wagner *et al.*, 1970; Kang and Prevec, 1971). Whether it is this or some other step in maturation that is responsible, there is an accumulation of ribonucleoprotein structures within the infected cell. These structures, which contain viral RNA as well as proteins N, L, and some NS, resemble by electron microscopic examination the ribonucleoprotein cores isolated from virions. It has been shown in the author's laboratory that these intracellular ribonucleoprotein structures can utilize ribonucleoside triphosphates *in vitro* to synthesize virus-specific RNA (Galet *et al.*, 1973) that may have associated polyadenylic acid residues (Galet and Prevec, 1973). While it seems likely that the intracellular ribonucleoproteins represent precursors to mature virus, there is still no evidence to indicate the biochemical mechanism by which this process occurs. The occurrence of two or more complexes in cells infected under conditions in which little or no defective particles are being produced (Kiley and Wagner, 1972; Galet *et al.*, 1973) remains to be explained. It is also clear from unpublished work done in the author's laboratory that L cells infected with VSV of the Indiana serotype accumulate, within their cytoplasm, ribonucleoprotein well in excess of that required to produce the maximal yield of infectious virus. We shall discuss below a similar situation in cells coinfected with defective particles.

As well as the single-stranded RNA species within the infected cell, a number of double-stranded or partially double-stranded RNA species have been identified (Stampfer *et al.*, 1969; Schincariol and Howatson, 1970; Mudd and Summers, 1970b). Whether these represent replicative

complexes or intermediates in transcriptive complexes remains to be determined. The mechanism by which replication of viral RNA occurs in VSV infection is not known.

IV. Replication of T Particles

Experiments employing relatively pure populations of T particles isolated by rate zonal centrifugation suggest that little or no virus-specific biosynthetic activity occurs in cells infected with these particles (Stampfer *et al.*, 1969; Schaffer *et al.*, 1969; C. Y. Kang, unpublished studies). Huang and Manders (1972) have shown that primary transcription (i.e., intracellular synthesis of mRNA by the infecting virus in the presence of inhibitors of protein synthesis) does not occur in cells infected with standard Indiana T particles nor do the T particles have any effect on primary transcription by coinfecting B particles. This result is in agreement with observations that suggest that the standard T particles of Indiana or New Jersey virus do not have *in vitro* transcriptase activity (Bishop and Roy, 1971; Perrault and Holland, 1972b). The diversity of T particles that can be isolated after mutagenesis of a common parental virus together with the fact that T particles arise in a virus stock originally obtained by plaquing is very strong evidence that they are produced during B particle replication and are not merely contaminating satellite viruses. Considering what is known regarding the genome of VSV B particles and assuming that all of the virus specific proteins are essential to virus replication, a T particle stock could be infectious only if different particles contained different part of the B particle genome in such a way that the total information could be presented to a cell in high multiplicity infection. This is the situation in some of the multipartite virus systems of plants (Lane and Kaesberg, 1971; Bancroft, 1972). The absence of significant T particle replication even at high multiplicity infection together with rather specific hybridization with particular virus-specific complementary RNA species (Schaffer *et al.*, 1969; Schincariol and Howatson, 1972) suggest that the T particles carry a specific portion of the B particle genome. We (Prevec and Kang, 1970) have previously speculated on the genome composition of LT and ST particles. This speculation, presented in modified form in Fig. 6 and discussed later, is in general agreement with the hybridization data of Schincariol and Howatson (1972). These workers showed that messenger species purified from polyacrylamide gel and of the size to code for G, M, and N or

NS, respectively, hybridized well with RNA from the LT particle, while the latter messenger species hybridized to a far lesser extent with RNA from ST particles. T particles then are essentially deletion mutants of B particles and require helper B particles for replication.

T particle genomes can serve as templates for their own replication. This was shown by Prevec and Kang (1970) who infected cells with LT or ST particles in the presence of the nonhomologous parental Indiana B particles and showed that the T particles produced were the same size as those added. This result was greatly strengthened by the work of Wild (1972), who showed, using a T particle derived from the Brazil strain and having a characteristic genome of 23 S RNA, that defective particles produced in cells coinfected with this T particle and helper B particles of the Indiana strain contained the 23 S RNA genome in particles that were antigenically of Indiana virus origin.

Cells coinfected with and replicating T particles along with B particles show some characteristic changes. In particular, ribonucleoprotein complexes containing RNA species characteristic of the defective particle being replicated are found in the cell cytoplasm (Petric and Prevec, 1970; Wild, 1971; Kiley and Wagner, 1972).

Coincident with defective particle replication in infected cells there is a decrease in the yield of infectious particles by that cell. This process is called "interference." When T particles of one virus strain interfere with B particles of the same serotype the interference is "homotypic," when the B and T particles are of different serotype the interference is "heterotypic."

Huang and Wagner (1966) showed that the standard T particles of the Indiana serotype of VSV were much less effective in interfering with the New Jersey serotype than in the homotypic situation. Crick and Brown (1973) suggested that the degree of "heterotypic interference" could be used as a measure of the relatedness of the mammalian rhabdoviruses. Using the defective particles produced by the strains of viruses present in their laboratory, they showed that the T particle of the Indiana serotype could interfere with infectious particles of the Brazil, Argentina, and Cocal serotypes but not with New Jersey, Piry, or Chandipura. T particles of these latter three types exhibited only "homotypic interference." These authors suggested on the basis of their results together with a consideration of the immunological data that the viruses Indiana, Cocal/Argentina, and Brazil form a group distantly related to the New Jersey virus and completely distinct from the viruses Piry and Chandipura.

As we have previously reported (Prevec and Kang, 1970) in contrast to the result described above for the ST particle, the LT defective particle of the Indiana serotype is as effective in "heterotypic interference"

with the New Jersey serotype as it is in "homotypic interference." This result is unlikely to be due to any gross difference in the mechanism by which LT particles produce interference, since, as for ST homotypic interference (Huang and Wagner, 1966), we have shown that LT particle interference occurs only if LT particles are added in the first 2 hours of infection, is prevented if LT particles are UV irradiated, and is not inhibited by actinomycin D (H. Galet, unpublished results).

Figure 3 shows an extension of this comparison to the viruses Cocal, Piry, and Chandipura. As described previously, interference results plotted in this manner show a region of T particle concentrations in which the logarithm of the yield of infectious virus is inversely related to the T particle concentration. The slope of this experimental curve can be used as a measure of interference. At very high T particle concentrations a plateau value is reached. The significance of this plateau value or the reason for its existence is not known, although it would seem to represent a lower limit of interference for that particular defective-helper combination.

In agreement with the results of Crick and Brown (1973) ST particles of the Indiana serotype show heterotypic interference only with Cocal virus and not at all with New Jersey, Piry, or Chandipura strains. The LT particles, on the other hand, show heterotypic interference with

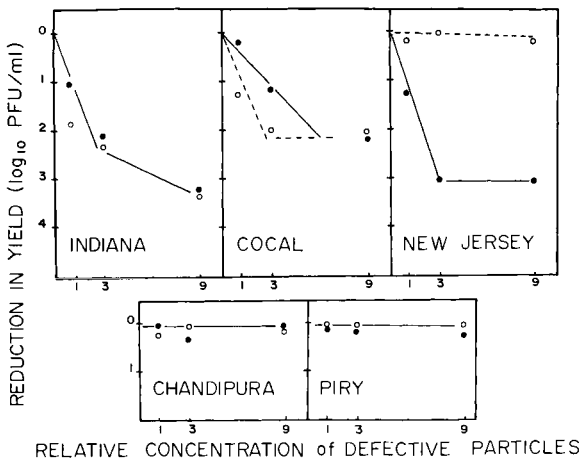

FIG. 3. Reduction in infectious virus yield of different viruses as a result of coinfection with LT and ST particles of the Indiana serotype. L cell monolayers were infected at a multiplicity of 1 PFU/cell in the absence or presence of appropriately diluted ST or LT particles as described by Prevec and Kang (1970). The yield of infectious virus was determined after 18 hours. The reduction in infectious virus yield (in log_{10} units) is plotted against the relative concentration of T particles for ST (○) and LT (●) particles.

Cocal and New Jersey strains but not with Piry and Chandipura. It is interesting that the LT particle interference with Cocal virus seems to be somewhat less effective, as measured by initial slope, than the New Jersey virus. If the extent of interference by LT particles was used as a measure of similarity, we might conclude that the New Jersey serotype is more closely related to the Indiana serotype in some characteristics than is Cocal virus. In order to gain some feeling for the possible significance of this result, it is necessary to examine very briefly the possible mechanisms of interference. No attempt will be made to cover all aspects of the interference phenomena since very current reviews are available on this topic (see, for example, Huang, 1973).

V. Intracellular Events during Interference

At least two plausible hypotheses have been advanced to explain interference by defective particles. One of these, which I shall refer to as "the inhibition hypothesis," suggests that a product of the T particle genome serves as an inhibitor of some function essential to the production of infectious virions. The inhibitor could, for example, be an RNA transcript of the T particle genome or a protein coded by such a transcript. Interaction of this inhibitor with the B particle genome or product of the B particle genome in such a way as to interfere with the synthesis or function of some gene absent from the defective particle could inhibit the viral replicative process within the cell.

The inhibitor hypothesis fails to explain the apparent selective advantage for replication of T particles over B particles during homologous interference. Furthermore this hypothesis requires that the information in the T particle genome be translated either into an RNA transcript or even further into specific proteins. While evidence for replication of the T particle genome was presented previously, there is no evidence to support transcription or translation of the genome even in the presence of helper virus. The absence of *in vitro* transcriptase activity in standard T particles and the inability of these particles to complement temperature-sensitive mutant B particles (Reichmann *et al.*, 1971) are evidence against a possible involvement of the T particle genomes in transcription or translation. Recent, unpublished reports from a number of laboratories, including our own, that some RNA synthesis may be directed by the ribonucleoprotein component of defective particles may cause a reexamination of this hypothesis. On the basis of the inhibitor hypothesis, heterotypic interference would necessitate sufficient complementarity of nucleic acid or structural similarity of protein of the two strains to allow interaction and inhibition to occur.

The second hypothesis of interference, "the competitive hypothesis," suggests that interference is a consequence of the natural selective advantage of the T particle genome in competition with the B particle genome for replicase molecules. The selective advantage of the T particle genome could simply be the consequence of its shorter length, which for a constant rate of synthesis allows more defective than infectious particles to be replicated in unit time. If replicase itself is coded in whole or in part from information present only in the B particle genome then as the number of these genomes is kept low by effective competition of T particles, the total amount of available replicase is also low. In this way the total yield of particles, infectious and defective, is reduced.

The "competitive hypothesis" would predict that the extent of interference is a function of the number of defective particles added to a cell, that under conditions of low to moderate interference the replication of defective particle genomes would take place at the expense of B particle genomes, and that under conditions of high interference the synthesis of both infectious and defective particles would be greatly reduced. These predictions seem to be borne out in studies of homotypic interference.

The one apparent exception to these postulates occurs in the heterotypic interference of New Jersey virus by LT particles of the Indiana serotype. As reported by Prevec and Kang (1970) little or no production of complete LT particles occurs under any condition of interference with New Jersey B particle production. This result could be due to the lack of maturation or release of LT particles under these conditions rather than to the absence of competitive type of interference. If so it should be possible to determine whether there is intracellular evidence of LT genome replication. As mentioned previously the replication of defective particle genomes within the infected cell can be readily detected by the presence in the cytoplasm of ribonucleoprotein particles characteristic of the defective particles. This is seen in the left panels of Fig. 4 during homotypic interference of Indiana VSV by LT particles. The large ribonucleoprotein complexes observed in cells infected with infectious virus alone are replaced during interference by a low molecular weight complex characteristic of the LT particle (Petric and Prevec, 1970). In contrast to the Indiana VSV, cells infected with the New Jersey serotype alone do not accumulate large amounts of viral ribonucleoprotein. Despite this, however, when LT particles are present together with New Jersey B particles, there is accumulation within the cell of ribonucleoprotein characteristic of LT particles. Thus replication of the LT genome does indeed occur in this case and could therefore be responsible for interference.

The absence of significant LT particle maturation even though genome replication occurs is of some interest. Whether this is due to the

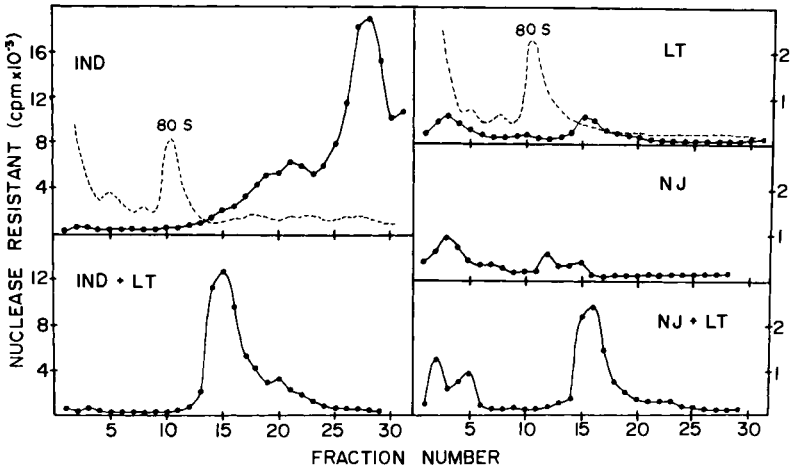

FIG. 4. Sucrose gradient analysis of the ribonucleoprotein components within the cytoplasm of VSV infected cells. L cells in suspension culture were infected with Indiana B particles alone (IND) or together with LT particles (IND + LT), with New Jersey B particles alone (NJ) or together with LT particles (NJ + LT), and with LT particles alone (LT). Actinomycin D (2 μg/ml) was added to all cultures at 1 hour postinfection (PI), and [^{3}H]uridine (2 μCi/ml) was added at 2 hours PI. All cultures were harvested at 5 hours PI, the cells homogenized gently, the nuclei removed by centrifugation, and the cytoplasmic fraction analyzed on 15 to 30% sucrose gradients all as described by Petric and Prevec (1970). Each successive gradient fraction after monitoring for optical density (dashed line) was treated at 37°C for 30 minutes with 20 μg/ml of pancreatic ribonuclease and then acid precipitated and the resistant radioactivity plotted. The direction of sedimentation was from left to right, and the position of the 80 S ribosome is indicated.

specific absence of membrane-associated proteins and hence potential maturation sites as a result of interference or to some other pecularity of this system remains to be determined.

The presence or absence of interference in heterotypic combinations should, on the basis of the competition hypothesis, be determined by the ability of the infectious virus replicase to recognize and use as template the genome of the interfering defective particle.

VI. Nucleic Acid Homology to Indiana VSV

In addition to antigenicity, protein size, electrophoretic mobility, and interference, the extent of biochemical homology in the viral genome as measured by RNA–RNA hybridization should be an indication of the

genetic relatedness of two strains. The availability of reasonably well characterized mRNA molecules complementary in base-pairing sequence to the viral genome allows comparisons of the extent of homology in different genomes. For the experiments to be reported here we fractionated radioactively labeled mRNA purified from Indiana VSV-infected cells into the 28 S species and the 10 S to 16 S species. The first of these should provide an indication of the homology of the genome coding for L protein in each of the strains, while the latter class presumably contains messengers for proteins G, N, NS, and M. Yet another class of RNA complementary in base sequence to the viral genome is the *in vitro* transcription product synthesized by the complexes isolated from infected cells (Galet *et al.*, 1973). While the exact portion of the viral genome transcribed by this enzyme at 37°C still remains to be identified, it is known that the product is totally complementary to a base sequence present in the LT particle genome but shares no complementarity with the ST particle genome (Fig. 5).

The two size classes of mRNA and the *in vitro* transcript, all specified by Indiana VSV, were incubated with the indicated viral RNA under conditions that allowed virtually total hybrid formation, as measured by resistance to digestion by pancreatic ribonuclease, with the homologous Indiana RNA. As seen in Table I, no hybrid formation in excess of

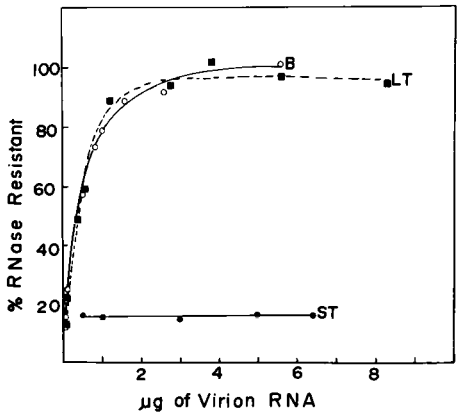

FIG. 5. Hybridization of RNA synthesized *in vitro* by a polymerase complex from Indiana virus infected cells. The [^{3}H]GTP labeled product synthesized at 37°C as described by Galet *et al.* (1973) was purified by phenol extraction. Unlabeled RNA was similarly extracted from sucrose gradient purified B, LT, and ST particles of the Indiana serotype. The labeled product together with the appropriate concentration of virion RNA was allowed to anneal at 65°C for 2.5 hours, and the amount of RNA resistant to pancreatic nuclease was then determined and plotted, all as described by Galet *et al.* (1973).

TABLE I

Hybridization of Indiana Complementary RNA to RNA from Purified Virus of Different Strains[a]

RNA source	*In vitro* product[b]	28 S messenger[b]	10 S–16 S messenger[b]
Indiana	93	89	76
Cocal	23	27	16
New Jersey	32	32	17
Piry	7	27	9
Chandipura	8	26	10
No viral RNA	9	20	7

[a] Messenger RNA labeled with [^{3}H]uridine was purified from L cells infected with Indiana VSV by dissociation from polyribosomes with EDTA, followed by phenol extraction and reisolation of the 28 S fraction and the 10 S–16 S fraction from sucrose gradients. The *in vitro* product was phenol-extracted RNA synthesized by the intracellular transcriptive complex with [^{3}H]GTP as one precursor (Galet *et al.*, 1973). Hybridization was as described in Fig. 5 with 5 μg/ml of unlabeled RNA extracted from gradient purified virions of the named virus strain.

[b] Percent of ribonuclease-resistant material after hybridization.

background (i.e., no viral RNA added) was observed with either Piry or Chandipura viruses with the *in vitro* product or the 28 S mRNA class. In contrast however both the Cocal and New Jersey strains showed significant hybrid formation with both of these classes. It is perhaps significant that the *in vitro* product RNA, which has complete homology with LT particles, appears to show somewhat greater homology with RNA from New Jersey virus than with Cocal virus.

Because of the high background after hybridization of 28 S RNA, it is not possible to assess the significance of the hybridization result with this RNA class. It can be said that all four heterologous strains showed roughly the same homology or lack of homology with this class.

VII. Conclusions and Speculations

The results and discussion presented in this chapter serve to highlight some features of the relationship between members of the VSV group of rhabdoviruses. Interference by ST particles suggests that Indiana and Cocal virus are closely related, while the viruses New Jersey, Piry, and Chandipura are very distantly if at all related to the first two. In contrast, interference by Indiana LT particles suggests that the New Jersey strain is as closely related to the Indiana strain as is Cocal.

Immunological studies, while by no means unanimous, suggest that

the Indiana, Cocal and New Jersey strains share a cross-reacting complement-fixing antigen, while Cocal and Indiana viruses also share a common determinant for neutralizing antibody. The work of Kang and Prevec (1970), Cartwright and Brown (1972), and Kelley *et al.* (1972) shows that the cross-reacting complement-fixing determinant is carried by the nucleoprotein N, whereas neutralizing antibody is directed to the glycoprotein G. On this basis it might be expected that the RNA sequence coding for protein N may have some similarity in the strains Indiana, Cocal, and New Jersey, and the sequence coding for protein G may be somewhat similar in Indiana and Cocal viruses.

The possibility that the proteins L, NS, or M also share common antigenic and structural features in different strains has not yet been excluded, since these proteins may not elicit significant antibody in inoculated animals. This could be due to the relatively small amount of L and NS present in the virion and to the masked structural location or association with lipid of protein M in the virion.

If 28 S RNA is indeed the messenger for L protein, then the hybridization data presented in Table I would suggest that the L proteins of the four strains examined have very little in common with the L protein of the Indiana serotype. If it should turn out, as seems entirely possible from the results of Emerson and Wagner (1972), that protein L serves as part of the transcriptive complex of the virion, it might be expected that the enzyme active site would be structurally similar in the different virus strains. The above result would suggest then that the bulk of this large protein is strain-specific. Since RNA transcription and perhaps replication in the VS viruses could involve interaction between proteins L, NS and the RNA-N protein complex (Emerson and Wagner, 1972; Bishop and Roy, 1972), it would seem reasonable that strain differences in protein L may prevent interstrain complementation by this protein. The same reasoning might be applied regarding interstrain differences in proteins NS and N.

Examination of the hybridization and interference data presented in this chapter, in the light of a speculative model of the genomic constitution of LT and ST particles, points out some interesting possibilities for further research. The original model proposed by Prevec and Kang (1970) from a consideration of the size of the then known viral proteins is presented again in slightly updated fashion in Fig. 6. No polarity or order of genes is implied; only the nature of the coding potential in each particle is suggested. Portions of the genomes of heterologous serotypes that may have some homology with Indiana virus are shown as solid segments, while regions of unknown or little homology are presented as broken lines. Examination of this model shows that interference by ST

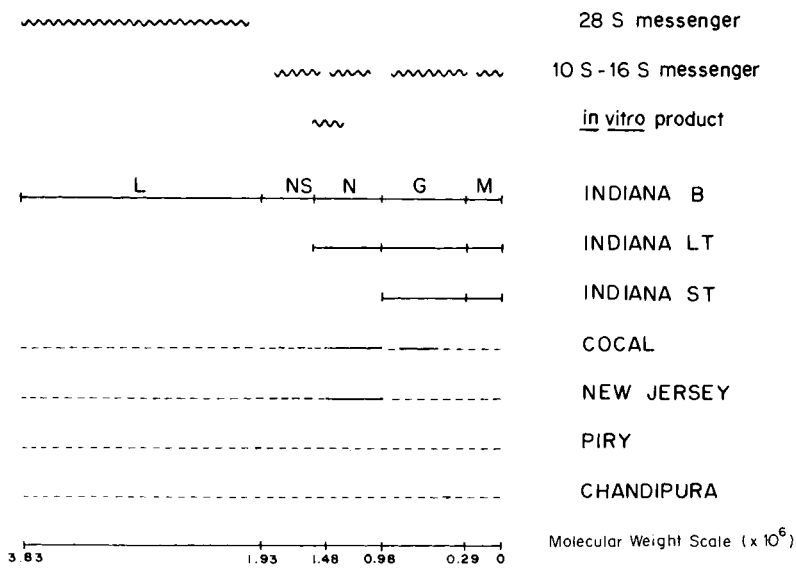

FIG. 6. A speculative model of the coding potential and nucleic acid homology of different virions of the VS group. The genomes of Indiana virus and RNA sequences with potential homology to the Indiana genome are shown as solid lines. The broken line indicates regions of no or unknown homology. The wavy lines represent RNA species that show complementarity to the designated regions of the Indiana virus genome.

or LT particles was effective only against virus strains that had a homologous genome segment with the defective particle. This concept of genetic homology is strengthened by the hybridization experiments employing *in vitro* synthesized complementary RNA, which as shown in the figure must be a transcript of that portion of the Indiana B particle genome present in the LT particle but absent from ST. This complementary RNA hybridizes to a good extent with both Cocal and New Jersey virus but not with Piry and Chandipura. While the model suggests that this RNA species is a transcript of RNA sequences coding for N protein, this remains to be proved.

How does this necessity for genetic homology tie in with the models of interference presented earlier? The inhibitor hypothesis fits this model very easily, since we need only assume that transcripts or translation products of T particle genomes can be inhibitory to the helper virus only if they have some identity with the proteins or RNA of the helper virus.

It is much more difficult to explain the results of heterotypic interference using the competitive hypothesis. If we look at the primary events associated with LT and ST particle infection, both particles contain the same Indiana proteins and can function as templates for Indiana B par-

ticle replicase. Since the LT particle can also be recognized as a template for the New Jersey replicase, it is difficult to envisage why the ST particle would fail to function similarly in this initial step. Let us suppose that the first round of replication is successfully carried out in this heterotypic infection. The newly synthesized T particle RNA must now presumably associate with nucleocapsid proteins of the New Jersey serotype (Wild, 1972) before it too can replicate and continue the competitive process. Since LT particles appear to successfully complete this process in heterotypic interference, if ST particles fail to do so we must postulate that some homology of RNA sequence between the defective particle and the helper is essential for proper nucleic acid–protein combination. While it is possible to postulate a number of schemes whereby such a possibility could arise, it is evident that no simple answer to this problem is available.

In summary it would seem that while a competitive interference model best explains the results of homotypic interference, it is much easier to understand heterotypic interference on the basis of an inhibitor type model. This conclusion, while it leaves unanswered any specific postulates concerning the mechanism of inhibition, suggests that a search for such controls may be in order.

Finally it is hoped that the experiments and discussion presented in this chapter will serve in part to show why this unique group of viruses, with relative biochemical simplicity yet diverse biological interaction, should continue to provide a very fruitful research area for the molecular biologists and biochemists being attracted to this field.

ACKNOWLEDGMENTS

The author would like to express his heartfelt thanks to his former and current graduate students, in particular, Dr. C. Y. Kang, Dr. M. Petric, Dr. H. Galet, and Mr. D. Hallett for their effort and stimulation in this research area and to his technicians Mr. D. Takayesu and Mrs. Mary Ann Eastman for their assistance. Special thanks are also due Dr. J. Obijeski, Centre for Disease Control, Atlanta from whom the Cocal, Piry and Chandipura viruses were obtained and who provided us with purified Piry and Chandipura viruses from which RNA was obtained for the initial hybridization experiments.

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CHAPTER 26

Evolution of Rhabdovirus Tropisms

FREDERICK A. MURPHY

I. Introduction

Rhabdoviruses spread among susceptible vertebrate populations in nature by two known patterns. One pattern, typified by vesicular stomatitis viruses (VSV), consists of epizootic infection sweeping through a population at risk and then disappearing. A second pattern, typified by rabies virus, is also epizootic, but the spread may be so slow that it appears enzootic. Undoubtedly, many viral growth characteristics and ecologic factors contribute to these patterns, but variance in tissue tropism and in target cell cytopathology also seem to have had central roles in the evolution of rhabdovirus diseases.

In considering those events of virus infection in an individual animal which relate to invasion, replication, *in vivo* spread, and shedding, the most primeval cycle hypothetically would involve an epitheliotropic, cytopathic infection wherein virus is rapidly shed from the site initially invaded. The next more sophisticated infection cycle would require systemic invasion and viremia en route to infection of sites necessary for

transmission. Both of these infection cycles must successfully challenge the rate of initiation of the host immune response to allow transmission before termination of infection. They must also depend upon a continuing availability and turnover of a susceptible host population. If such primeval, tenuous viral infection cycles survived despite continually challenging the host immune mechanism (with its ultimate threat of producing an effectively immune population), then evolutionary selective influences might have favored any series of variances toward circumvention of the recognition-triggering mechanism of the humoral and/or cellular immune systems. The neural pathway from the site of viral invasion [through the central nervous system (CNS)] to the organs involved with transmission represents a most sophisticated means of avoidance of viral contact with elements of the reticuloendothelial system and consequent immune triggering. Because neurotropism and intraneural spread of virus are associated with the completion of slower infection cycles in individual hosts, they offer the survival advantage of slower spread through host populations.

If rhabdoviruses have evolved from a single biologic archetype, it is intriguing to interpret the pathogenetic patterns of presently known animal viruses in terms of their probable sequential positions along an evolutionary path, or alternatively, their varying divergence from the common ancestor. From this standpoint VSV infection (in a rodent model) has been chosen as representing the simple primeval pattern, and rabies virus infection, the sophisticated descendent.

II. Vesicular Stomatitis Virus Tropism in Experimental Animals

Many aspects of the pathogenesis of VSV (VSV-Indiana; Cocal; Alagoas; VSV-New Jersey; Piry; and Chandipura) in species primarily involved in nature are still unknown. In ruminants and horses the direct relationship between site of experimental or natural entry and site of lesion development, the rapidity of development of epithelial lesions, the release of virus from the epithelial lesions, the absence of viremia, and the explosive spread of infection among contact animals (Jonkers, 1967; Hanson, 1968) all suggest the most primitive epitheliotropic infection cycle completing itself near the site of invasion. This has never been proved, and the premise does not lend itself easily to the techniques of experimental pathology. Even when the importance of spread by direct contact is conceded, the participation of arthropods and wild animals is presumed to explain the spread of VSV over long distances (an "ar-

bovirus" cycle: Mussgay and Suarez, 1962; Bergold *et al.*, 1968). This further complicates attempts to predict pathogenetic characteristics in individuals from epizootiologic observations of populations (Tesh *et al.*, 1970, 1972; Donaldson, 1970). Tesh *et al.* (1972) concluded that very young wild animals could not be important in completing an arthropod transmission cycle despite their susceptibility to infection and viremia. They considered that the high mortality and the effective protection conferred via maternal antibody would rapidly abort the cycle. Nevertheless, other "arboviruses" flourish through similar cycles, and characterization of VSV infections and tropisms in very susceptible hosts is of interest otherwise because it exemplifies the primitive infection pattern that requires systemic infection before viral transmission.

Pathogenetic studies of VSV infections in young hamsters and mice have shown (by organ titration and immunofluorescence) that viral replication, in particular in thoracic and abdominal organs (liver, kidney, spleen-thymus-reticuloendothelial elements, and lung), precedes and contributes to a viremia, which then results in CNS invasion and encephalitis (Cox and Olitsky, 1933; Sabin and Olitsky, 1937; Falke and Rowe, 1965a,b; Bruno-Lobo *et al.*, 1968a,b). Typically, target organs of young rodents undergo severe necrotic changes; when older rodents are infected, the limitation of necrosis to CNS tissues coincides with very low titers in extraneural organs and high titers in brain and spinal cord (Miyoshi *et al.*, 1971).

Experimental Observations

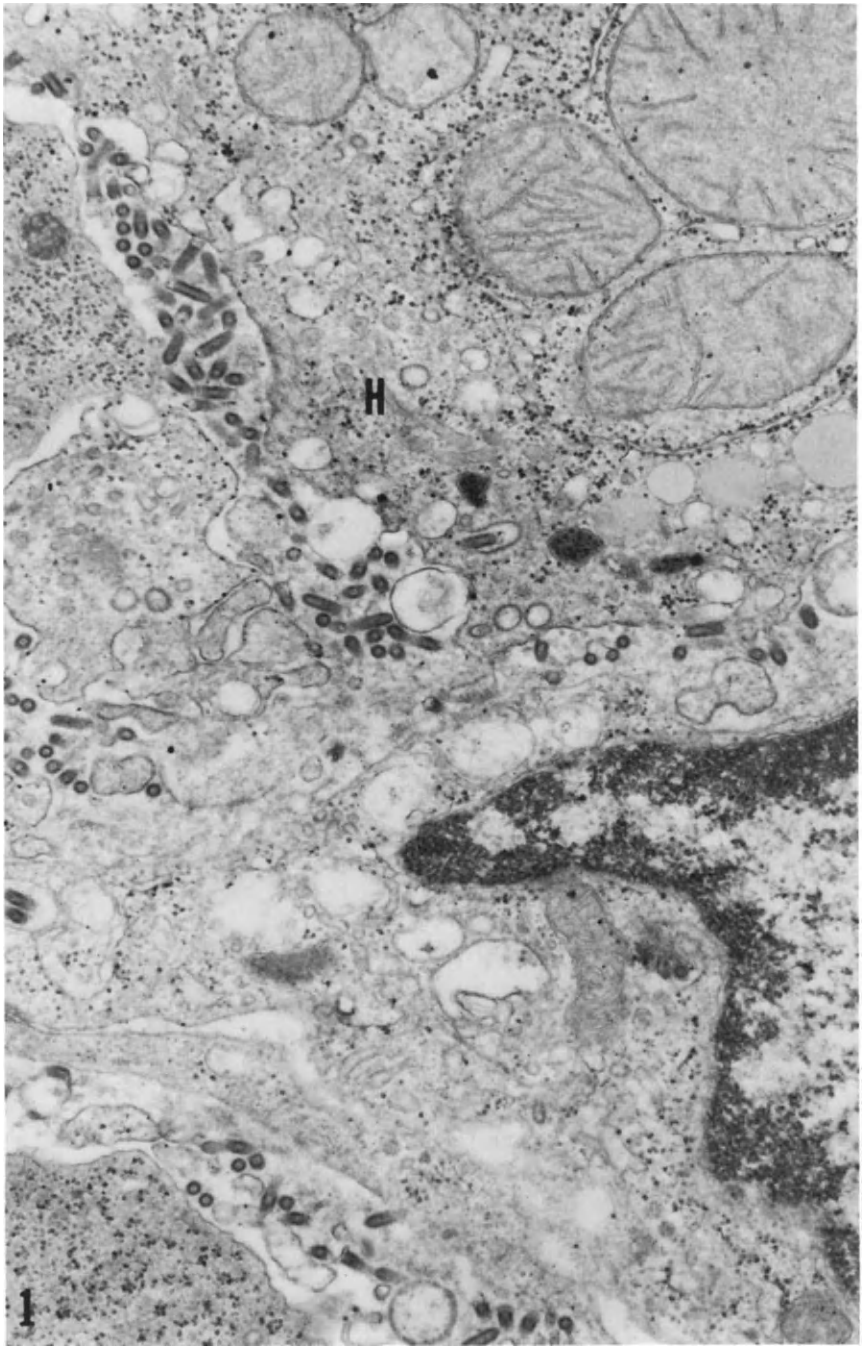
In our laboratory, we have used light, immunofluorescent, and electron microscopy to compare the extraneural and neural phases of infection in young mice and hamsters inoculated intramuscularly or intracerebrally with low-passage isolates of VSV-New Jersey and VSV-Indiana viruses [strain designations: VSV-NJ, GMO-110, suckling mouse (SM) passage 4; VSV-Ind., NM5-7M, SM4] (Sudia *et al.*, 1967). The particular tropism of these viruses for liver and kidney after peripheral inoculation was clearly defined by frozen-section immunofluorescence. These organs, as well as (1) reticuloendothelial elements (macrophages) of the peritoneal and pleural cavities, (2) muscle (striated and cardiac), (3) brown fat, and (4) fibroblastic cells of connective tissue in subcutaneous, intermuscular, and perivascular spaces, all fluoresced specifically despite the progression to a moribund state by 24 hours. In many of these animals, viral antigen was absent or extremely sparse in brain and spinal cord at the time of death. When older animals (mice,

4–21 days of age) were inoculated, extraneural immunofluorescence was restricted to trace levels in kidney, liver, and macrophages, and, in contrast, neural fluorescence was intensified especially in the thalamus, pons, and cerebellum. These observations are consistent with those reported by Bruno-Lobo *et al.* (1968a,b).

Electron microscopic observations of liver and kidney of these moribund mice and hamsters confirmed the fulminating, lethal, necrotic hepatitis and nephritis observed by light microscopy. Hepatocytes were focally destroyed through stages of vacuolation, cytoplasmic rarifaction, and lysis (Fig. 1). Kupffer cells were even more susceptible, being completely destroyed throughout the liver (Fig. 2). Both of these cell types were very productive of virus particles, with almost all budding occurring upon plasma membranes and the remainder upon endoplasmic reticulum membranes. Kidney tubule infection was also focal, very productive, and necrotizing (Fig. 3). In both organs, virus particles appeared very homogeneous in length and structure – characteristics which we have associated with high infectivity/particle ratios. In contrast, brains of animals inoculated intramuscularly showed minimal infection at time of death, and the ultrastructural character of CNS infection after intracerebral inoculation was marked by maintenance of neuronal cell body and axon-dendrite architecture until very late (relative to virus particle production). Typically, the exuberant budding upon plasma membranes of these neuronal cell bodies and processes resulted in packing of the normally collapsed intercellular spaces with virus particles (Fig. 4). Other cell types (glia and endothelium) were infected but yielded few particles. Interstitial edema and degenerative changes of neuronal cytoplasmic organelles were very focal. In these foci the progression of infection was also marked by formation of anomalously long virus particles and viral inclusions (Fig. 5). At the same locations a few neurons budded large numbers of virus particles only upon intracytoplasmic membranes, a phenomenon never seen in liver or kidney; this budding was associated with cytopathic changes (Fig. 6). The relative resistance of neurons to the necrotizing effects of VSV infection (with concomitant high virus yield) was observed with both serotypes; similar findings were made previously with Piry and Chandipura virus infections of newborn mice (F. A. Murphy and R. E. Shope, unpublished study).

Although parallel ultrastructural studies of the effects upon VSV

FIG. 1. Vesicular stomatitis virus (New Jersey serotype; VSV-NJ) in pericapillary space adjoining a sinusoid in the liver of a moribund suckling mouse 24 hours after intramuscular inoculation. Budding from plasma membranes of hepatocytes (H) resulted in high concentrations of extracellular virus particles preceding the vicious parenchymal necrosis. ×27,000.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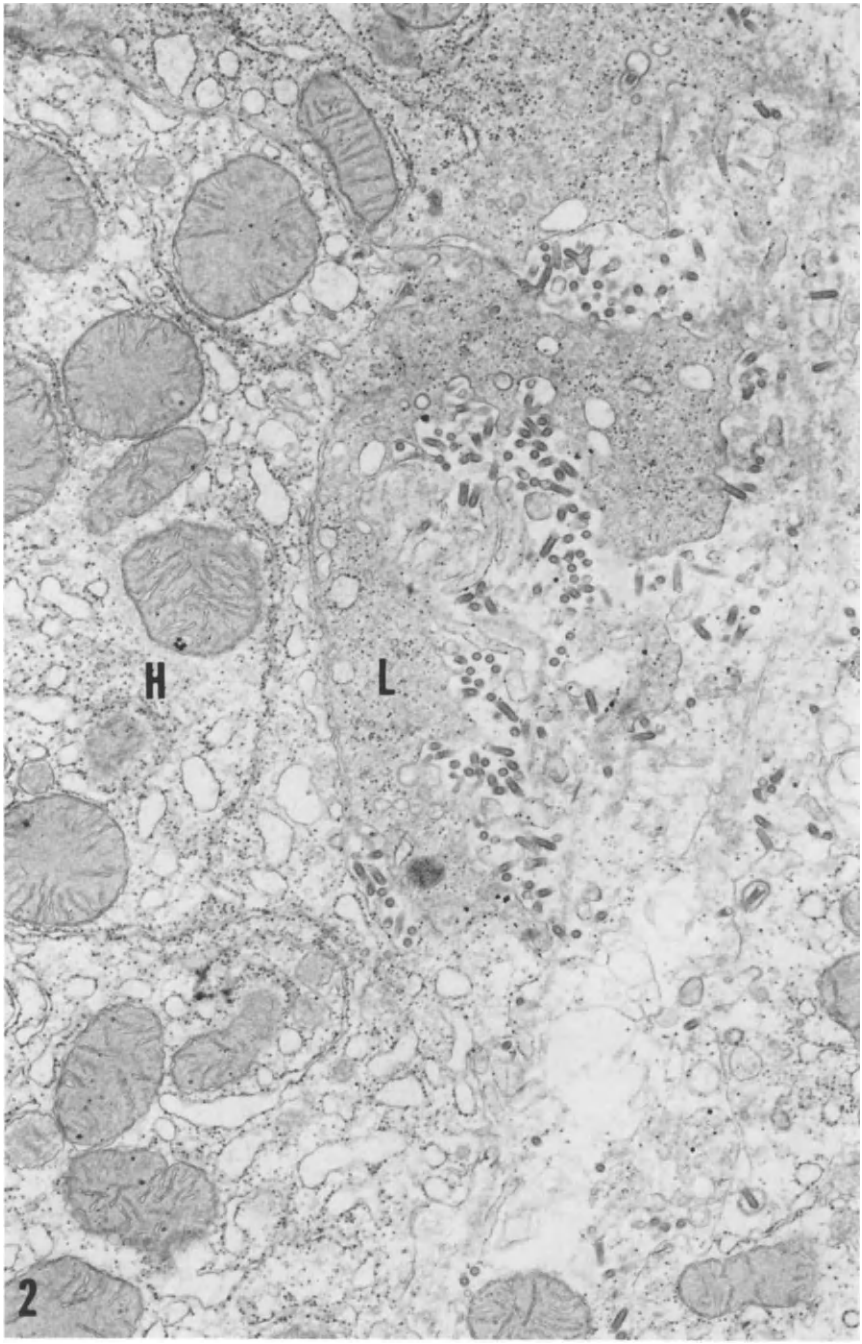


FIG. 2. VSV-NJ particles budding into a liver sinusoid from a littoral cell (L) which is undergoing necrotic changes. Hepatic necrosis seemed to be the cause of the peracute death in peripherally inoculated young hamsters and mice. H, hepatocytes. ×18,600.

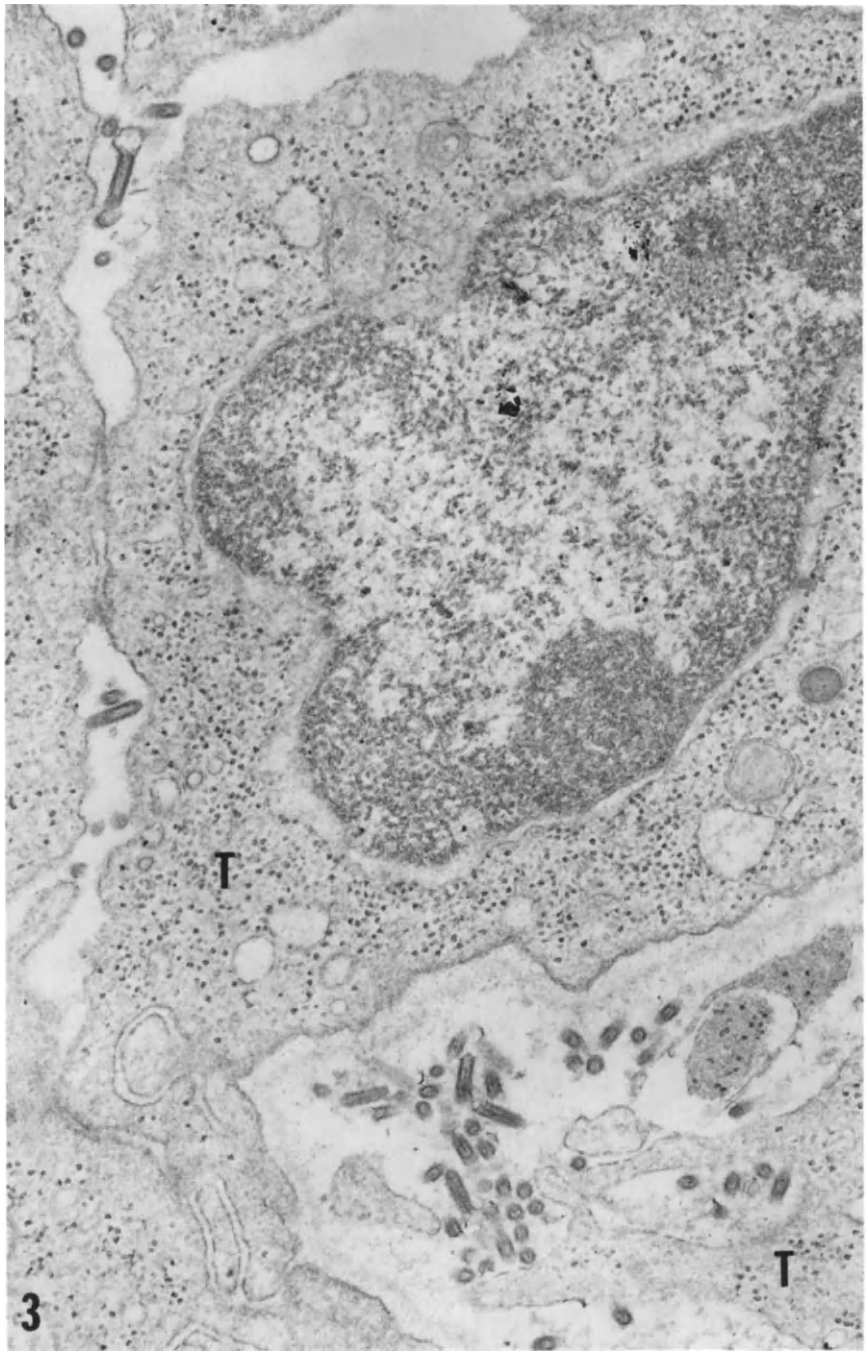
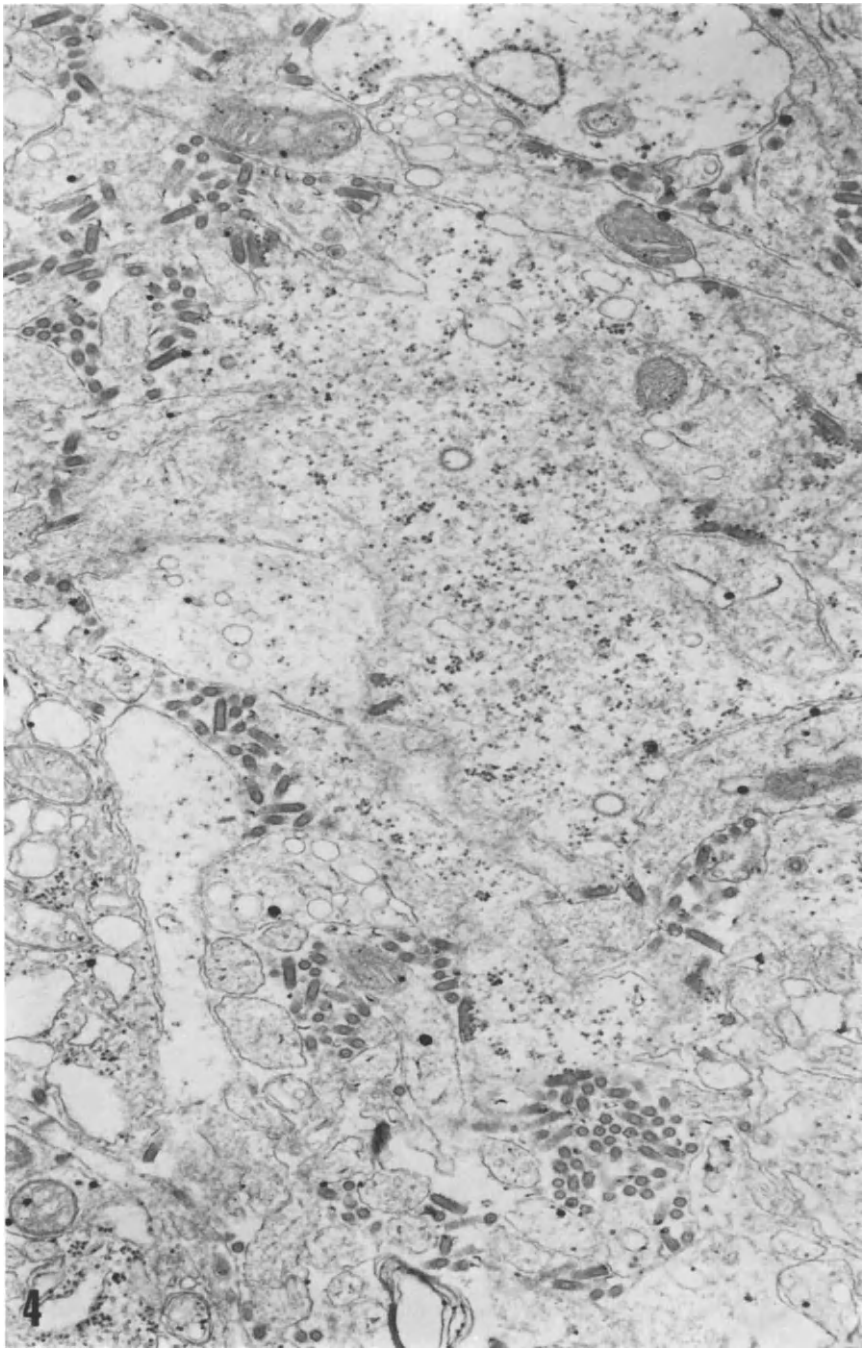


FIG. 3. VSV-NJ infection of kidney tubule cells (T) of a suckling mouse. When animals were moribund tubular necrosis was severe but focal, and most virus was associated with cells which were still intact. ×35,000.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tropism of variations in (1) virus passage level, (2) age, and (3) species of animal still need to be undertaken, it seems clear that the potential for modification of the infection pattern, primarily by restriction of viscerotropism and epitheliotropism and maintenance of neurotropism, is an inherent characteristic of the VSV genome. Such modification is in the direction of the rabies infection pattern.

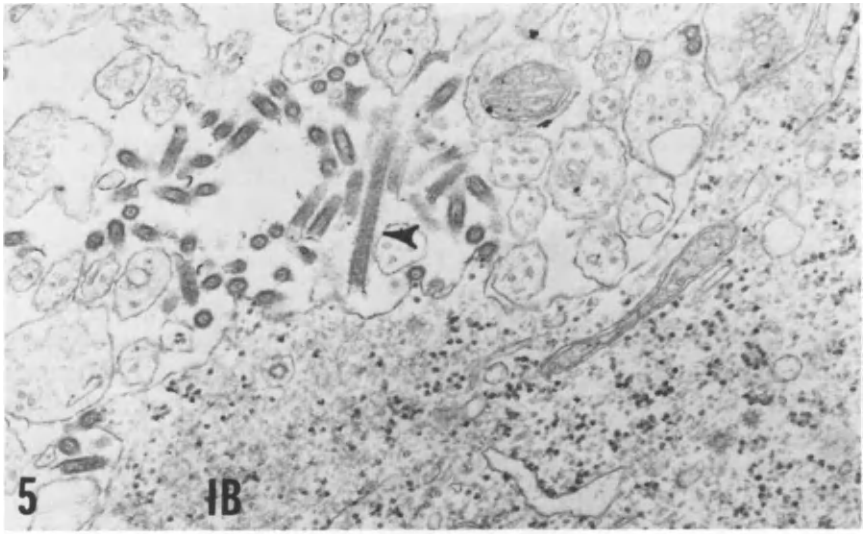
III. Rabies and Rabies-Like Virus Tropism in Experimental Animals

Street rabies virus strains in the species of most importance in North America (fox, skunk, and dog) are extremely neurotropic and lethal; the extraneural phase of infection is primarily epitheliotropic, with infection of salivary gland secretory epithelium being a requirement for transmission. Although a neural route from the site of viral entry to the CNS and a neural route centrifugally to the salivary gland and other organs involved terminally has been considered for many years (reviewed by Johnson, 1971), experimental proof has required use of more susceptible species (mice, rats, and hamsters) which support higher infectivity titers and exhibit less individual variation than feral animals (Dean *et al.*, 1963; Baer *et al.*, 1965). In the absence of any early cytopathology or inflammatory infiltration and an insensitivity of organ titration techniques, understanding of the sequential tropism of rabies virus has depended entirely upon immunofluorescence and electron microscopy (Fischman, 1969; Schneider, 1969; Matsumoto, 1970).

Experimental Observations

In our laboratory, we have studied the pathogenesis of three rabies virus strains (strain designations: vampire bat isolate, passage history uncertain; CVS-Pasteur, more than 2000 laboratory passages; Arctic fox isolate, mouse passage 2) and two African rabies-like viruses (Mokola, mouse passage 4; Lagos bat, mouse passage 12) (Shope *et al.*, 1970; Tignor and Shope, 1972; Murphy *et al.*, 1973a,b). After intramuscular inoculation of these viruses into hindlimbs of newborn hamsters, the first immunofluorescent evidence of viral proliferation was found at 36 to 40 hours in striated muscle cells near the site of inocula-

FIG. 4. VSV-NJ particles budding from the plasma membranes of neuronal processes into the normally collapsed intercellular spaces of the brain of a suckling mouse 24 hours after intracerebral inoculation. The absence of interstitial edema and of normal cytoplasmic architecture indicate some neuronal resistance to the typical cytopathic effects of VSV infection. ×26,000.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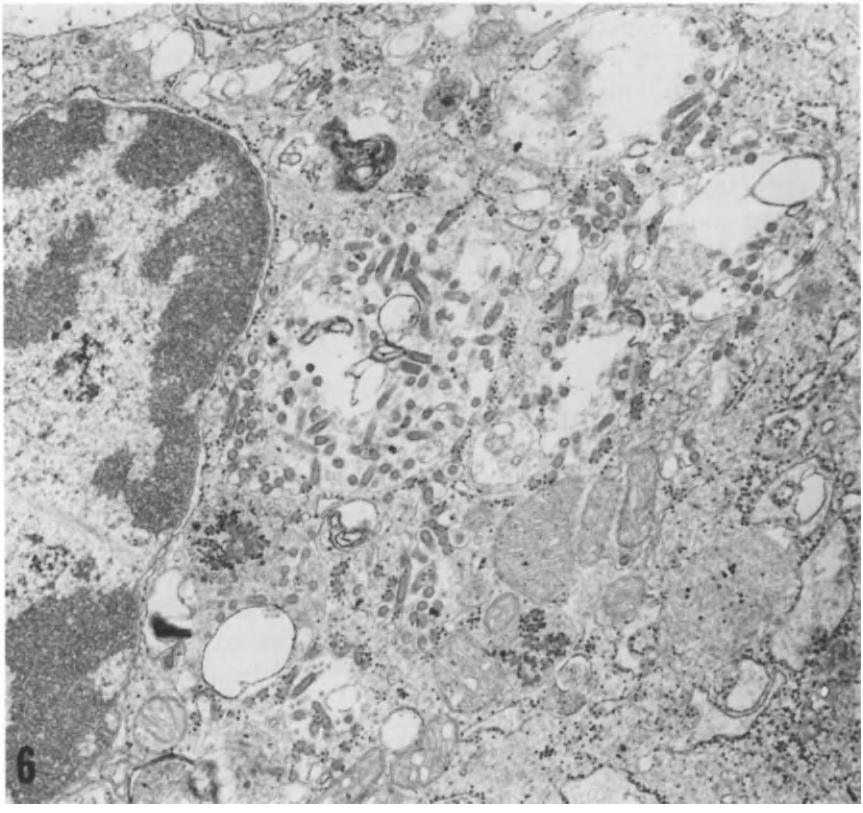
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tion (Fig. 7). (Arctic fox rabies infection progressed much slower at all stages.) Infection progressed to involve individual and focal groups of muscle cells at many distant sites. By electron microscopy, typical rhabdovirus morphogenesis was observed upon sarcoplasmic reticulum (Fig. 8) and plasma membranes, and inclusion body (massed viral ribonucleocapsid strands) formation occurred within the cytoplasm of myocytes (Fig. 9).

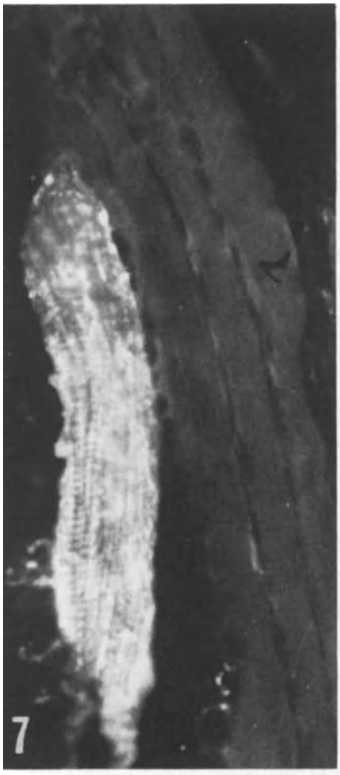
Next infected were neuromuscular spindles within the inoculated limb muscle (Fig. 10). By 48 hours these neuromuscular sensory stretch receptors contained viral antigen in their modified muscle cells and associated nerves. Peripheral nerve fluorescence started at 60 hours, at the same time as in ipsilateral spinal ganglia. Involved cells in nerves were not identifiable (Fig. 11) except by electron microscopy. This clearly showed viral maturation only in axons of nerve cells—never in supportive tissues (Fig. 12) (Jenson *et al.*, 1969). Virus particle budding was prominent upon internal membranes where axons contained organelles (near nodes of Ranvier) and in other areas only upon plasma membranes (Fig. 13). Inclusion bodies were small in axon cylinders.

Dorsal root spinal ganglia, from 60–72 hours onward, exhibited large accumulations of viral antigen in fine and aggregate form (Fig. 14). Ultrastructurally, these antigen masses were resolved as varyingly sized viral nucleocapsid inclusions with relatively few associated virus particles. Viral antigen was detected in lumbar spinal cord neurons at 60–72 hours and in the brainstem only a few hours later. The ascending infection of brain appeared to involve all parenchymal cells as the animals became sick (Fig. 15), but as in ganglia, electron microscopy indicated that only neurons and not glia contained viral products. The nucleocapsid masses and associated virus particles were found in all parts of the perikaryon and processes of neurons without any associated cytopathic changes (Fig. 16). With rare exceptions, virus particle budding in the CNS took place upon intracytoplasmic membranes. Likewise, the compact brain architecture was intact, and only in very few instances in terminal specimens were inflammatory cells found by light microscopy.

The strict neurotropism of rabies and rabies-like viruses in the CNS changed with the centrifugal spread of virus via the peripheral nervous

FIG. 5. VSV-NJ infection in suckling mouse brain at 24 hours. Neuronal infection, in few instances, was marked by formation of anomalously long virus particles (arrowhead), intracytoplasmic inclusion bodies (IB), interstitial edema (intercellular space), and cytopathic changes. ×42,000.

FIG. 6. VSV-NJ infection of a mouse brain neuron at 24 hours. This exceptional occurrence of viral particle maturation upon intracytoplasmic membranes was associated with cell destruction. ×20,000.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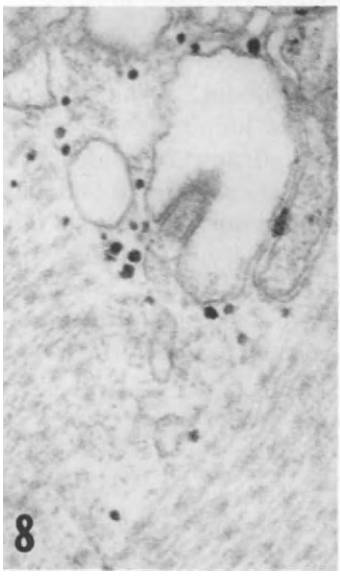
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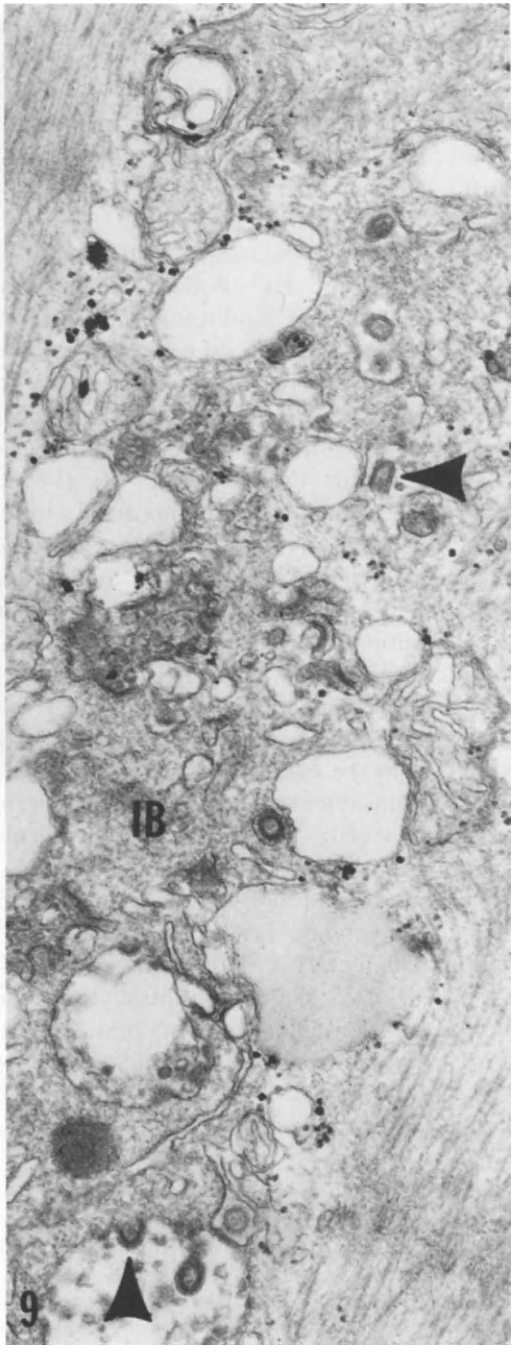
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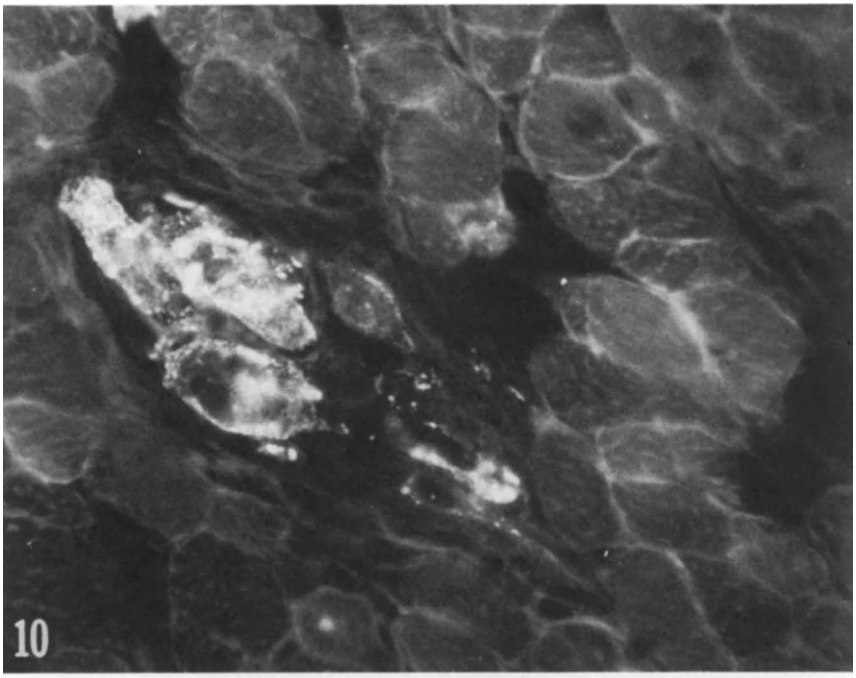
system to terminally involved target organs. Immunofluorescent antigen was contained in the retina, autonomic nerves of the intestine, adrenal gland, brown fat, pancreas, and heart. Of those tissues with a potential for yielding infectious oral secretions, salivary gland epithelium was found to be only occasionally infected (Fig. 17), but many sensory nerve endings in the oronasal cavities contained massive amounts of antigen. These included taste buds (Fig. 18), the neuroepithelium of the olfactory end organ, and the wide range of other sensory end organs of the mucosal and skin surfaces. This distribution of rabies antigen was extremely precise—other organs never contained antigen. With the exception of the relative sparing of hamster salivary gland tissue and probable dependence upon sensory end organ infection for viral transmission, rabies tropism in experimental animals is the same as that in feral animals (Debbie and Trimarchi, 1970). There is no doubt that salivary gland is the primary source of virus for transmission by bite in the species of importance in nature (Dierks *et al.*, 1969); in the absence of cytopathic changes in infected mucous acinar cells, viral budding upon luminal plasma membranes leads to efficient shedding. This predilection for plasma membranes as the site for rabies virus maturation is so similar to that of VSV in all target organs that it is the internal budding of rabies virus in neurons that must be considered exceptional.

Sites of viral maturation and kinetics of viral synthesis at each site determine, in part, the exposure of antigens to the reticuloendothelial system and, therefore, must be considered in any attempt to explain the patterns of rabies disease. Early phase neurotropism, noncytopathic infection, and minimal plasma membrane budding (except in salivary gland), would effectively delay triggering of an effective immune response. Furthermore, clinical evidence of a delayed, often mild inflammatory response (Johnson, 1965) and a typically insufficient antibody response is consistent with this point. The similar difficulty animals and man exhibit in terminating other viral infections that spread by the neural route (e.g., herpes simplex, varicella-zoster) (Dillard *et al.*, 1972; Cook and Stevens, 1973) also support the hypothesis that circumvention

FIG. 7. Rabies-like virus (Mokola) in striated muscle of a hamster at 6 days after inoculation. Immunofluorescent antigen is located between myofibrillar bundles in the sarcoplasm. This was the initial site of antigen localization in animals infected peripherally with each of five rabies and rabies-like viruses. ×600.

FIG. 8. Rabies-like virus (Mokola) in leg muscle of hamster at 6 days. The single virus particle is budding from the sarcoplasmic reticulum; virus also budded from plasma membranes of muscle cells. ×77,000.

FIG. 9. Rabies-like virus (Mokola) in a striated muscle cell at 6 days. The cytoplasm adjoining myofibrils is displaced by a viral inclusion body (IB) which is actually massed nucleocapsid material and associated virus particles (arrowheads). ×45,000.



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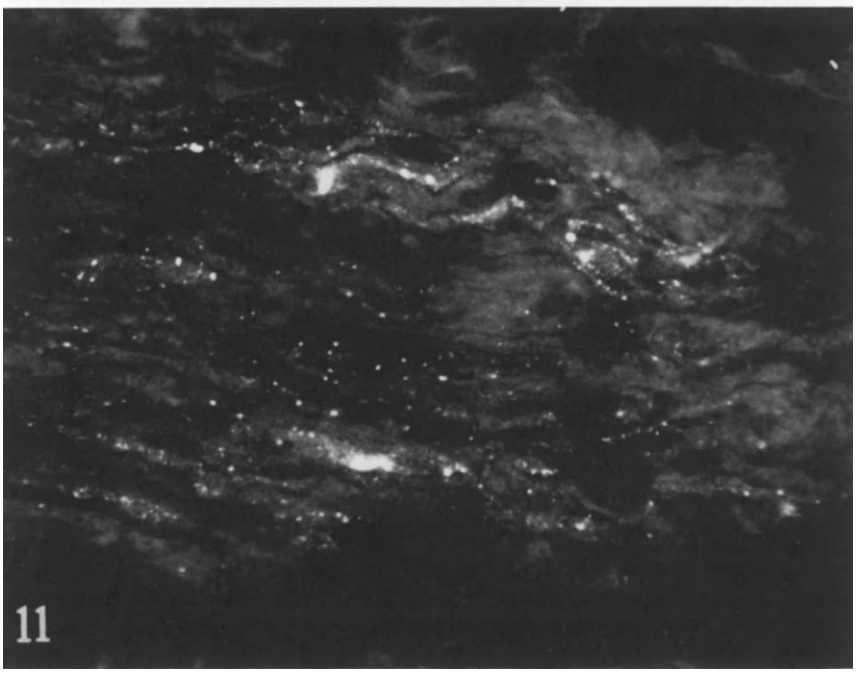

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of immune triggering by early sequestration and avoidance of the immune environment by late sequestration are evolutionarily sophisticated infection patterns that favor virus over hosts.

IV. Tropism of Other Rhabdoviruses of Animals

A subgrouping of the rhabdoviruses of animals based upon serologic cross-reactivity and on a composite of morphologic characteristics as determined from studies in our laboratory is presented as Table I. All animal rhabdoviruses known at this time are included (Knudson, 1973; Karabatsos *et al.*, 1973). Superimposition of the preferential site of virion maturation in experimentally infected animals (mice, hamsters) and known patterns of organ tropisms emphasize in only few instances precise similarities between the less studied rhabdoviruses and rabies or vesicular stomatitis viruses. Serologically related viruses do have similar sites of virion maturation and similar organ tropisms in experimental animals, but disease in nature has, in too many instances, not been studied well enough to make predictions of disease patterns in populations. Moreover, the pathogenetic patterns in individuals are in many instances moot. For example, with several viruses a neurotropism in mice is the only pathogenetic characteristic known (e.g., Kern Canyon or Klamath virus); in some cases this is because only high passage neuroadapted virus has been studied (e.g., bovine ephemeral fever virus: Murphy *et al.*, 1972). Exceptional differences between rabies and Kotonkan or Obodhiang viruses (despite a serologic relation through Mokola virus) are presently under investigation in several laboratories (Kemp *et al.*, 1973). All in all, much more comparative pathogenetic investigation of rhabdoviruses needs to be done to determine patterns and predict disease potential of obscure isolates. The recent emergence of rabies-like viruses from Africa (Shope *et al.*, 1970; Meredith, 1971) confirms the need for such investigations.

V. Evolutionary Significance of Varying Tropisms

Evolution of diverse rhabdovirus infections of animals may be proceeding toward particular host–parasite relationships in which strict

FIG. 10. Rabies virus (vampire bat isolate) in a neuromuscular spindle within an inoculated leg muscle of a hamster at 4 days. These deep sensory end organs in inoculated limbs were infected after nearby muscle cells, but before evidence of neural spread toward the CNS. ×1000.

FIG. 11. Rabies virus (CVS strain) in a peripheral nerve of a hamster at 4 days. It was not possible to identify the tropism of rabies in nerves by immunofluorescence. ×600.

TABLE I
Rhabdoviruses of Animals[a,b] (Infection Patterns)

Serologic grouping[c]		Virus	Particle length	*In vivo* site of budding[d]	Organ tropism[d]	Disease pattern[d]
A	1	Vesicular stomatitis-Indiana (prototype)	178	PM	E/V/N	Ex
	2	Cocal (VSV-Argentina)	188	PM	E/V/N	Ex
	3	Alagoas (VSV-Brazil)	–	–	–	Ex
	4	Vesicular stomatitis-New Jersey	188	PM	E/V/N	Ex
	5	Piry	188	PM	V/N	–
	6	Chandipura (and IbAn 9978)	180	PM	E/V/N	Ex
B	1	Rabies	180	ER/PM	N/E	S
	2	Lagos bat	180	ER/PM	N/E	–
	3	Mokola (IbAn 27377)	180	ER/PM	N/E	–
	4	Nigerian horse (H 28)	–	–	–	–
	5	Duvenhage	180	–	–	–
	6	Kotonkan (IbAr 23380)	180	PM	N	–
	7	Obodhiang (SudAr 1154–64)	180	PM	N	–
C		Bovine ephemeral fever	185	PM	E/N	Ex
D		Kern canyon	132	PM	N	–
E		Klamath (M 1056)	167	ER	N	–
F		I 6235	149	PM	N	–
G	1	Flanders	218	PM	N	–
	2	Hart Park	218	PM	N	–
H		Mount Elgon bat	230	PM	N	–
I		Joinjakaka (MK 7937)	197	PM	N	–
J		Navarro (Col. Cali 874)	220	ER/PM	N	–
K		Kwatta (Tr 58603)	230	ER/PM	N	–
L		Mossuril	–	–	–	–
?M		Marburg	665n	PM	V	–

[a] Horizontal lines distinguish morphological subgroupings based upon virus particle length, shape, and morphogenetic characteristics.

[b] Mention of previously undescribed viruses in this table is not intended to constitute priority publication.

[c] Letters distinguish independent serologic subgroups and numbers the individual member viruses of these subgroups.

[d] PM, plasma membrane; ER, endoplasmic reticulum; E, epitheliotropic; V, viscerotropic; N, neurotropic; Ex, explosive epizootic; S, slowly epizootic–appearing enzootic.

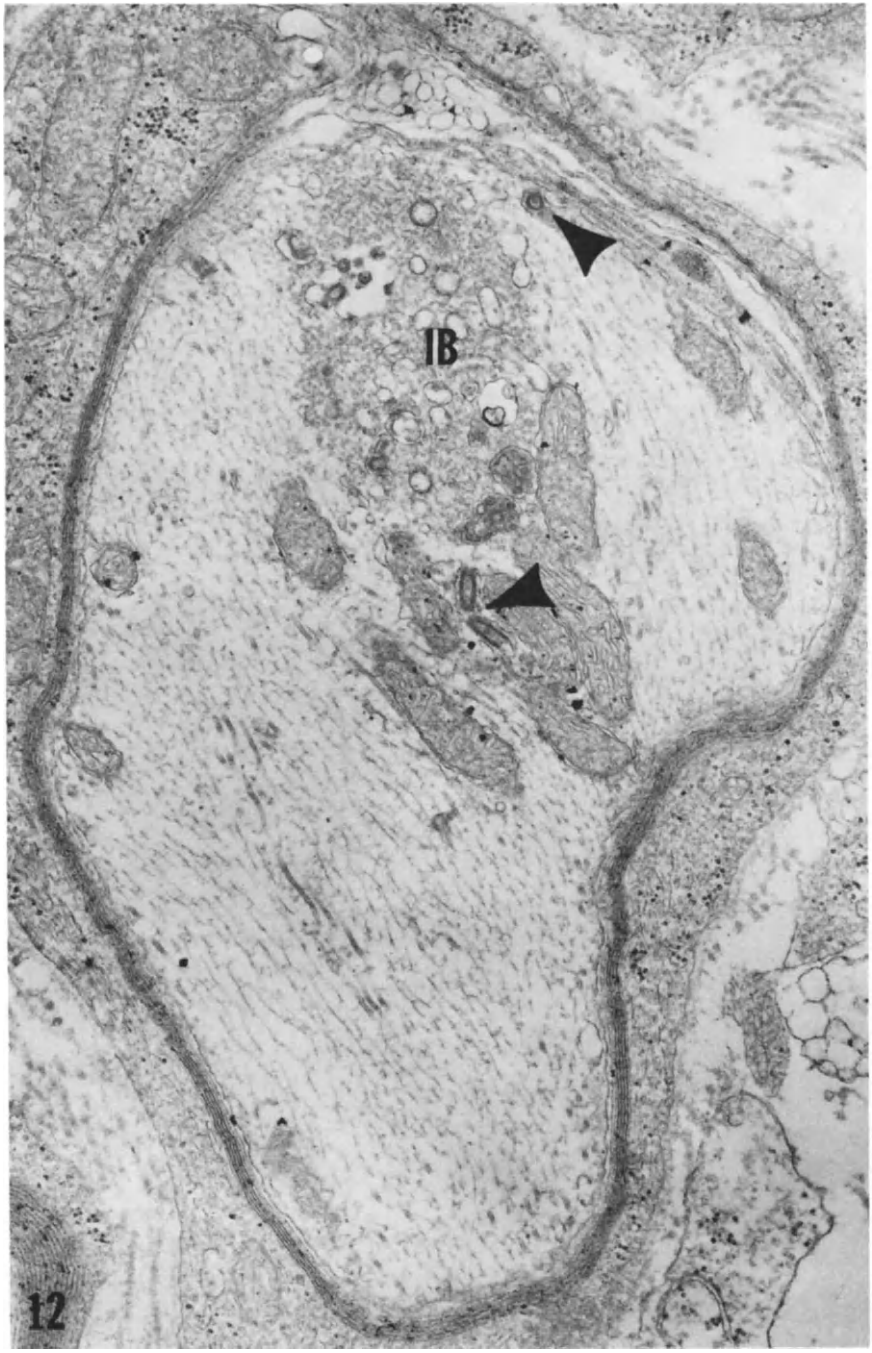
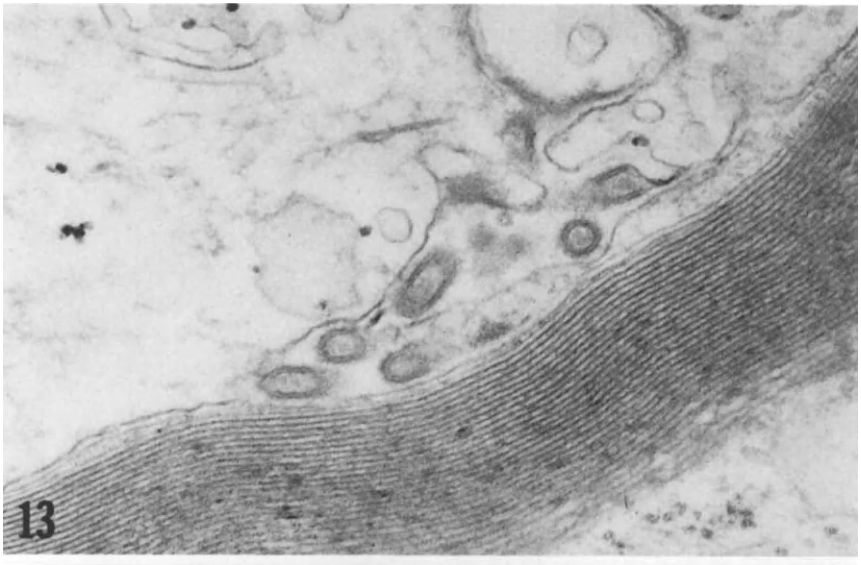


FIG. 12. Rabies virus (vampire bat isolate) in a myelinated nerve of a hamster at 6 days. Viral inclusion bodies (IB) were contained in nerve cell axoplasm, and virus particles (arrowheads) budded upon axonal membranes only; Schwann cells were never infected. ×32,000.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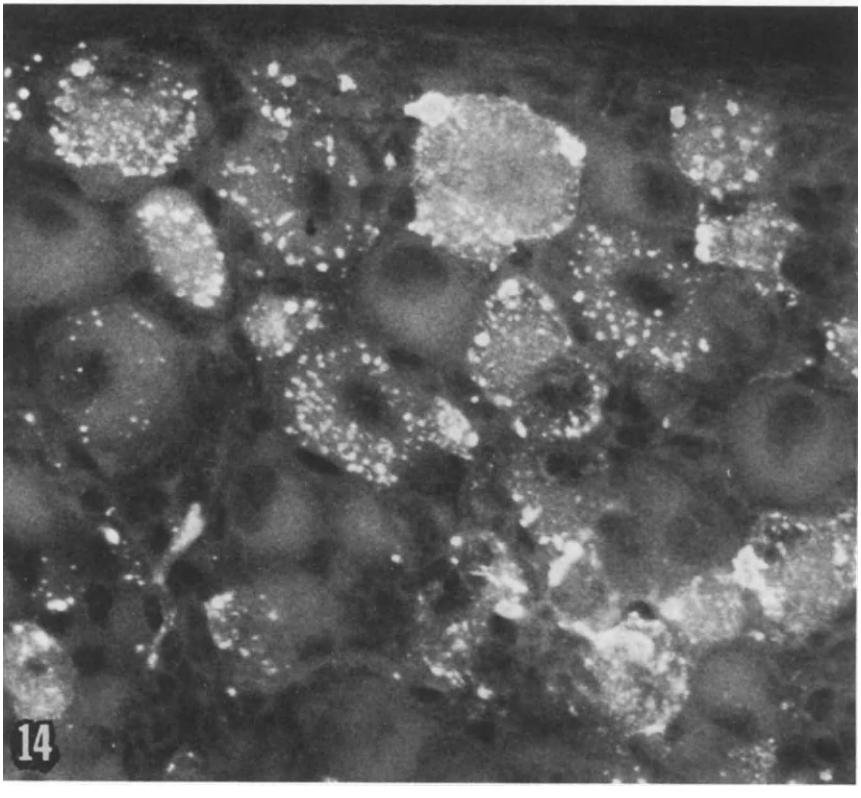
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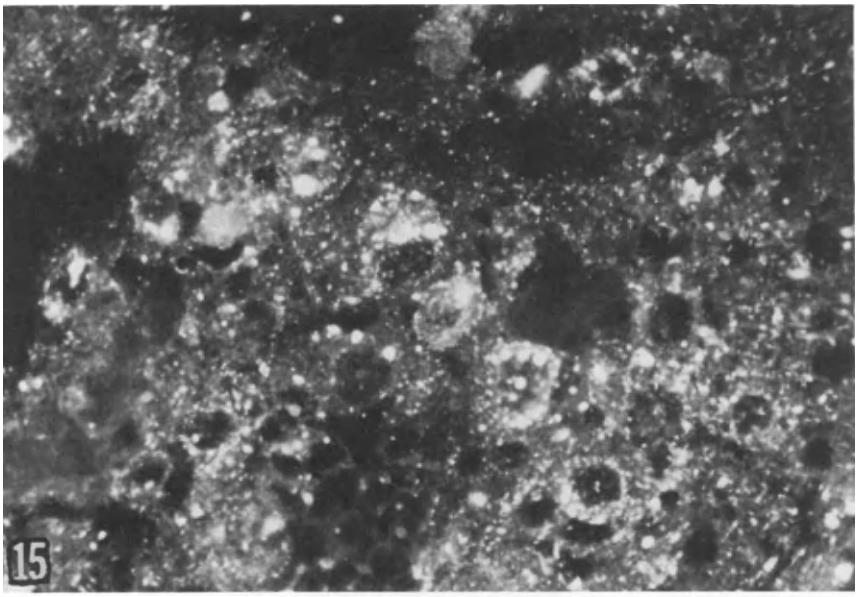
neurotropism favors the virus at the expense of the host. In considering this matter, however, a bias in the point of view of medical and veterinary sciences must be recognized. We focus on the pathogenetic characteristics of those viruses that still cause serious disease problems—the examples of failures of professional intervention in individuals (vaccines, chemotherapeutics, etc.) or populations (quarantine, eradication programs, etc.). Pursuing this bias, we conclude that, rather than failure of research effort, it is the sophistication of infection patterns which is responsible for the refractoriness of these viral diseases. Infections that circumvent immune responsiveness are, in this sense, good examples of such sophistication. It is tempting—and the basis of this chapter—to continue this bias and argue that the observed spectrum of rhabdovirus viscerotropism-neurotropism may be taken as evidence of an evolutionary progression based upon selection of variant viruses that induce insufficient immune response. Rapid immune response is the key to much disease "control" in nature and, of course, the key to most control instigated by man.

The fatal outcome of most rabies infections (and VSV infections of newborns of many species) may be a factor in viral survival and spread. By removing individuals from a niche, which is then quickly refilled by susceptibles, lethality might be considered to be of evolutionary survival advantage. In contrast, virus survival after a nonlethal epidemic-epizootic (with its consequent immunization of the population) might be more difficult. The relative merits of lethality versus nonlethality of infection might also vary with the turnover rate of animal host populations. If rabies has evolved into a highly lethal infection from a more temperate one, then the survival advantage of this evolutionary direction needs to be considered in predicting the pathogenic potential of other viruses.

Participation of an arthropod vector in the transmission of some rhabdoviruses (Tesh *et al.*, 1972) may be most significant in long distance movement of virus and only complementary to rapid, direct spread among contact animals (Jonkers, 1967). Persistence of infection for the life span of the arthropod and multiple transmissions to animals would

FIG. 13. Rabies-like virus (Mokola) particles lying within the intercellular space between an axon and its myelin sheath. Along most of the length of nerves, where axons have few membranous organelles, virus budded primarily from plasma membranes into this space. ×66,000.

FIG. 14. Rabies-like virus (Lagos bat) in a lumbar dorsal root ganglion of a hamster at 6 days. The cell bodies of the large ganglionic neurons, but not their satellite cells, accumulated massive amounts of viral antigen after the centripetal neural movement of virus to the spinal cord. ×400.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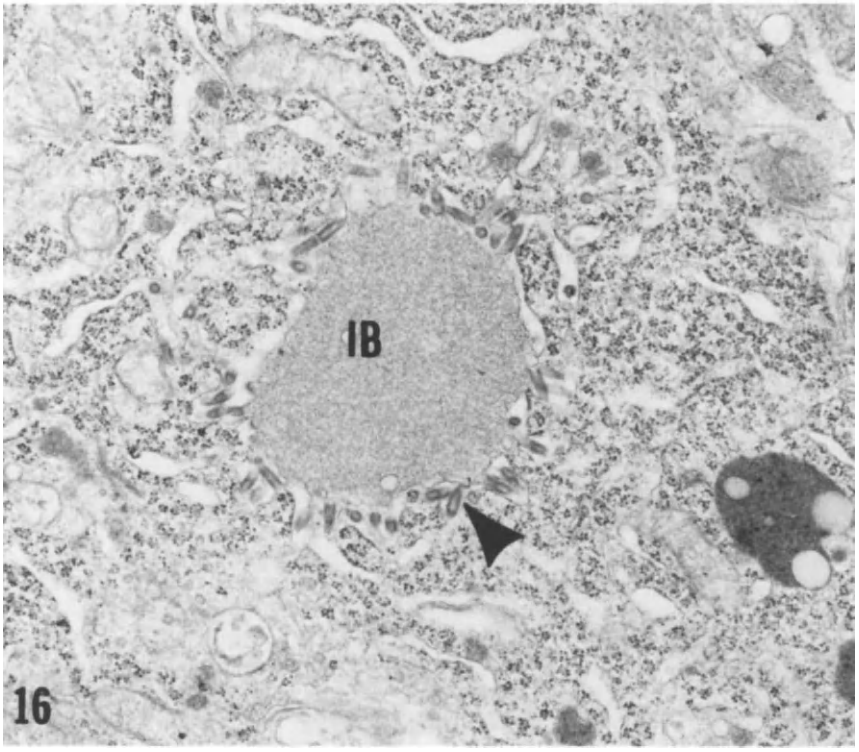
IB
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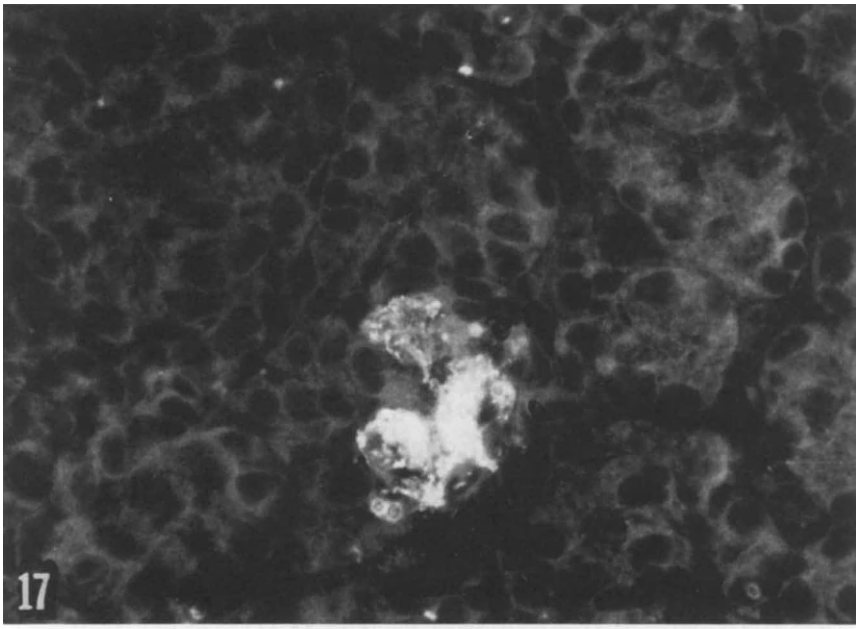
be clearly advantageous to the survival of the virus (Bergold *et al.*, 1968); transovarial transmission of virus to new generations of arthropods would further this advantage (Tesh *et al.*, 1972). An arbovirus must cause viremia in its vertebrate host in order to provide its arthropod host with an infected blood meal; the neural pathway of rabies virus in its vertebrate host precludes any possibility of arthropod involvement. Thus, VSV and rabies viruses seem to have evolved very different means of "survival insurance"—an arbovirus cycle and the potential for a protracted incubation in mammalian hosts, respectively. Unifying evolutionary concepts on this point seem moot—both seem to be sophisticated evolutionary derivatives and little may be added from knowledge of viremia levels or arthropod susceptibility of other rhabdoviruses.

All variations associated with improved capacity for transmission from one host to another must have had central roles in viral evolution. The capacity for transmission of rabies by bite depends on the interaction of the infection in two organs, the brain and the salivary gland. Referring to the brain, Johnson has reflected: "The greater localization to limbic system with relative sparing of neocortex provides a fascinating clinicopathologic correlate with the alertness, loss of natural timidity, aberrant sexual behavior and aggressiveness that may occur in clinical rabies. No other virus is so diabolically adapted to selective neuronal populations that it can drive the host in fury to transmit the virus to another host animal" (Johnson, 1971). This precise neuronal predilection occurs in species involved in rabies transmission in nature, but not in laboratory species where neurons in all parts of the brain become infected and there is no furious phase of clinical disease. In the salivary gland, large amounts of virus are shed freely into saliva because rabies virus buds from the plasma membranes of infected mucogenic acinar cells (Dierks *et al.*, 1969). The exclusive orientation of this budding upon luminal plasma membranes may represent another evolutionary sophistication with the purpose of protecting virus from antibody. Late in rabies infection when antibody is often formed in high titer, the intact basement membrane and salivary acinar structure would

FIG. 15. Rabies-like virus (Lagos bat) in the midbrain of a hamster at 6 days. The progressive accumulation of antigen of each of the five rabies and rabies-like viruses in hamster brain was striking, but the strict neurotropism of brain infection was not evident by immunofluorescence. ×400.

FIG. 16. Rabies-like virus (Lagos bat) in hippocampus of a hamster at 6 days. Despite the nearby ubiquitous occurrence of multiple viral inclusion bodies (IB) with associated virus particles (arrowhead) in brain neurons, cell architecture remained essentially normal, even in dying animals. ×22,000.



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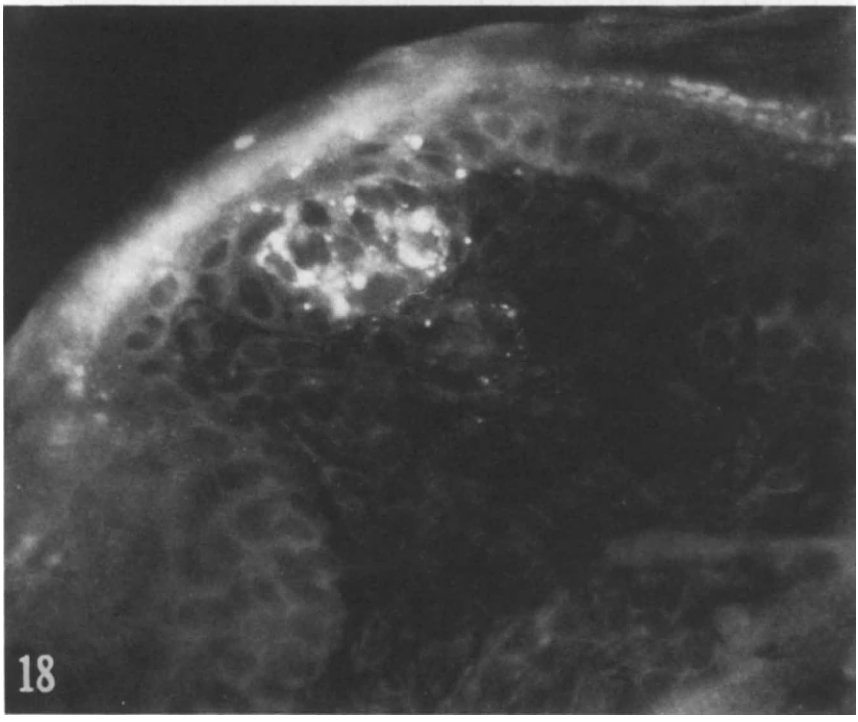

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serve to sequester virus in saliva. Salivary secretions could thereby remain more highly infectious for a longer time; this sequestration is not complete as evidenced by the usual terminal decline in saliva titer in moribund animals and man (G. Baer, personal communication). Because the transmission efficiency of rabies virus is favored by (1) the neurotropism resulting in furious clinical disease and (2) the shedding of virus in saliva throughout the course of this clinical disease, the development of both characteristics may be considered as evolutionary derivatives. Since neither would offer clear survival advantage without the other, the two characteristics might be considered as parallel, interacting or integrated derivatives—as such their coincident evolutionary development is all the more remarkable.

Particular viral tropisms portend varying severity of disease and ultimate outcome. Neurotropism, with its capacity for critical functional loss of nondividing cells, is generally a most serious viral characteristic and associated with fatality in many instances (Johnson and Mims, 1968). If the central premise of this chapter is correct, that evolution toward neurotropism offers survival advantages to viruses by minimizing the efficacy of early immune responsiveness, then an intriguing question may be asked. Is rabies virus (like herpes simplex, varicella-zoster, and Measles-SSPE) an evolutionary forerunner of neurotropic variants, which may emerge from other pathogenic viruses?

ACKNOWLEDGMENT

The collaboration of Alyne K. Harrison, Washington C. Winn, Jr., and Sally P. Bauer in the experimental studies carried out in this laboratory is gratefully acknowledged.

FIG. 17. Rabies virus (vampire bat isolate) in several contiguous mucous cells of a single acinus in the salivary gland of a hamster at 6 days. Typically, salivary glands were not infected or contained very few infected cells in hamsters and mice infected with rabies or rabies-like viruses; this contrasts with the massive salivary gland infection of foxes, skunks, and dogs. ×600.

FIG. 18. Rabies-like virus (Mokola) in a taste bud on the tongue of a hamster at 7 days. In hamsters and mice the source of virus in the infectious oral secretions necessary for transmission via bite is most likely the diverse sensory nervous end organs of the oronasal cavity; many types of these were infected with the rabies and rabies-like viruses studied. Cornified epithelium is autofluorescent. ×600.

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CHAPTER 27

The Biological Activity of Heterogeneous Particle Types of Plant Viruses

ROBERT W. FULTON

The desire to obtain homogeneity in purified preparations of plant viruses originated from the need to associate infectivity with a specific kind of particle. Heterogeneity, fortunately, was recognized as possible in purified preparations. Rice *et al.* (1955) and Steere (1959) pointed out

the possibility that more than one kind of particle may be involved in virus infection.

The type of heterogeneity in plant viruses that is of particular interest is that which results in particles differing in sedimentation rate, or in buoyant density. This is associated in most instances with differences in nucleic acid content. Electrophoretic heterogeneity is known for a number of plant viruses (Bancroft, 1962, 1968b), but this apparently reflects differences in coat protein or its assembly.

It is the differences in genetic complement that make viruses with heterogeneous particles of particular interest. Most such viruses that have been adequately tested require, for replication, genetic material from two or more complementary particles. Obviously this will permit reassortment of genetic material and greatly increase the probability of variation. The complexity of the infection process is thus greater than with viruses existing as homogeneous groups of particles. The present review attempts to describe recent work on the nature of differences among particles of certain viruses and their effect on biological properties of the viruses. Recent reviews of work with multiparticulate viruses, or with specific viruses, have been made by Bancroft (1968a), Hull (1969), Kassanis (1968), Lister (1969), Sänger (1968b), and Van Kammen (1972).

I. Terminology

Various terms have been used to describe viruses existing as a group of heterogeneous particles. The term "defective" was used by Bancroft (1968a) in the sense that one kind of particle was unable to infect alone, but depended on another kind of particle for a complete set of factors for replication. "Dependence" also is a characteristic of satellite viruses, which multiply only in conjunction with a specific virus. These will not be considered in this chapter. The term "defective" does not seem particularly appropriate now that it has become apparent that heterogeneity may well confer an advantage on a virus, rather than constituting a defect.

"Multicomponent" is the term most commonly used to refer to viruses with heterogeneous particle populations. This seems somewhat less desirable than the term "multiparticulate" because of prior usage of the word "component" in relation to the protein and nucleic acid parts of viruses.

Van Kammen (1972) has used the term "divided genome" to refer to multiparticulate viruses. This emphasizes a fundamental difference among particles of such viruses. The term should not be assumed to imply, however, that no duplication of genetic material exists among or

between differing particle types. Although evidence for cowpea mosaic virus suggests that there is little or no redundance (Van Griensven *et al.*, 1973), evidence for some other viruses suggests that there may be redundance among particle types.

Van der Want (1969) questioned the suitability of the term "virion" in relation to multiparticulate viruses because a single particle does not represent the total potentiality of these viruses. The definition of Luria and Darnell (1967), however, does not seem to exclude viruses with more than one kind of particle, each of which contains only a part of the viral genome.

The differing particles are most often referred to as "top" (T), "middle" (M), and "bottom" (B) in reference to their position after centrifugation in density gradient columns. The terms are not completely satisfactory, but no better are at hand. Top particles of some multiparticulate viruses contain no nucleic acid and thus differ functionally from top particles which do contain nucleic acid. Another disadvantage of the terms is that it becomes awkward to designate more than three particle types. Additional types occur, and these have been referred to by letter subdivisions of one or other term.

Particle types of tobacco streak virus were referred to originally as T (top), a (middle), and b (bottom) (Fulton, 1970) because minor particle types were apparent, some of which sedimented less rapidly than top and some more rapidly than bottom particles. No biological activity could be demonstrated for these, however, and uniformity in terminology with other multiparticulate viruses seemed desirable, so the terms top, middle, and bottom have been used recently.

When two nucleoprotein particle types exist for a virus these are commonly designated middle and bottom, the assumption being made that the virus lacks a top particle type, which often contain no nucleic acid. The two particle types of tobacco rattle virus have been designated L (long) and S (short), based on apparent size differences.

The word "cross" has come to be used to designate the technique of mixing different particle types to produce "hybrid" clones of virus presumed or proven to result from the cooperation and replication of particle types from two or more parental virus clones. This has usually involved inoculating mixtures diluted appropriately so that any lesion resulting has a high probability of having originated from the cooperation of heterologous particles. Transfers from single lesions perpetuate the virus clones. Hybrid clones have also been originated by inoculating dilute mixtures to a host which becomes systemically infected. This method suffers a disadvantage in that the virus clone found may be the dominant one in a host systemically infected by more than one kind of hybrid virus clone.

II. The Basis of Heterogeneity

Heterogeneity in sedimentation characteristics occurs in several taxonomically distinct groups of viruses (Table I). It is a characteristic of the tobravirus group (tobacco rattle and pea early browning viruses), which exist as thick (22–25 nm), straight rods predominantly of two lengths (Harrison and Woods, 1966; Harrison, 1970). A third, very short particle occurs with some isolates, but appears to have no biological significance and occurs only in small amounts (Lister, 1969). Three zones thus may appear upon density gradient centrifugation, the rate of sedimentation being related to particle length. The functional particles are referred to as long (L) and short (S), corresponding functionally to bottom and middle particles of viruses not exhibiting apparent morphological differences.

The lengths of L particles of tobravirus isolates are all close to 190 nm. The shorter particles range, with different isolates, from 45 nm for the nonfunctional top particle to 55–114 nm for S (short) particles. While the length of S particles may differ among strains, biologically different isolates may have S particles of the same length. Isolates may occur appearing to have two kinds of S particles, but such isolates are separable, by local lesion isolation, into two strains, each with an S particle of a different length (Semancik, 1966).

Alfalfa mosaic virus also exists as a group of morphologically different particles. Particle width is uniformly about 18 nm; lengths of the round ended rods are 58 nm for bottom (B); 49 nm for middle (M); 38 nm for top b (Tb); 29 nm for top a (Ta); and 19 nm for top z (Tz). Another top component, To, has been described (Hull, 1969); it and Tz appear to have no function. Hull (1969) also describes two additional components, present in old infections, which sediment more rapidly than B. They are present in only small amounts, and their significance is not known. Although there are conflicting reports (Kelley and Kaesberg, 1962; Bancroft and Kaesberg, 1960), evidence indicates that the major particle types each contain about 18% RNA (Hull *et al.*, 1969b). As with tobacco rattle virus, differences in sedimentation rates reflect differences in size of the major particles.

Another multiparticulate group of viruses is the comovirus group (cowpea mosaic, bean pod mottle, broad bean stain, radish mosaic, red clover mottle, and squash mosaic viruses) (Van Kammen, 1972). These are small, isometric viruses, particles of which sediment at different rates but do not differ in size. Top particles characteristically contain no nucleic acid and have a sedimentation coefficient about 0.8 that of the corresponding bottom particles.

Two viruses in the group, radish mosaic (Campbell, 1964) and true broadbean mosaic (Paul, 1963) have been reported to produce no top particles. Certain strains of some of the other viruses in the group also produce no top particles (Bruening, 1969; Valenta and Marcinka, 1968).

The nucleic acid content of the particle types of the comovirus group is significantly different. M particles contain only about 25% RNA, while B particles contain about 35%. Thus in this group of multiparticulate viruses differences in sedimentation rates occur because of differences in weights of the particles rather than differences in size.

NEPO viruses are another group of isometric multiparticulate viruses of uniform diameter (28–30 nm) with two or three particle types. Most members of the group produce a top particle lacking nucleic acid and a bottom particle containing, as far as investigated, 38–40% nucleic acid. M particles have been described for most of the viruses, and these have, where measured, 28–31% nucleic acid. Tomato ringspot virus apparently produces no M particles, while grapevine chrome mosaic virus produces only M and B particles. Heterogeneity among the NEPO viruses is due to different amounts of nucleic acid in various particles, which are not reflected in differences in size.

The bromovirus group (brome mosaic, broadbean mottle, and cowpea chlorotic mottle viruses) do not show appreciable heterogeneity upon analytical or sucrose density gradient centrifugation under ionic and pH conditions in which they are stable. Brome mosaic virus, however, when subjected to buoyant density centrifugation in CsCl solutions was heterogeneous (Lane and Kaesberg, 1971), and the heterogeneity was associated with differences in nucleic acid content. Similar heterogeneity has been demonstrated for cowpea chlorotic mottle (Bancroft, 1971; Bancroft and Flack, 1972) and broadbean mottle viruses (Aronson and Bancroft, 1962). Fowlks and Young (1970) reported that broadbean mottle virus contained RNA strands of three sizes, 1.1, 1.0, and 0.3×10^6 daltons.

A number of viruses, not at present taxonomically grouped, also are multiparticulate (Table I). Tobacco streak virus exists as three main kinds of nucleoprotein particles; the nucleic acid content of each particle is near 14%. Differences in sedimentation rates result from differences in size according to Lister *et al.* (1972), who found modal values of 28, 30, and 35 nm for T, M, and B particles. Presumably these differences reflect structural alterations conditioned by relatively small differences in nucleic acid content. Similar size differences have been reported for the two particle types of the isometric pea enation mosaic virus (Hull and Lane, 1973).

Size differences have been reported also for apple mosaic virus par-

TABLE I

Viruses Reported as Being Multiparticulate

Virus	Particle types (No.)	Sedimentation coefficients of particles (S)	Infectivity enhancement	References
Alfalfa mosaic	5	53, 68, 76, 88, 99	Yes	Van Vloten-Doting *et al.*, 1970
Apple mosaic	3	88, (103), 117	?	De Sequeira, 1967; Fulton, 1967b
Arabis mosaic	3	53[a], 93, 126	?	Mayo *et al.*, 1971
Barley stripe mosaic	3	180, 189, 199	Yes[b]	Jackson *et al.*, 1972
Bean pod mottle	3	54[a], 91, 112	Yes	Bancroft, 1962
Belladonna mottle	2	53, 113[c]	No	Paul, 1969
Black raspberry latent	3	81, 89, 98	?	Converse and Lister, 1969
Broadbean mottle	2[c]	*d*	Yes	Fowlks and Young, 1970; Hull, 1972
Broadbean stain	3	60[a], 100, 127	?	Gibbs *et al.*, 1968
Broadbean wilt	3	63[a], 100, 126	?	Taylor *et al.*, 1968
Brome mosaic	3[c]	*d*	Yes	Lane and Kaesberg, 1971
Cacao yellow mosaic	2	49[a], 108	?	Brunt, 1970
Carnation mottle	2			Waterworth and Kaper, 1972
Cherry leafroll	3	52[a], 114, 132	Yes	Jones and Mayo, 1972b; Walkey *et al.*, 1973
Chichory yellow mottle	3		?	Voulas and Martelli, 1971
Cocoa necrosis	2	101, 129	?	Kenten, 1972
Cowpea chlorotic mottle	3	*d*	Yes[b]	Fowlks and Young, 1970
Cowpea mosaic	3	58[a], 95, 115	Yes	Van Kammen, 1968
Cucumber necrosis	2	50, 136	No	Tremaine, 1972
Cucumber mosaic	2	*d*	Yes[b]	Lot and Kaper, 1973; Peden and Symons, 1973; Kaper *et al.*, 1965
Elderberry latent	2	48, 112	?	Jones, 1972a
Echtes Ackerbohnemosaik	2	98, 119	?	Paul, 1963
Elm mottle	2		?	Jones, 1972b
Grapevine chrome mosaic	2	92, 117	?	Martelli and Quacquarelli, 1972

Pea early browning	2	(87.5 nm, 225 nm)	*e*	Lister, 1968; Huttinga, 1969
Pea enation mosaic	2	99, 112	Yes	Bozarth and Chow, 1966; Gonsalves and Shepherd, 1972; Hull and Lane, 1973
Plum line pattern	2		?	Paulsen and Fulton, 1969
Prune dwarf	2		?	R. W. Fulton (unpublished)
Prunus necrotic ringspot	3	72, 95, 117	Yes	Tolin, 1965; L. S. Loesch (unpublished)
Quailpea mosaic	3	57, 93, 112	?	Moore, 1973
Radish mosaic	3	57[a], 97, 116	Yes	Campbell, 1973
Raspberry bushy dwarf	2	111, 116	?	Barnett and Murant, 1970
Raspberry ringspot	3	52[a], 92, 130	Yes	Murant *et al.*, 1972
Red clover mottle	3	60, 101, 127	Yes	Valenta and Marcinka, 1968; Mahmood *et al.*, 1972
Scrophularia mottle	2	54, 116		Bercks *et al.*, 1971
Squash mosaic	3	57, 95, 118	?	Mazzone *et al.*, 1962; Lastra and Munz, 1969
Strawberry latent ringspot	2	49, 130	?	Richter and Proll, 1970
Tobacco rattle	2	155–243, 300	*e*	Lister, 1966; Frost *et al.*, 1967; Sänger, 1968a
Tobacco ringspot	3	53[a], 91, 126	(Yes)	Stace-Smith, 1970
Tobacco streak	3	90, 100, 113	Yes	Lister and Bancroft, 1970
Tomato black ring	3	55[a], 97, 121	?	Murant, 1970
Tomato ringspot	2	55[a], 128	?	Stace-Smith, 1966
Tomato top necrosis	3	52[a], 102, 126	Yes	Bancroft, 1968b
Tulare apple mosaic	3		Yes	R. W. Fulton (unpublished)
Turnip yellow mosaic	5	54[a], 117[f]	No	Faed *et al.*, 1972; Matthews, 1959, 1960
Wild cucumber mosaic	3	53[a], 62.5, 119	?	Yamazaki and Kaesberg, 1960

[a] Protein only.
[b] By mixing RNA's.
[c] Density heterogeneity.
[d] RNA is heterogeneous.
[e] Cooperate to produce complete virus.
[f] Minor particles separable by buoyant density differences.

ticles with different sedimentation rates (De Sequeira, 1967). Such differences may well exist among particles of other viruses with heterogeneous sedimentation rates, but these have not been thoroughly investigated.

For a number of other viruses not yet taxonomically grouped (Table I), two or three particle types have been reported. It may be suspected that some reports of two particle types will eventually be corrected to three or more with longer times of centrifugation and better methods for detecting and collecting zones in gradient tubes.

On the other hand, it may be that the multiparticulate viruses investigated so far are merely those with differences sufficiently large that particles sediment at different rates, or have different buoyant densities. There would seem to be no reason why particle heterogeneity could not involve qualitative differences in nucleic acid content without quantitative differences detectable by present techniques.

III. Techniques of Particle Separation

The conventional method of separating heterogeneous particles has been by rate density gradient centrifugation in sucrose columns. Visible zones can be removed from swing-out tubes by the somewhat inefficient technique of inserting a needle into or slightly below the zone and withdrawing material. Collection may involve puncturing the side or bottom of the tube, or it may be done by lowering a needle with a U-shaped tip from the top of the tube. The use of a fractionating apparatus which moves the entire contents of the tube slowly up through a UV scanner permits collecting zones not visible otherwise and is considerably more efficient than collecting through a needle.

The differences in sedimentation rates of different particle types may be rather small, which results in zones which overlap, so that complete separation is not possible in one centrifugation run. Virus collected from gradient tubes must be concentrated by high speed centrifugation and the density gradient centrifugation repeated. Two to four successive cycles of density gradient centrifugation have been used to obtain reasonably pure preparations of particle types of a number of viruses (Bol *et al.*, 1971; Fulton, 1970; Van Kammen, 1968).

The relatively low capacity of swinging bucket tubes has led to the use of zonal rotors capable of handling much more material. Van Kammen (1968) and others have used such rotors for preliminary separation of particles, which were then further purified by centrifugation in rate zonal tubes or by equilibrium zonal centrifugation. Van Kammen and Van

Griensven (1970) reported that M particles of cowpea mosaic virus were obtained free of infectivity after two runs in a zonal rotor. B particles, however, required equilibrium density gradient centrifugation in 40% CsCl to eliminate M particles.

Centrifugation to equilibrium in salt solutions, such as CsCl, separates particles on the basis of differences in buoyant densities rather than differences in sedimentation velocities. Particles with sedimentation rates too close for separation by rate zonal centrifugation can be separated in this way (Bruening, 1969; Hull, 1972). Heterogeneity in brome mosaic virus was demonstrated in this way (Lane and Kaesberg, 1971); the virus sediments as a single particle type in analytical centrifugation. It should be noted, however, that particles of different sizes may have the same buoyant density, as shown by Semancik (1966) and Cooper and Mayo (1972) for tobacco rattle virus.

Desjardins and Steere (1969) recommended combined density gradient and differential centrifugation as being more efficient in separating particles of alfalfa mosaic virus than density gradient centrifugation alone.

Other differences in properties of virus particles have been exploited in separating them. The larger particles of alfalfa mosaic virus (B, M, and Tb, but not Ta) precipitate in the presence of Mg^{2+} (0.05 *M*) (Kelley and Kaesberg, 1962), and this has been used as a preliminary step to remove Ta particles from a preparation (Gillaspie and Bancroft, 1965a; Moed and Veldstra, 1968; Van Vloten-Doting and Jaspars, 1967; Hull *et al.*, 1969b; Majorana, 1968). Other divalent cations (Sr^{2+}, Ca^{2+}) also precipitate the larger alfalfa mosaic virus particles (Hull, 1969).

Polyethylene glycol (PEG 6000) has been used as a precipitant, in the presence of NaCl, for many viruses. Clark (1968) used the differential response to PEG of different size particles of alfalfa mosaic virus to separate them. Precipitated virus was incorporated in a Kieselguhr column which was washed with decreasing concentrations of PEG. Smallest particles were eluted first, largest last.

A somewhat different application involved gradients of PEG concentrations decreasing from top to bottom of centrifuge tubes (Clark, 1970; Clark and Lister, 1971a). The PEG gradients were stabilized by gradients of sucrose increasing in concentration from the top to the bottom of the tubes. Precipitated virus, layered on the tubes, sedimented upon low speed centrifugation until reaching a PEG concentration at which a specific particle type was soluble. It then remained at that position in a discrete zone.

Inverse PEG gradients have given zones with tobacco streak virus in our laboratory that appeared sufficiently well separated so that there was

no overlapping. When material from single zones was centrifuged on conventional sucrose gradients, however, it contained a higher proportion of heterologous particles than zones derived from sucrose gradients. Hull and Lane (1973) could not separate the two particle types of pea enation mosaic virus with this technique.

Differential precipitation by PEG was used by Huttinga (1973) to separate L and S particles of tobacco rattle virus. The L particles precipitated at 3% PEG and 0.1 *M* NaCl, leaving S particles in suspension.

A chromatographic purification method was described by Rialdi and Zunino (1969) in which virus was retained by a column of magnesium pyrophosphate and Kieselguhr and then eluted by an increasing concentration of phosphate buffer. Sequential elution of short and long particles of TMV suggests that the method could be adapted to virus particle separation where size differences exist.

IV. Variation in Proportion of Particle Types

It has been a common observation that the proportion of T, M, and B particles varied among different virus isolates (Bruening, 1969; Debrot, 1964; de Jager and Van Kammen, 1970; Fulton, 1967a,b; Harrison and Woods, 1966; Matsumoto and Murayama, 1971; Paul, 1963; Schneider and Diener, 1966; Schwenck *et al.*, 1971; Wood, 1972). The proportion of particle types was reported not to vary with time with bean pod mottle virus (Gillaspie and Bancroft, 1965b), cherry leafroll virus (Jones and Mayo, 1972a), or tobacco streak virus (Fulton, 1967a). It was also shown with tobacco streak virus that host, temperature during virus increase, or symptom pattern did not affect the proportion of particle types. Differences in proportion of top particles of different strains of cowpea mosaic virus are genetically controlled characteristics (Bruening, 1969; De Jager and Van Kammen, 1970).

Despite the consistent association of characteristic particle ratios with specific strains of many multiparticulate viruses, variations in ratios associated with a variety of factors have been described. Schneider and Diener (1966) found that the proportion of M particles of tobacco ringspot virus decreased between 2.5 and 7 days after infection. Ladipo and De Zoeten (1972) found that particle type ratios of this virus varied with host and with season of the year. A similar decrease in proportion of M particles of alfalfa mosaic virus was reported by Matsumoto and Murayama (1971). A decrease in proportion of S particles of tobacco rattle virus with time was reported by Semancik and Kajiyama (1967). A decrease in the proportion of T particles of tobacco streak virus was

described by Fulton and Potter (1971) for a time period of 24 to 60 hours after inoculation.

For a number of viruses the host of propagation seems not to affect the ratio of particle types. One strain of tobacco streak virus, however, produced less T particles in *Nicotiana affinis* and *N. glutinosa* than in a number of other hosts (Fulton and Potter, 1971). An alteration in particle type ratio of *Prunus* necrotic ringspot virus with continuous culture in cucumber was described by Tolin (1965).

A somewhat different host effect was described by Lister and Bancroft (1970) for tobacco streak virus. The proportion of T particles was higher when infected *Chenopodium quinoa* was extracted at pH 5 than at pH 7. With infected *Phaseolus vulgaris* the reverse was true. Evidently host factors affected the selective loss or destruction of some particle types.

There are a number of other reports of differences in stability among particle types. Semancik and Bancroft (1965) found that M particles of bean pod mottle virus were less stable at pH 9.5 than B particles. Verhoyen (1972) reported that B and M particles of alfalfa mosaic virus were degraded more rapidly by sonication than Tb particles. A partially selective, pH dependent destruction of M particles of cowpea mosaic virus by heat was described by Duncan and Bruening (1971). Freezing increased the proportion of T particles of chicory yellow mottle virus (Quacquarelli *et al.*, 1972).

The observations of differential stability of virus particle types indicate the need for caution in using proportion of types as a genetic marker. For most viruses no evidence has been presented to show whether different purification procedures affect the proportion of particle types.

V. Role of Particle Types in Initiating Infection

A fundamental characteristic of multiparticulate viruses is that a single particle type is either noninfectious or weakly infectious by itself, or, in the case of tobacco rattle virus, results in a different type of infection than a mixture of particle types. The possibility that more than one particle may be required to initiate infection was considered by Lauffer and Price (1945), and was suggested by Rice *et al.* (1955) when they demonstrated the multiparticulate nature of squash mosaic virus.

A requirement for two or more particles to initiate infection can be demonstrated by mixing different kinds of particles and comparing the infectivity of the mixture with the infectivity of each particle type alone

at the same concentration as the mixture. The increase in infectivity on mixing has been referred to as enhancement, and has been reported for only about a third of the multiparticulate viruses listed in Table I. For most of the remaining viruses, infectivity tests have either not been reported for mixtures of particle types or infectivity was found for one or both of two types of nucleoprotein particles. For a number of well-investigated viruses the number of particles known to be biologically active has increased as separation methods have improved. It seems probable that many of the viruses listed in Table I will be found to have more particle types than are indicated and that enhancement will be found for mixtures of certain types.

The degree of increase in infectivity on mixing particle types depends, in part, on how complete the separation of types has been. It also depends on whether intrinsic infectivity is associated with one or more of the particle types. The relation of an excess of one particle type to total infectivity is poorly understood. Van Kammen (1968) found with cowpea mosaic virus that the concentration of the particle type present in lowest concentration determined the infectivity of the mixture. He also showed, however, that infectivity increased with excesses of one particle type up to four times the concentration of the other particle type. A somewhat similar behavior was found for tobacco streak virus (Fulton, 1970). Adding T particles (nucleoprotein) to a mixture of M and B particles resulted in infectivity increases proportional to the amount of added T particles over a range of up to five times as much T as the total of M and B.

When infection requires more than one particle type infectivity is a more sensitive indicator of unseparated mixtures than physical tests. Van Kammen (1968) reported that the particle types of cowpea mosaic virus were noninfectious when completely separated. In a subsequent paper Van Kammen and Van Griensven (1970) pointed out that while M particles can be obtained free of infectivity after two runs in a zonal rotor, it required equilibrium centrifugation in CsCl to eliminate M from B particles and to obtain noninfectious preparations of B. Similarly Van Vloten-Doting *et al.* (1970) reported that repeated cycles of density gradient centrifugation yielded particle types of alfalfa mosaic virus that were noninfectious until mixed.

A low level of infectivity, on the other hand, has been reported for the separated M and B particles of tobacco streak virus (Fulton, 1970). Because repeated density gradient centrifugation cycles did not eliminate infectivity, an effort was made to determine if it was intrinsic to the particle types or was due to residual complementary particles. Particle types of two strains were separated. One strain caused large red lesions

on *Vigna cylindrica,* and the other caused small red lesions. In mixtures of particle types, the lesion size was determined by the source of M particles. When M particles of the large lesion strain were mixed with B particles from the small lesion strain, 90% of the lesions produced were large. When the mixture was separated by two cycles of density gradient centrifugation, the B particles alone, when sufficiently concentrated, produced about 90% small lesions. If infectivity were due to incomplete separation, lesions should have been mainly large, reflecting the lesion type of the isolate supplying the M particles with which B had been mixed. Since this result was not obtained, it seemed likely that a low level of infectivity was intrinsic to the particle types of TSV.

The infectivity of separated particle types of TSV is low; to obtain significant numbers of lesions requires inoculum concentrations higher than that of mixed particle types. Infectivity might be due to the presence of a few "complete" virus particles containing all factors necessary for replication. It seems unlikely, however, that such particles would sediment with particles lacking some essential factors. On the other hand, there may be heterogeneity within single particle types that is not reflected in differences in sedimentation rates. The possibility of intrinsic infectivity of a single particle type needs to be considered in interpreting results of genetic experiments.

VI. Multiple-Hit Dilution Curves

One result of a requirement for two or more particles to initiate infection is a "multiple-hit" dilution-infectivity curve, which shows a disproportionately large decrease in infectivity with dilution. Data analyzed by Lauffer and Price (1945) showed such a dilution curve for alfalfa mosaic virus, which was eventually shown to be multiparticulate.

The steep, multiple-hit dilution curves are best explained on the basis that they fit Poisson distribution. The probability of two particles occurring in a small volume, assumed to represent the required contact with an infection site, decreases more rapidly with dilution than the probability of finding one particle in such a volume. Multiple-hit dilution curves have been reported for a number of viruses shown to be multiparticulate (*Prunus* necrotic ringspot and prune dwarf viruses, Fulton, 1962; alfalfa mosaic virus, Havránek and Zavada, 1967; tobacco streak virus, Fulton, 1967a; radish mosaic virus, Kassanis *et al.*, 1973; tobacco rattle virus unstable form, Sänger, 1968a).

A peculiar behavior of tobacco rattle virus, related to multiple-hit ef-

fects is that the proportion of "unstable form" lesions, i.e., those containing only nucleic acid, increases with dilution (Brandenburg *et al.*, 1959). This results, as pointed out by Sänger (1968a), from the decreased probability of finding both a long and a short particle at an infection site, which would produce "stable form" lesions containing complete virus.

Failure to exhibit multiple-hit infectivity dilution curves has been reported for cucumber mosaic virus (Peden and Symons, 1973), brome mosaic virus (Bockstahler and Kaesberg, 1965), tobacco streak virus in *Chenopodium quinoa* (Clark and Lister, 1971b), and others, where they might be expected. It cannot, therefore, be assumed that all multiparticulate viruses will show multiple-hit dilution infectivity curves. Lane and Kaesberg (1971) suggest that variations in susceptibility of some infection sites may alter the shape of the dilution curve, but it is difficult to see how the presence of sites requiring more than one particle for the initiation of infection would alter a curve in a direction to resemble a single-hit curve. Nevertheless, the Poisson formula may not be appropriately applied to some systems. It assumes that all pairs (or larger groups) of particles at an infection site are equally likely to initiate infection. With multiparticulate viruses, however, only groups containing complementary particles are able to initiate infection. While pairs or groups of identical particles might be nonfunctional and thus without effect on the rest of the system, they also might block infection sites or otherwise interfere with infection (Morris and Semancik, 1973).

Disparity in amount of each of two or more essential particle types might affect the infectivity of the system in unknown ways. Presumably a marked excess of one particle type would condition a single-hit curve for a certain range of concentrations in which infection sites would be nearly saturated with one particle type.

VII. Genetic Aspects of the Multiparticulate State

An intriguing aspect of the partition of nucleic acid among two or more particles is that determinants for different virus characteristics may be carried in different particles. Thus infection originating from a heterologous combination of particles might result in a combination of virus characteristics that are different from the parent strains. Associating specific viral characters with specific particle types can be done relatively easily experimentally; finding and identifying characters suitable for experimental manipulation is often more laborious. Because individual viruses vary in the manner of inheritance of various characters, they will be discussed individually or by related groups.

A. Tobacco Rattle Virus (TRV)

1. Essential Particles

This and the related pea early browning virus are unique in that infection may be initiated by one particle type alone (L particles), but such infection results in the production of only nucleic acid, not complete virus. Inoculation with unfractionated virus results in production of nucleoprotein virus particles of the two characteristic lengths. Lesions containing only nucleic acid differ in appearance from those containing complete virus. Köhler (1956) applied the terms "summer type" and "winter type," and Cadman and Harrison (1959) used the terms "multiplying" (M) and "nonmultiplying" (NM) to refer to the different types of lesions because transfers from NM lesions were difficult.

Harrison and Nixon (1959) found, after separating the particle types, that only the long particles were infective. They also pointed out that the nucleic acid, prepared by phenol disruption of TRV, was more infective than nucleic acid preparations of other viruses. Sänger and Brandenburg (1961) found that phenol extraction of plants with "winter type" symptoms (NM) resulted in more infectious preparations than conventional techniques. They suggested that this type of infection might be due to nucleic acid alone.

The relationship between particle type of TRV and function was first clearly described by Lister (1966), who found that subcultures of lesions produced by L particles were almost entirely of the "winter type" (NM). Any lesions formed from inoculations with S particles were "summer type" (M). Such lesions apparently resulted from the presence of a few long particles in preparations of short particles. The results indicated that production of stable type TRV occurred only when both long and short particles were present in the inoculum. The RNA of the long particles, while able to replicate itself, is deficient in information to code for coat protein. This information is evidently contained in the short particle RNA which, alone, is unable to replicate itself.

The division of functions of TRV between L and S particles has been repeatedly confirmed. Frost *et al.* (1967) separated long and short particles of TRV and showed that only long particles were infective, and that the lesions they produced contained nucleic acid, but not virus particles. When short particles were mixed with long particles of the same isolate and inoculated, the number of lesions was not altered, but some of them now contained tubular particles of the two characteristic lengths. Infectivity associated with such lesions was stable to freezing and thawing, whereas infectivity of lesions containing only nucleic acid was not. The proportion of lesions with stable infective material increased as the proportion of short particles in the inoculum increased.

Similar results were obtained with the RNA's of long and short particles. Interaction of long and short particles to produce stable infections was not obtained with heterologous mixtures of two strains which were only distantly related serologically, or their RNA's. Similar results were obtained by Lister (1968) with other isolates of TRV and by Lister (1968) and Huttinga (1969) with pea early browning virus.

Semancik and Kajiyama (1968) found that short particles of two North American strains enhanced stable form infection when mixed with long particles of Lister's PRN isolate as well as when mixed with each other. The sedimentation profiles of the stable type infections showed a short particle type characteristic of the strain supplying the short particles to the heterologous mixture.

2. *Association of Viral Characters with Specific Particles*

Evidence that short particle RNA coded for protein production was strengthened by Sänger's (1968a) demonstration that heterologous mixtures of long and short particles of a German and an American isolate resulted in stable form infections with the serological specificity of the strain supplying the short particles. Two other characters, length of short particles and lesion type were also contributed by the short particles. Lister and Bracker (1969) also found that particle-length heterogeneity and symptomatology were characters determined by the short particles. Semancik (1970) showed that the short particles of two different isolates, although they differed in length, coded for the same protein. Ghabrial and Lister (1973) found that the amino acid composition of protein derived from a hybrid TRV strain was essentially identical with protein of the strain supplying the short particles.

The long particles of TRV presumably code for replicase necessary for both their own and for short particle RNA replication. The capacity for producing necrotic (instead of yellow) lesions in petunia is also conditioned by the long particles (Ghabrial and Lister, 1973). Differences in size, buoyant density, and protein have been described for isolates of TRV and pea early browning virus, but their association with specific particle types is not yet known (Cooper and Mayo, 1972).

There are fairly numerous instances of the failure of short particles of one isolate to promote production of complete virus when mixed with long particles of a serologically distinct strain. Both Ghabrial and Lister (1973) and Sänger (1968a), however, have obtained this interaction with serologically different isolates. Ghabrial and Lister (1973) speculate that a "recognition factor" is involved, so that long particles mediate replicase activity appropriate for themselves, but effective for only a limited range of short particle RNA's.

The existence of a range of sizes of short particles of TRV indicates that some must contain genetic information not carried by others. It is not certain that each factor involved in RNA replication and protein synthesis is invariably carried in its specific particle type. Thus the failure of heterologous long and short particles to interact might result, in heterologous mixtures, from a deficiency of an essential factor not carried by either particle.

B. Alfalfa Mosaic Virus (AMV)

1. Essential Particles

As is the case with many of the other multiparticulate viruses, the apparent complexity of interrelationships of the various particles of AMV has increased with improvements in techniques of particle separation. The early reports of infectivity associated with one (Bancroft, 1961) or each of several particle types (Gibbs *et al.*, 1963) were supplanted by data showing that two (Van Vloten-Doting and Jaspars, 1967; Wood and Bancroft, 1965) or three (Van Vloten-Doting *et al.*, 1970) particle types or their nucleic acids were involved in infection.

The number of particle types of AMV has been revised upward from three (Bancroft and Kaesberg, 1960) to four major types and a number of minor ones. Hull (1969) lists bottom (B), middle (M), top b (Tb), and top a (Ta) as major particle types. Top o and Top z occur as minor particle types; other minor types sedimenting more rapidly than B occur only in old infections.

Improved separation methods revealed a somewhat complicated system involving cooperation among B, M, and Tb particle types (Van Vloten-Doting and Jaspars, 1967; Van Vloten-Doting *et al.*, 1968, 1970). Bol *et al.* (1971) found that, although mixtures of B, M, and Tb particles were required for infectivity, mixtures of the three corresponding RNA's were not infectious. These RNA mixtures did become infective when Ta RNA was added. Infectivity also occurred upon the addition of small amounts of AMV protein to mixtures of B, M, and Tb RNA's. Thus infection can be started with three different kinds of mixtures (Bol *et al.*, 1971): (1) B, M, and Tb nucleoprotein particles, (2) B, M, and Tb nucleic acid plus coat protein, and (3) B, M, Tb, and Ta nucleic acid.

Bol *et al.* (1971) found that activation of mixture (2) by coat protein required only 4–8 subunits per RNA molecule, suggesting that the function of the protein might be enzymatic. Van Vloten-Doting and Jaspars (1972) found that adding AMV RNA to virus resulted in the uncoating

of the virus particles. The reaction was specific for the virus RNA and the protein. The authors postulated that there must be sites on the RNA with a high affinity for coat protein and that the sites might be related to the biological role of the coat protein.

2. *RNA Content of AMV Particle Types*

The nucleic acid of AMV consists of four pieces, each associated with a specific particle type (Hull *et al.*, 1969a; Bos and Jaspars, 1971; Pinck and Hirth, 1972). The S values and molecular weights of the RNA's are the following: B, 24.3 S, 1.3×10^6 daltons; M, 20.0 S, 0.86×10^6 daltons; Tb, 17.3 S, 0.63×10^6 daltons; Ta, 12.7 S, 0.33×10^6 daltons. Ta particles probably contain two 12.7 S pieces of RNA. Pinck and Hirth (1972) presented evidence indicating the presence in infected plants of double-stranded replicative RNA corresponding to each of the four pieces of AMV RNA.

3. *Association of Viral Characters with Specific Particles*

When it was thought that mixtures of B RNA and Ta RNA were infectious, heterologous mixtures from two strains gave rise to a hybrid strain which produced symptoms on tobacco, had the particle type proportion, and reacted serologically like the parent strain from which the Ta RNA had been obtained (Van Vloten-Doting *et al.*, 1968). The sedimentation rate of B RNA and the symptoms on bean were characteristic of the strain supplying the B RNA. Majorana and Paul (1969) described the production of hybrid strains of virus by mixtures of particle types of two AMV strains, the symptoms caused being different from those of either parent. Evidence derived from a study of the translation of Ta RNA in an *Escherichia coli* cell-free system (Van Ravenswaaij Claasen *et al.*, 1967) also indicated that the code for coat protein was carried by the Ta RNA.

Subsequent to these investigations, however, Bol and Van Vloten-Doting (1973) found that what they had previously referred to as 27 S RNA (B RNA) was actually a mixture of B RNA, M RNA, and Tb RNA. Attempts to determine whether Ta RNA contributed genetically when added to a mixture of Tb, M, and B RNA's from serologically and biologically distinct strains failed to show any hybrid character typical of the strain supplying the Ta-RNA. Since Ta particles are produced from inoculum of Tb, M, and B, the genetic information in Ta must be carried in another particle, probably Tb.

In a study of two strains of AMV, Dingjan-Versteegh *et al.* (1972) confirmed that coat protein (serotype) was coded for by Tb nucleic acid.

The type of symptom on tobacco (chlorotic or necrotic), relative proportion of particle types, and sensitivity to cycloheximide were also determined by factors carried in Tb particles. The type of symptom in bean (local necrotic or chlorotic, becoming systemic) was determined by factors carried in M particles. No genetic markers were found in B particles.

Dingjan-Versteegh *et al.* (1972) point out that the genetic information carried in Ta is redundant. Ta RNA could be formed either by degradation or partial transcription of Tb RNA. They suggest the possibility that there may be redundant information in M and B particles. They also point out that the location of the coat protein code in the smallest RNA (Tb) of AMV corresponds with the location of the coat protein code in the smallest particles of tobacco rattle virus (Sänger, 1968a; Lister, 1966), bean pod mottle virus (Moore and Scott, 1971), and cowpea chlorotic mottle virus (Bancroft, 1972). As will be seen, however, this is not true of certain other viruses.

C. Cowpea Mosaic Virus (CPMV)

1. Essential Particles

This virus is isometric (28 nm) and consists of two kinds of nucleoprotein particles, often with a top particle devoid of nucleic acid. Wood and Bancroft (1965) and Bruening and Agrawal (1967) showed that mixtures of M and B particles, separated by zonal sucrose gradient centrifugation, were more infectious than equal amounts of M or B alone. Repeated zonal density gradient centrifugation or equilibrium density centrifugation in CsCl resulted in better separation (Van Kammen, 1968). These completely separated particle types were noninfectious alone, but were infectious when mixed.

The infectivity of mixtures of M and B particles of CPMV depended on the concentration of the particle type present in the lowest amount (Van Kammen, 1968). Infectivity increased, however, when the concentration of either particle type was increased beyond an amount equal to that of the other particle type.

Wood and Bancroft (1965), who were evidently working with B particle preparations of cowpea yellow mosaic virus that contained some M particles, found that the infection-activating property of M particles was sensitive to UV irradiation. They also found that the proportion of B particles that could be activated by M particles was increased by a heat treatment. Van Kammen (1968) could not confirm this, but found that a proportion of B or M particles were damaged by heat. When heated

preparations were used in mixtures, fewer lesions resulted than with unheated preparations.

2. *RNA Content of CPMV Particle Types*

Middle particles contain 23% RNA; bottom contain 32% RNA (Van Kammen, 1967). Van Kammen (1968) found that the RNA of CPMV functioned similarly in enhancing infection as did the particle types. There was, however, a strain specificity; infectivity increases were not obtained except with homologous mixtures (Wood and Bancroft, 1965; Van Kammen, 1968). As will be seen this is not true of all strains.

Van Kammen and Van Griensven (1970) found that M particles had 26 S RNA (1.45×10^6 daltons) and that B particles had 34 S RNA (2.58×10^6 daltons). The base composition of the two RNA's differed, B particle RNA having more guanylic acid and less cytidylic acid than M particle RNA.

The presence in CPMV-infected cowpeas of double-stranded, replicative RNA of two sizes (2.8×10^6 and 5.0×10^6 daltons) corresponding to the M and B particle RNA was described by Van Griensven and Van Kammen (1969). In an extension of this work Van Griensven *et al.* (1973) and Van Kammen (1971) found that labeled CPMV RNA hybridized specifically with the minus strands of the corresponding double-stranded RNA. There appeared to be no overlapping of base sequences. They concluded that M RNA and B RNA do not have genes in common and that the total genome is divided among the two pieces of RNA. It is not certain, however, that this technique is adequate to detect duplication of base sequences involved in a single gene.

3. *Association of Viral Characters with Specific Particles*

Genetic analysis of CPMV has been hampered by the inability to obtain enhancement between M and B particles of a number of strains differing in biological characteristics. This may reflect a lack of redundance in genetic information, coupled with some differences, however slight, in the division of genetic information between particles of different strains.

Bruening (1969), by subculturing single lesions of a strain of CPMV which did not produce a top particle type, obtained several strains which did produce top particles. Heterologous mixtures of particles of one such strain and its parent strain were highly infectious. Whether or not a top particle type was formed was determined by the characteristics of the strain supplying the M particles.

De Jager and Van Kammen (1970), by nitrous acid treatment, obtained mutants of the Sb strain of CPMV that, unlike the parent, failed

to infect bean systemically at 22°–24°C. This mutant also produced a much higher proportion of T particles than the parent strain. When heterologous mixtures of particle types were inoculated, specific properties of the mutants were found only in combinations containing M particles of the mutant. At 30°C the mutant became systemic in bean as readily as did the parent strain, and the formation of T particles was reduced at 30°C to nearly the same proportion formed by the parent strain. The authors suggested that the formation of excess top particles and the failure to become systemic in bean may have a basic mechanism in common.

Wood (1972), also by nitrous acid treatment, obtained mutants of CPMV differing in type of lesion in bean and in proportion of nucleoprotein particles. Strains of heterologous, mixed particle origin exhibited the mutant lesion type and mutant nucleoprotein particle proportion if the B particles were derived from a mutant strain. Backcrosses of hybrid strains to the parent strains showed that the B and M particles of the hybrid strains had the same genetic constitution as the original mutants. In these crosses, lesion type and particle proportion never segregated, suggesting that these characters were specified by closely linked (or the same) genetic determinant(s).

D. Bean Pod Mottle Virus (BPMV)

This virus, a member of the cowpea mosaic virus group, is similar to CPMV in having three particle types, two of which are nucleoprotein (Bancroft, 1962). M and B particle types differ in base composition; B particles have a higher proportion of guanylic and uridylic acids than B particles (Semancik and Bancroft, 1964). The virus is unusual in showing two particle types upon electrophoresis (Bancroft, 1962). Each electrophoretic type contained at least both M and B particles and was infectious. Each of the centrifugal particle types contained both electrophoretic types.

A strain of BPMV was described by Moore and Scott (1971) that differed serologically from the type strain. The serological reaction of cultures arising from heterologous mixtures of particle types was the same as the strain providing the M particles, indicating that genetic information specifying coat protein type is carried in the M particles.

E. Radish Mosaic Virus (RdMV)

This virus, also a member of the comovirus group, has particle types resembling those of cowpea mosaic virus (Kassanis *et al.*, 1973). Com-

plementation occurred between M and B particles of the same strain and in heterologous combinations with one, but not another strain. Kassanis *et al.* (1973) made crosses between two strains which differed in that one contained an antigen lacking in the other and also formed aggregates which the other strain did not. Regardless of the source of M or B particles, all offspring virus clones lacked the specific antigen and failed to form aggregates. The authors conclude that the coat protein is determined by both M and B particle types. In this respect the virus differs from bean pod mottle virus (Moore and Scott, 1971) in which the coat protein determinant is carried in the M particles, and from tobacco streak virus (Fulton, 1972), in which the coat protein determinant is carried in three particle types. It is not easy to understand, however, why the strain-specific antigen of one parent failed to appear in any of the RdMV progeny clones. The fact that there are two coat proteins involved for members of the comovirus group (Wu and Bruening, 1971) indicates that genetic factors for each need to be accounted for.

F. Raspberry Ringspot (RRV) and Tobacco Ringspot (TRSV) Viruses

The genetics of these viruses has been investigated more extensively than other NEPO viruses. The number of particle types of RRV is known to vary from two to three with different isolates (Debrot, 1964). The three types contain 0, 30, and about 44% RNA (Harrison *et al.*, 1972a). The B particles are infective alone, but infectivity is increased by adding M particles.

Polyacrylamide gel analysis of RNA preparations showed two predominant RNA species with molecular weights of 2.4×10^6 (RNA-1) and 1.4×10^6 (RNA-2) (Murant *et al.*, 1972). M particles of RRV contained one molecule of RNA-2, while B particles contained either one molecule of RNA-1 or two molecules of RNA-2. Confirmation of the latter condition was obtained by UV irradiation of intact B particles, which produced an RNA slightly greater in size than RNA-1. This was considered to be a dimer of RNA-2, and it was not formed by irradiating the free RNA or M particles (Mayo *et al.*, 1973).

Preparations of RNA-1 were infective, but preparations of RNA-2 were not. The addition of RNA-2 to RNA-1 greatly increased infectivity, however (Harrison *et al.*, 1972a). RNA-1 and RNA-2 of TRSV behaved like the corresponding RNA's of RRV in infectivity tests, but there was no heterologous complementation with RRV nucleic acids.

Genetic determinants carried by RNA-2 were demonstrated with strains of RRV that differed serologically and in symptoms on petunia (Harrison *et al.*, 1972b). Single lesion isolates from heterologous mix-

tures of RNA-1 and RNA-2 were serologically like the isolate supplying the RNA-2, and produced symptoms on petunia typical of the isolate supplying the RNA-2.

The relationship between nucleic acid, particle types, and infectivity of TRSV is apparently similar to that of RRV. Stace-Smith *et al.* (1965) found three particles types, two of which contained nucleic acid. Only B particles were infectious. Diener and Schneider (1966) found that nucleic acid from the noninfectious M particles was noninfectious, while nucleic acid from B particles consisted of one infectious and one noninfectious species. The noninfectious nucleic acid from B particles appeared to be the same size as the nucleic acid from M particles.

Murant *et al.* (1972) also found two kinds of nucleic acid in TRSV. The RNA-2 was the same size as the RNA-2 of RRV, but the RNA-1 was smaller. The B particles of TRSV formed two buoyant density classes, indicating a heterogeneity in RNA content. Infectivity of RNA of TRSV paralleled that of RRV, ie., RNA-1 was infectious and the addition of RNA-2 enhanced infectivity.

G. Tobacco Streak Virus (TSV)

1. Essential Particles

Tobacco streak virus exists as three main particle types. Additional particle types have been described (Mink *et al.*, 1966; Fulton, 1970), but only T, M, and B particles have shown biological activity. T particles are nucleoprotein, but are not infectious. M and B particles are weakly infectious alone, but highly infectious when mixed (Fulton, 1970). Addition of T particles to mixtures of M and B particles further increases infectivity, the increase being about proportional to the amount of T added.

2. RNA Content of Particle Types

Clark and Lister (1971b) point out that TSV RNA seems unusually prone to degradation, which makes difficult the precise determination of molecular sizes of the RNA and the association of specific sizes with specific nucleoprotein particles. They described, from a grapevine isolate of TSV, RNA of five sizes: 1.3, 1.06, 0.78, 0.72, and 0.36×10^6 daltons. B particles contained the largest piece of RNA. M particles contained the next largest piece and, in addition, a smaller piece of RNA which was also carried in T particles. T particles contained the smallest pieces of RNA and more than one of these per particle. Results in our laboratory, however, with other strains of TSV, have shown only three

different pieces of RNA, one associated with each particle type (L. S. Loesch, unpublished).

3. Association of Viral Characters with Particle Types

TSV has a very wide natural host range and occurs in many strains causing different symptoms. One useful character has been the type of lesion induced in *Vigna cylindrica,* which may be reddish and either 1 mm or less in diameter or 3–4 mm in diameter 3 to 4 days after inoculation. Ring-like lesions are also produced by some isolates on this host (Fulton, 1970), but these tend to intergrade with the large reddish lesions and cannot always be reliably differentiated. An isolate from Brazil causes chlorotic lesions on *V. cylindrica.*

Another type of symptom markedly varying with different isolates occurs on leaves of Turkish tobacco produced after systemic necrosis occurs. These leaves may be markedly toothed or, with another strain, they may appear healthy. With another strain the upper leaves of Turkish tobacco are markedly stunted and thickened. With still other strains, infected plants never produce leaves without necrosis.

Serological differences also occur among isolates of TSV. An isolate from Brazil contains antigenic group(s) not present in North American isolates and lacks some antigenic group(s) present in North American isolates. By heterologous cross-absorption of antisera it was possible to prepare antisera reacting specifically with each strain (Fulton, 1972).

Characteristics of hybrid clones of virus were determined by first inoculating mixtures of particle types to *Vigna cylindrica* at such dilutions that 2–10 well-separated lesions were produced per half-leaf. Lesions were then punched out and inoculated individually to Xanthi-nc (Turkish) tobacco.

Determinants for lesion type occur in T and M particles of TSV, but not in B particles (Fulton, 1970). Some evidence was obtained (Fulton, 1972) that lesion size was determined by a different factor than lesion color.

The toothed symptom on Turkish tobacco was inherited through any of the three particle types (Fulton, 1972). In crosses between strains inducing toothed leaves and those inducing normal or "entire" leaves, progeny were about equally divided among those causing toothed leaves and those causing entire leaves. Single lesion subcultures from such hybrid clones always resembled the clone from which they were derived, and in backcrosses there was no evidence for the occurrence of any of these determinants except that typical for the symptoms expressed.

The dwarfing symptom on tobacco depended on the presence of two determinants, one from M and one from B particles. Hybrid clones car-

rying only one such determinant induced toothed leaves, indicating that the dwarfing strain carried a toothing determinant which was masked. When hybrid clones carrying one dwarfing determinant were backcrossed to the dwarfing parent, all offspring virus clones were dwarfing if the combination of M and B particles provided two dwarfing determinants. Hybrid clones with one dwarfing determinant usually carried it in the particle type that had provided it in the original cross. In some hybrids, however, the single dwarfing determinant appeared to be carried in part of the M particles and in part of the B particles. These strains could be detected only by backcrossing to the dwarfing parent; dwarfing offspring did not appear among selfed progeny.

The determinant for "nonrecovering" leaves was carried only in the T particles, and evidently only in part of them. The symptom type was never produced by virus clones originating from only M and B particles. This determinant was unstable and progressively disappeared *in vitro* from particle preparations stored at 2°C.

The determinant for serological type (coat protein) was carried in each of the three particle types. In crosses involving serological markers, offspring clones were of one serological type or the other. There was no evidence in backcrosses for the presence of any serological determinant except that typical for the coat protein of the hybrid strain. There appeared to be a linkage between the dwarfing phenotype and the North American antigenic type, although some recombinations did occur.

Recombinations of other characteristics occurred about as frequently as would be expected on the basis of randomly assorting factors. Some recombinations represented new combinations of M and B particles; others occurred within particle types. For example, from a parent whose M particles carried factors for toothed (masked), dwarfing, and North American antigen, offspring virus was obtained whose M particles carried the toothed determinant, lacked the dwarfing factor, and carried the factor for Brazilian antigen.

H. Pea Enation Mosaic Virus (PEMV)

This virus resembles tobacco streak virus in having isometric particles of different sizes (Hull and Lane, 1973). Three different pieces of nucleic acid have been found in preparations from unfractionated virus (Gonsalves and Shepherd, 1972; Hull and Lane, 1973). Infectivity enhancement by mixing particle types was reported by Hull and Lane (1973) but was not found by Gonsalves and Shepherd (1972). Hull and Lane (1973) described B particles as containing RNA-1, but not RNA-2, thus disagreeing with Gonsalves and Shepherd (1972). Evidence that coat protein

determinants were carried in B particles was presented by Hull and Lane (1973).

I. Barley Stripe Mosaic Virus (BSMV)

McKinney and Greely (1965) have pointed out the great variability of this virus. It is composed of short, straight rods of varying lengths. Harrison *et al.* (1965) found that the most frequent length was 128 nm; other distribution peaks occurred at 148 and 111 nm. Particles shorter than 111 nm were not infective, but the infectivity of the other individual length classes could not be determined because they could not be separated.

RNA Heterogeneity

Jackson *et al.* (1972) found more than one RNA species in all BSMV isolates examined. Certain strains contained RNA species which were not represented in other strains, although a number of strains seemed to have the same RNA species. More than one species of RNA was necessary for infectivity, although the role of individual RNA species was not determined. Replicative RNA's corresponding to two of the RNA species were described by Pring (1972).

Jackson and Brakke (1973) found that all strains of BSMV tested had a prominent 21.3 S RNA, which separated into two kinds upon gel electrophoresis. Certain strains had an additional 19.5 S RNA and some preparations had variable amounts of minor components sedimenting at about 30 S. Homologous or heterologous mixtures of the separated RNA components were more infectious than single components, indicating that more than one particle type was involved in infection.

J. Brome Mosaic Virus (BMV)

1. RNA Heterogeneity

The multiparticulate nature of brome mosaic virus is not apparent upon density gradient centrifugation. There are, however, RNA species of different sizes (Bockstahler and Kaesberg, 1965; Lane and Kaesberg, 1971). Lane and Kaesberg (1971) differentiated three types of structurally similar virions by their buoyant density differences in CsCl. The most dense particle contained an RNA of 1.09×10^6 daltons (RNA-1); the next most dense particle contained two RNA molecules, 0.75×10^6 daltons (RNA-3) and 0.28×10^6 daltons (RNA-4). The least dense par-

ticle type contained one RNA molecule of molecular weight 0.99×10^6 daltons (RNA-2).

Separation of particle types by buoyant density differences was not sufficiently complete to make it possible to determine the contribution of each particle type to infectivity. Mixtures of partially separated particle types, however, were more infectious than each type alone.

When the different nucleic acids were separated it was found that each of the three larger kinds (RNA-1, -2, -3) was required for infectivity. Deletion of any of these (but not the smallest nucleic acid) reduced infectivity to very low levels.

2. Association of Viral Characters with RNA Species

Lane and Kaesberg (1971) investigated a number of isolates of BMV some of which differed in electrophoretic mobility. Isolates derived from heterologous mixtures of nucleic acid species exhibited the same electrophoretic mobility as the isolate supplying the third largest nucleic acid (RNA-3).

A series of lesion type mutants of BMV, produced by nitrous acid treatment, were found to carry the mutant gene on RNA-3 (Bancroft and Lane, 1973). A naturally occurring mutant of the electrophoretic variant described by Lane and Kaesberg (1971), migrating even more slowly, was also found to carry the mutated gene on RNA-3. Both these mutants had decreased amounts of RNA-3 and RNA-4, and the altered ratios were also determined by RNA-3. Another naturally occurring mutant described by Bancroft and Lane (1973) differed from the wild type in lesion appearance and in having 15% of its particles smaller than normal. Factors conditioning both these characters appeared to be carried on RNA-2.

K. Cowpea Chlorotic Mottle Virus (CCMV)

1. RNA Heterogeneity

Like brome mosaic virus, CCMV sediments as a single kind of particle. The RNA extracted from it, however, is heterogeneous, with four sizes detectable when subjected to electrophoresis on polyacrylamide gels (Fowlks and Young, 1970). Infectivity depended on a mixture of at least RNA-1 and RNA-2 (Bancroft, 1971). Inclusion of RNA-3 in the mixture further increased lesion numbers. Subsequent work by Bancroft and Flack (1972) indicated that when the RNA's were completely separated, RNA-1, -2, and -3 were all essential to infectivity. RNA-4 had no effect on infectivity.

The association of specific RNA's with nucleoprotein particles differing in buoyant density in CsCl was shown by Bancroft and Flack (1972). The heaviest particles contained RNA-1 (1.15×10^6 daltons), the lightest particles contained RNA-2 (1.00×10^6 daltons), and the medium density particles apparently contained RNA-3 (0.85×10^6 daltons), and RNA-4 (0.32×10^6 daltons).

2. Association of Viral Characters with RNA Species

CCMV and BMV are grouped together taxonomically (Harrison *et al.*, 1971) and resemble one another in having a genome divided among four sizes of RNA. Bancroft (1972) constructed a hybrid virus from RNA-1 and RNA-2 of BMV and RNA-3 of CCMV. This hybrid did not infect barley or cowpea, but did infect hosts common to both parent viruses. It reacted with CCMV antiserum, indicating, as had work with CCMV, that the coat protein cistron is carried by RNA-3. In the density distribution of nucleoprotein particles the hybrid resembled BBMV. Peculiarly, RNA-3 could not be detected in the hybrid. Backcrosses to the parent types showed that RNA-1 and RNA-2 were not changed from the parental types.

Bancroft and Lane (1973) selected several temperature-sensitive mutants of CCMV, induced by nitrous acid. One of these (ts) differed from the wild type in having milder symptoms and decreased yield at high temperature, lower specific infectivity, decreased thermal stability of coat protein, and altered nucleoprotein particle ratio in CsCl. Progeny from a cross involving RNA-1 and RNA-2 from the wild type and RNA-3 from the mutant showed all of the characters of the mutant parent, indicating that the genetic factor or factors involved are carried on RNA-3.

Another temperature sensitive mutant differed from the wild type in having thermally unstable protein and in causing mild symptoms in cowpea. These characters were also inherited through RNA-3, as they were for the first temperature sensitive mutant. The second mutant also differed from the wild type in causing smaller and less necrotic lesions in *Chenopodium hybridum*. This character was inherited through RNA-2.

Another isolate was described by Bancroft and Lane (1973) as having the genetic determinant for small lesions on RNA-2, but at least one additional mutation was involved which prevented lesion production in cowpea. Another small lesion mutant was also temperature sensitive, producing only 10% as many lesions on *C. hybridum* at 32°C as the wild type. The lesion type determinant was carried in RNA-2, and temperature sensitivity in RNA-1.

VIII. Comments

It is clear that for a number of plant viruses the genetic material is divided among two or more types of particles. The separation of the particle types, or the nucleic acid molecules, permits experimental manipulation of genetic characters of a group of isolates. For this it is essential to have available specific characters by which one isolate can be readily differentiated from another. The possible number of these characters is not known, but is probably small. Perhaps the most obvious character is the type of primary lesion. The type of systemic symptom is also a characteristic that varies among isolates. The kind of coat protein is a useful character and, as pointed out by Bancroft and Lane (1973), is one related directly to genotype.

In assessing virus characters as genetic markers it is important to know which phenotypic characters are expressions of the same genetic determinant. Does an alteration in coat protein, for example, always result also in an alteration of symptoms? Where two different characters separate in hybrids, and can be recombined in crosses of hybrids, it is obvious that different genetic determinants are involved. A consistent association of apparently unrelated characters may represent different effects of the same gene. This may provide a useful insight into the mechanisms of pathogenesis. On the other hand, two genes may be involved that are closely linked on the same RNA strand and do not readily separate.

It may be expected that some of these questions will be resolved as more virus characters are investigated. And it is probable that the genetic systems will be found to differ considerably among the various viruses. Evidence for tobacco streak virus, for example, suggests that there is a good deal of redundance in the genetic information carried by the three particle types. This is not apparent for a number of other viruses. Demonstration that a specific viral character is always associated only with one specific particle or specific RNA molecule requires an investigation of a significant number of hybrid clones to detect redundance.

The great inefficiency of mechanical inoculation of plant viruses has long been recognized. Whether this results entirely from host insusceptibility or entirely from functional inadequacy of most of the virus particles inoculated is not known. Some evidence suggests, however, that a significant part of a population of virus particles is nonfunctional (Furumoto and Wildman, 1963). If this is so, it is not difficult to see how monoparticulate viruses might evolve into multiparticulate viruses. A

selection pressure favoring particular variants produced by a fortuitous association of complementing particles would result in a multiparticulate virus. If this sort of evolution occurs, it might be supposed that the most highly evolved viruses would be those with little or no redundance of genetic material in different particle types. Conversely the least highly evolved viruses would be those most similar to the monoparticulate state, that is, with much redundance in genetic determinants carried in the different particle types.

The manner in which the differing strands of RNA are replicated is of significance in relation to genetic patterns. If, as some data indicates, replication occurs essentially independently, then any linkage groups should correspond to the specific RNA molecules. Recombination of viral characters in hybrid clones would only reflect reassortment of specific RNA from dissimilar parents. On the other hand, there is evidence for recombinations of determinants (Fulton, 1972) which are carried in a single RNA molecule of tobacco streak virus (L. S. Loesch, unpublished). The mechanism involved in this type of recombination is not apparent.

Bancroft and Lane (1973) pointed out that the ultimate aim of investigating the genetics of virus mutants is to establish a relationship of biological properties to specific proteins. It would seem that this would best be done with as wide a range of mutants and as many viruses as possible. Mutant types of many dissimilar viruses show many of the same sorts of variants: yellow mosaics, slow moving types, and host range variants, for example. It may be that the basic viral alteration conditioning these host reactions is similar for different viruses.

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CHAPTER 28

Viroids as Prototypes or Degeneration Products of Viruses

T. O. DIENER

I. Introduction

The term *viroid* has been introduced to designate a newly recognized class of pathogens. Viroids differ from viruses by the absence of a dormant phase (virions) and by genomes that are much smaller than those of known viruses (Diener, 1971b).

These distinguishing properties were first elucidated for the agent of the potato spindle tuber disease, a disease which, for many years, was believed to be virus-caused. Diener and Raymer (1967) reported that the infectious agent of the disease is a free RNA and that conventional virus particles are, apparently, not present in infected tissue; whereas Singh and Bagnall (1968) reported on some properties of infectious nucleic acid extracted from infected tissue.

Subsequent studies clarified a number of properties of the infectious RNA and showed that, *in vivo,* it is associated with the nuclei of infected cells (Diener and Raymer, 1969; Diener, 1971a). These earlier studies have been reviewed (Diener, 1971c). Later, a combination of sedimentation and gel electrophoretic analyses conclusively showed that the RNA has a very low molecular weight and that the agent, therefore, basically differs from conventional viruses (Diener, 1971b).

The recognition of viroids as a novel class of pathogens raises the question as to their evolutionary position and relationship, if any, with viruses. Unfortunately, present knowledge of viroids is far too incomplete to answer these questions; and all one can do, at this time, is to list the several possibilities, as they appear in light of present knowledge.

In this chapter, I wish first to summarize recent advances made with the potato spindle tuber viroid, then to discuss work done with other viroids, and finally to speculate on the evolutionary position of viroids with emphasis on their possible relationship with viruses.

II. Recent Progress with the Potato Spindle Tuber Viroid (PSTV)

A. Recognition of Low Molecular Weight

Until very recently, attempts to determine physical and chemical properties of PSTV were hampered by the fact that the RNA could be recognized only by virtue of its biological activity, and not as a physical entity. This handicap was particularly severe in efforts to determine the molecular weight of the RNA.

Although it had been known for some time that PSTV sediments at a lower rate than single-stranded viral RNA molecules (Diener and Raymer, 1969), no conclusions as to its molecular weight could be drawn from this observation, since the conformation of the RNA was unknown and since it was not known whether the RNA is single-, double-, or multistranded.

A principle elaborated by Loening (1967), however, made it possible

to obtain a molecular weight estimate based on biological activity alone. Loening reasoned that the effects of secondary structure of an RNA on its sedimentation properties are opposite to the effects of secondary structure on its electrophoretic mobility in polyacrylamide gels. Thus, a combination of the two analytical methods should be useful to distinguish differences in structure from differences in weights of RNA's. With either method, biological activity is the only parameter necessary for evaluation of the results.

Application of this principle to PSTV led to the unexpected conclusion that the RNA has a very low molecular weight (Diener, 1971b). An estimate of about 5×10^4 daltons was in accord with the experimental results. Further evidence for the very low molecular weight of PSTV came from the observation that the RNA was able to enter gels of high polyacrylamide concentration (i.e., small pore size), from which high molecular weight RNA's are excluded. In such gels, PSTV moves as a well-defined, homogeneous band (Fig. 1) with a rate of migration that is compatible with the above molecular weight estimate (Diener and Smith, 1971).

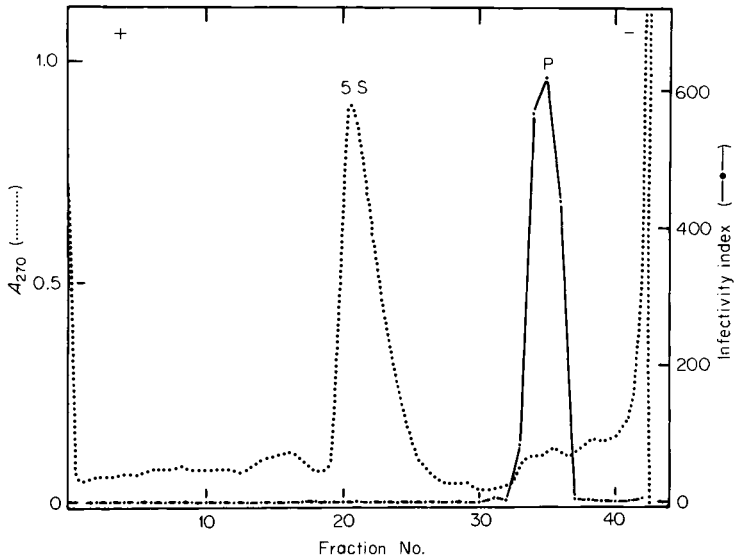

FIG. 1. Electrophoretic mobilities of 5 S ribosomal RNA (5 S) and of PSTV (P) in a 20% polyacrylamide gel. Ultraviolet absorption (····) and infectivity (—·—) profiles determined after electrophoresis for 4.5 hours at 4°C (5 mA per tube, constant current). A_{260}, absorbance at 260 nm. Electrophoretic movement from right to left. (From Diener and Smith, 1971.)

B. Identification as a Physical Entity

Although these experiments clearly demonstrated that PSTV is a low molecular weight RNA, further characterization of the molecule was not possible without isolation of the RNA as a physically recognizable entity. As a first step toward purification of PSTV, the RNA was extracted and purified from relatively large quantities of infected tomato leaves (Diener, 1972a). Identically extracted and purified preparations from healthy plants served as controls. Figure 2A shows the ultraviolet (UV) absorption profile of an RNA preparation from healthy leaves after electrophoresis in a 20% polyacrylamide gel. In addition to 5 S RNA, at

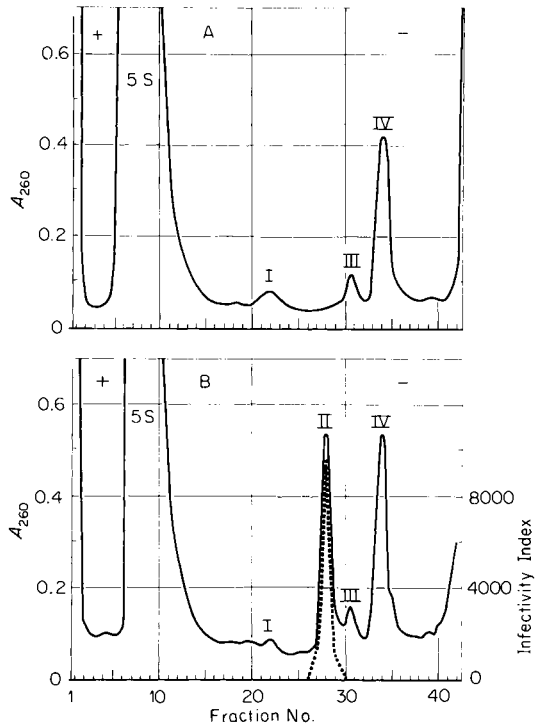

FIG. 2. (A) UV-absorption profile of RNA preparation from healthy tomato leaves after electrophoresis in a 20% polyacrylamide gel for 7.5 hours at 4°C (5 mA per tube, constant current). (B) UV-absorption (—) and infectivity distribution (---) of RNA preparation from PSTV-infected tomato leaves after electrophoresis in a 20% polyacrylamide gel [same conditions as in (A)]. 5 S, 5 S ribosomal RNA; I, III, IV, unidentified minor components of cellular RNA; II, PSTV; A_{260}, absorbance at 260 nm. Electrophoretic movement from right to left. (From Diener, 1972a.)

least three additional UV-absorbing components are discernible in the gel (I, III, and IV). Evidently, these components are minor low molecular weight constituents of cellular RNA.

Figure 2B shows the UV absorption and infectivity distribution profiles of an identically obtained RNA preparation from PSTV-infected leaves after electrophoresis in a 20% polyacrylamide gel. In addition to 5 S RNA, four UV-absorbing components are discernible. The positions in the gel of three of these (I, III, and IV) coincide with those of components found in the preparation from healthy leaves. In addition, another prominent component is discernible (II). Bioassay of individual gel slices demonstrated that infectivity coincides with this component (Fig. 2B). This coincidence, the high level of infectivity, and the fact that component II does not occur in preparations from healthy leaves constitutes strong evidence that component II is PSTV.

These results therefore confirmed—by physical means—earlier conclusions that were based entirely on infectivity assays (Diener, 1971b; Diener and Smith, 1971). Isolation of PSTV as a ribonuclease-sensitive, UV-absorbing component demonstrates that the infectious agent is, indeed, an RNA and, since this RNA is able to penetrate into 20% polyacrylamide gels and to move through such gels as a monodisperse, UV-absorbing band, the low molecular weight of the RNA was confirmed.

C. Purification

These observations made it possible to isolate and purify PSTV in amounts sufficient for biophysical and biochemical analyses. To purify PSTV, preparations from which DNA, ribosomal RNA's, tRNA, and polysaccharides had previously been removed (Diener, 1971b) were subjected to electrophoresis for 16 hours in 20% polyacrylamide gels. Under these conditions, PSTV migrates close to the bottom of the gel and is more completely separated from component III (Fig. 2). The gel portion containing PSTV was excised from each gel. PSTV-containing portions from many gels were combined, and the RNA was eluted by homogenization of the gel slices in buffer. PSTV was then separated from the gel particles (and reconcentrated) by centrifugation, followed by hydroxyapatite chromatography of the supernatant solution. An impurity that elutes from the gels together with PSTV was removed by a procedure that involved shaking of the preparations with methoxyethanol (Diener, 1973a).

Analysis of the final preparations by polyacrylamide gel electrophoresis revealed that, except for an occasional slight contamination with

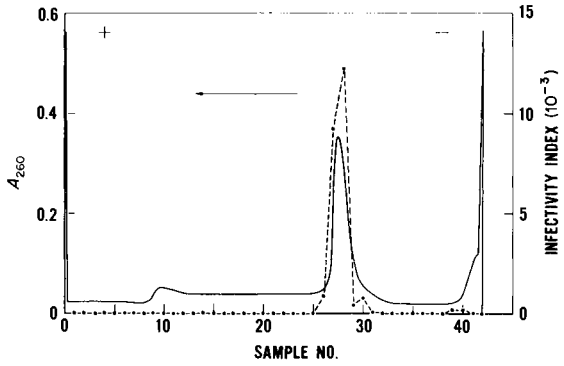

FIG. 3. UV-absorption (—) and infectivity distribution (●--●) profiles of purified PSTV after electrophoresis in a 20% polyacrylamide gel for 7.5 hours at 4°C (5 mA per tube, constant current). A_{260}, absorbance at 260 nm. Electrophoretic movement from right to left. (From Diener and Smith, 1973.)

what appears to be 5 S RNA (fractions 9-12), only one UV-absorbing component is consistently discernible in the gels (Fig. 3). Comparison of this component with PSTV in less highly purified preparations (Diener, 1972a) disclosed that both have identical electrophoretic mobilities. Furthermore, infectivity distribution in the gels coincides with the UV-absorbing component (Fig. 3). These observations indicate that the UV-absorbing component is PSTV and that the preparations are essentially free of contaminating nucleic acids.

D. Determination of Physical Properties

Although purification of PSTV is now feasible, the separation procedure is laborious and yields only microgram quantities of purified PSTV. With supplies so far available, the following physical properties of PSTV have been studied.

1. Molecular Weight

With the availability of purified PSTV, a redetermination of its molecular weight based, not on its biological activity, but on its absorption of UV light, became possible. For this purpose, a method described by Boedtker (1971) appeared particularly promising, as it permits the determination of the molecular weight of an RNA independent of its conformation.

With this method, RNA's are treated with formaldehyde at 63°C, a treatment which, according to Boedtker (1971), completely denatures

the RNA's, i.e., destroys their secondary and tertiary structure. Consequently, the electrophoretic migration of formylated RNA's in polyacrylamide gels is a function of their molecular weights, but not of their particular conformations.

To apply this method to PSTV, it was necessary to compare its rate of migration in gels with those of formylated RNA's of known molecular weights. Of all marker RNA's available, the electrophoretic mobility of formylated tobacco ringspot satellite virus (SAT) RNA (Schneider, 1971) was closest to that of formylated PSTV. Hence, this RNA was most useful as a molecular weight marker, particularly in gels with small pore size, from which high molecular weight RNA's are excluded.

Since the molecular weight of SAT RNA had so far been estimated only from its sedimentation coefficient by use of an empirical formula (Schneider, 1971), it was important to determine its molecular weight by gel electrophoresis of the RNA after treatment with formaldehyde.

Accordingly, formylated SAT RNA was analyzed by electrophoresis, in the presence of formaldehyde, using formylated tRNA, 5 S RNA, rRNA's, and carnation mottle virus (CarMV) RNA (Waterworth and Kaper, 1972) as molecular weight markers (Diener and Smith, 1973).

In Fig. 4, the results of such an analysis are given, and Fig. 5 illustrates the relation between the electrophoretic mobility of each RNA (expressed as the ratio of its movement in the gel to that of a visible marker, bromophenol blue dye) and its molecular weight. The range of electrophoretic mobilities of formylated SAT RNA is indicated in Fig. 5

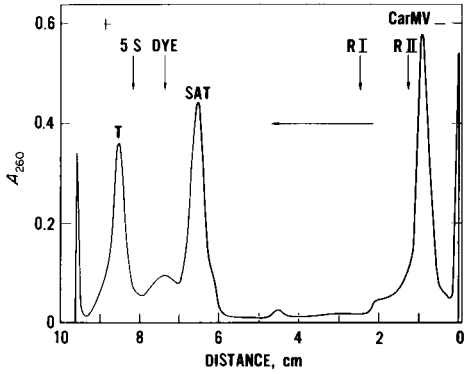

FIG. 4. UV-absorption profile of a sample containing formylated transfer RNA (T), formylated tobacco ringspot satellite virus RNA (SAT), and formylated carnation mottle virus RNA (CarMV) after electrophoresis (in the presence of formaldehyde) in a 2.4% polyacrylamide gel. The position of other formylated marker RNA's, determined in additional gels, is shown by arrows. 5 S, 5 S ribosomal RNA; R I and R II, ribosomal RNA's; A_{260}, absorbance at 260 nm. Electrophoretic movement from right to left. (From Diener and Smith, 1973).

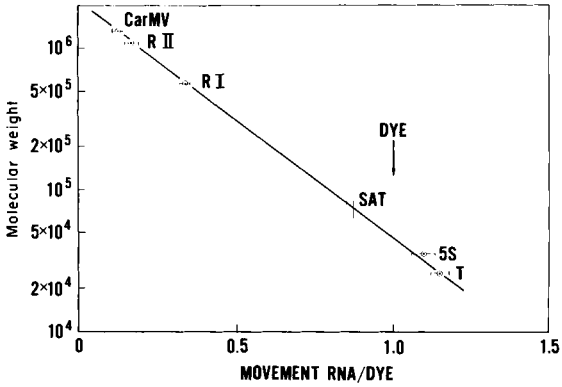

FIG. 5. Relation between electrophoretic mobility and log molecular weight of formylated marker RNA's and position of formylated tobacco ringspot satellite virus RNA (SAT). Horizontal bars represent range of values in all analyses made. Abbreviations as in Fig. 4. (From Diener and Smith, 1973.)

by vertical lines. The results indicate that the molecular weight of SAT RNA lies between 7.7×10^4 and 8.4×10^4 daltons.

Formylated PSTV was next analyzed by electrophoresis, together with formylated SAT RNA, 5 S RNA, and tRNA as markers.

Figure 6 shows the results of one analysis. The electrophoretic mobility of formylated PSTV is slightly larger than that of formylated SAT RNA. This result and those of additional experiments indicate that the

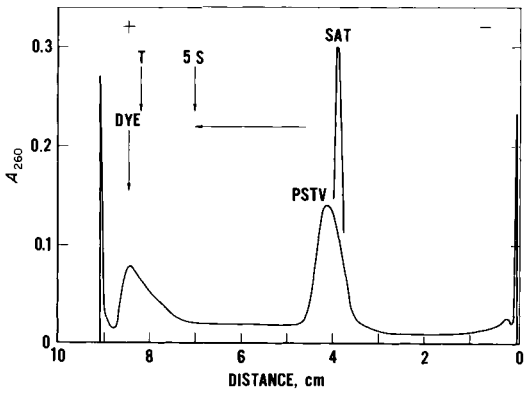

FIG. 6. UV-absorption profile of formylated PSTV after electrophoresis (in the presence of formaldehyde) in a 5% polyacrylamide gel. The position of formylated SAT RNA (SAT), as determined in separate gels, is indicated by the top of the UV-absorbing peak (shown at same scale as that of PSTV). The positions of other formylated marker RNA's are shown by arrows. Abbreviations as in Fig. 4. Electrophoretic movement from right to left. (From Diener and Smith, 1973.)

molecular weights of PSTV and SAT RNA are similar and that, therefore, the molecular weight of PSTV is within the range of 7.5×10^4 to 8.5×10^4 daltons (Diener and Smith, 1973).

2. *Thermal Denaturation Properties*

To determine whether PSTV is single- or double-stranded, thermal denaturation of the RNA in 0.01 × SSC was studied (Diener, 1972a). As shown in Fig. 7, the total hyperchromicity shift of PSTV in 0.01 × SSC is about 24% and the T_M about 50°C. The thermal denaturation curve indicates that PSTV is not a regularly base-paired structure, such as double-stranded RNA, since, in this case, denaturation would be expected to occur over a much narrower temperature range and at higher temperatures. The curve does not, however, rule out an irregularly base-paired single-stranded RNA molecule, similar to transfer RNA, in which single-stranded regions alternate with base-paired regions.

Determination of the thermal denaturation properties of PSTV dissolved in buffer of higher ionic strength (0.1 × SSC) confirmed this conclusion since, again, denaturation occurred over a wide temperature range. Under these conditions, T_M was about 54°C (T. O. Diener, unpublished).

3. *Inactivation by Ultraviolet Light*

In view of the low molecular weight of PSTV, it was of interest to determine its sensitivity to irradiation with UV light. Although one might

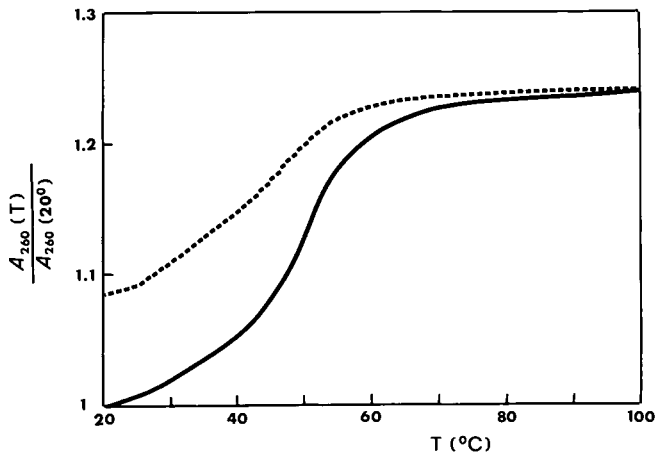

FIG. 7. Thermal denaturation curve of PSTV in 0.01 × SSC (—) and absorbance during slow cooling (---). (From Diener, 1972a.)

expect that a small molecule, such as PSTV, would be considerably more resistant to UV irradiation than a conventionally sized viral RNA or DNA, the effect of size on UV sensitivity of nucleic acids is not well understood (Adams, 1970).

Exposure of purified PSTV, of tobacco ringspot virus (TRSV), and of its satellite (SAT) to UV radiation of 254 nm showed that the inactivation dose of PSTV and SAT is 70 to 90 times as large as that of TRSV (Fig. 8). Although other explanations are possible (Diener *et al.*, 1973), this marked difference in sensitivity to UV radiation is most likely a consequence of the smaller size (smaller target volume) of PSTV and SAT RNA, as compared with TRSV RNA.

4. Electron Microscopy

In view of the purity of presently available PSTV preparations, it appeared feasible to visualize PSTV by electron microscopy and to deter-

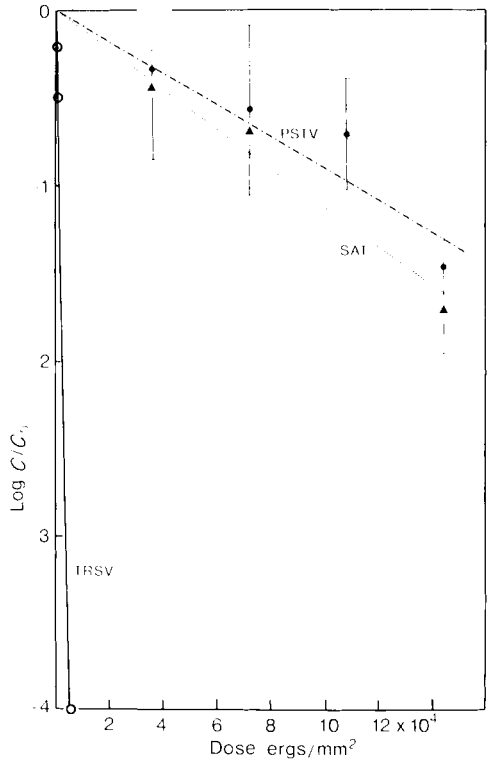

FIG. 8. Inactivation of PSTV, SAT, and TRSV by ultraviolet light of 254 nm. Logarithm of survival ratios (C/C_0) plotted as a function of incident dose. ●, purified PSTV; vertical bars, 95% fiducial limits for ratios to controls; ▲, purified SAT; ○, purified TRSV. (From Diener *et al.*, 1974).

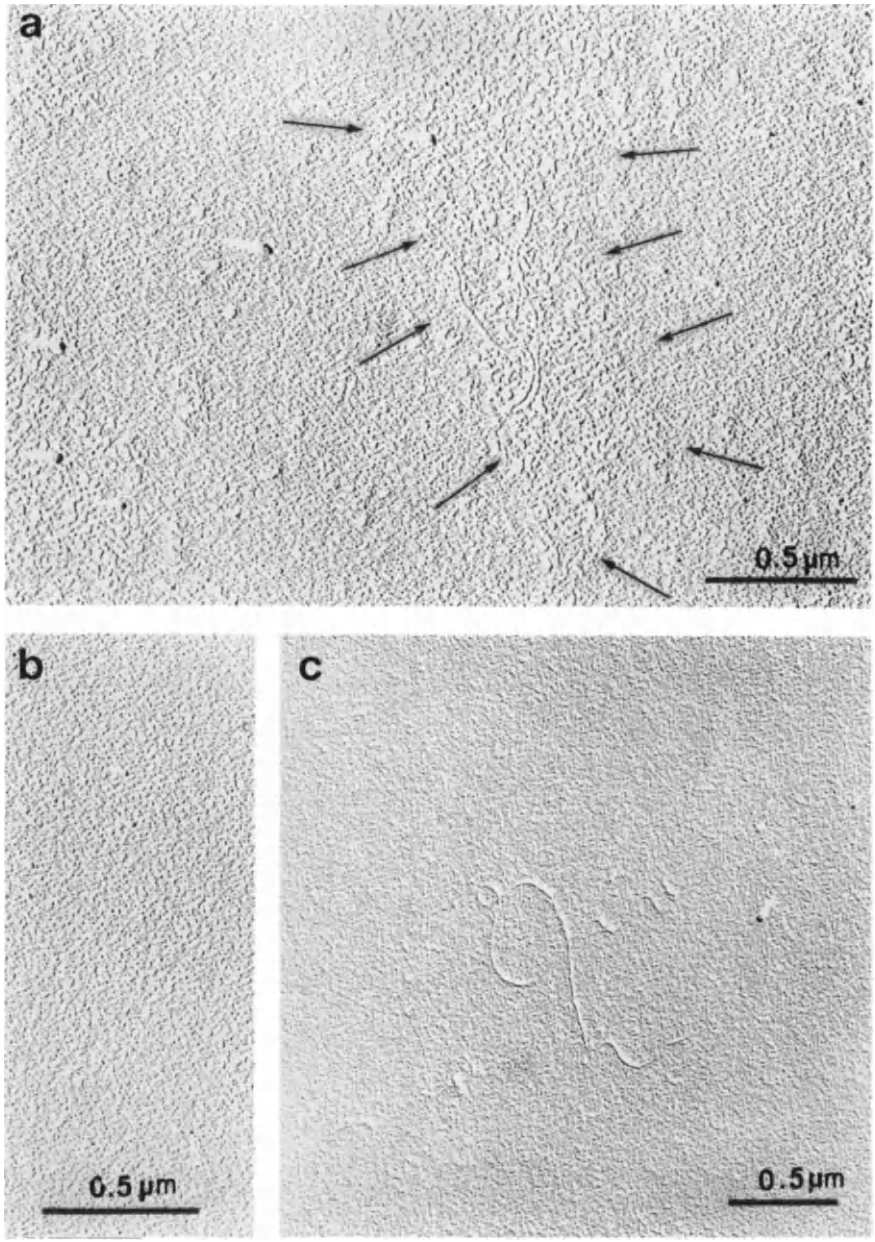

FIG. 9. Electron micrographs of preparations in 4 *M* sodium acetate, spread onto a hypophase of distilled water. Scale lines, 500 nm. (a) PSTV at a concentration of 5 µg/ml; arrows delineate the approximate edge of one large aggregate. (b) Control without RNA. (c) After treatment of PSTV with 10 µg/ml of RNase for 1 hour at 25°C. In (a) one recognizes large aggregates of short rod-like molecules, which are sensitive to RNase (c). Micrographs (a) and (c) show RNase-resistant filaments of varying length, presumably impurities from the polyacrylamide gels. (From Sogo *et al.*, 1973.)

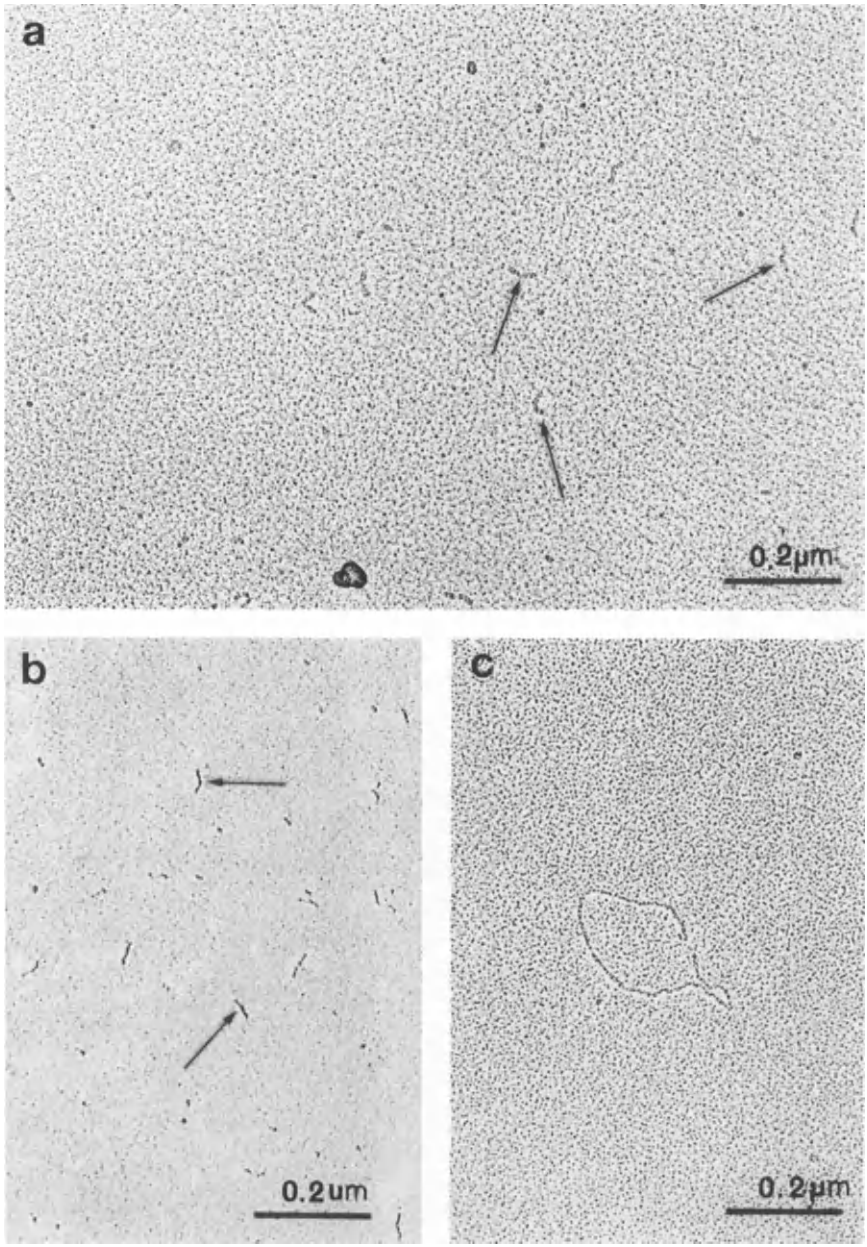

FIG. 10. Electron micrographs of PSTV spread in 0.1 *M* NaCl, 0.01 *M* EDTA, and 8 *M* urea at a concentration of 0.6 μg/ml onto a hypophase of 0.015 *M* ammonium acetate, pH 8. Scale lines, 200 nm. (a) Sample kept at 0°C. (b) Sample heated for 10 minutes at 63°C and then quenched in ice water. (c) Sample after treatment with 10 μg/ml RNase for 1 hour at 25°C. In micrographs (a) and (b), filamentous molecules with a length on the order

mine its molecular weight by direct length measurements of the RNA in electron micrographs. This, indeed, proved possible (Sogo *et al.*, 1973).

Purified PSTV preparations were processed for electron microscopy by the protein monolayer spreading technique of Kleinschmidt and Zahn (1959). When PSTV preparations in 4 *M* sodium acetate were spread onto a hypophase of distilled water, very short structures, mostly in compact aggregates, but occasionally as separate particles, were revealed (Fig. 9a). These structures could not be detected in nucleic acid-free controls (Fig. 9b), and they were absent from preparations treated with ribonuclease (Fig. 9c).

In addition to these short structures and aggregates, PSTV preparations occasionally contained a few long threads (Fig. 9a and c), which superficially resemble high molecular weight nucleic acids. These threads, however, were found to be resistant to both deoxy- and ribonuclease. Almost certainly, they are residual molecules of the gel impurity (see Section II,C), which were present in such small amounts that they could not be detected by UV spectrophotometry, but could be visualized by electron microscopy.

Because of the association of PSTV molecules in aggregates, conclusions as to their length and structure were difficult to draw from micrographs obtained with this method. Consequently, methods which promised to dissociate the aggregates and to make possible the visualization of individual PSTV molecules were investigated.

The method of Granboulan and Scherrer (1969) appeared suitable, since 8 *M* urea is known to suppress weak bonding forces. As shown in Fig. 10a, disaggregation indeed occurred with this method, and large numbers of individual short strands of relatively uniform length were visible. No such strands were discernible in the control samples or after treatment with ribonuclease (Fig. 10c).

To determine whether heating of PSTV in 8 *M* urea leads to unfolding of the molecules, preparations that had been heated for 10 minutes at 63°C, followed by quenching in ice water, were examined (Fig. 10b). Length measurements of over 2400 PSTV molecules showed, however, that the lengths of unheated and heated molecules did not differ significantly. Average length of molecules was found to be about 500 Å (Sogo *et al.*, 1973).

Since the mass per unit length is unknown, the lengths of PSTV molecules were compared with those of nucleic acids of known molecular

---

of 500 Å are recognizable (arrows). These molecules are sensitive to RNase (c). The lengths of heated and unheated molecules differ only slightly, presumably because of rapid renaturation after cooling. Looped strand in (c) is gel impurity. (From Sogo *et al.*, 1973.)

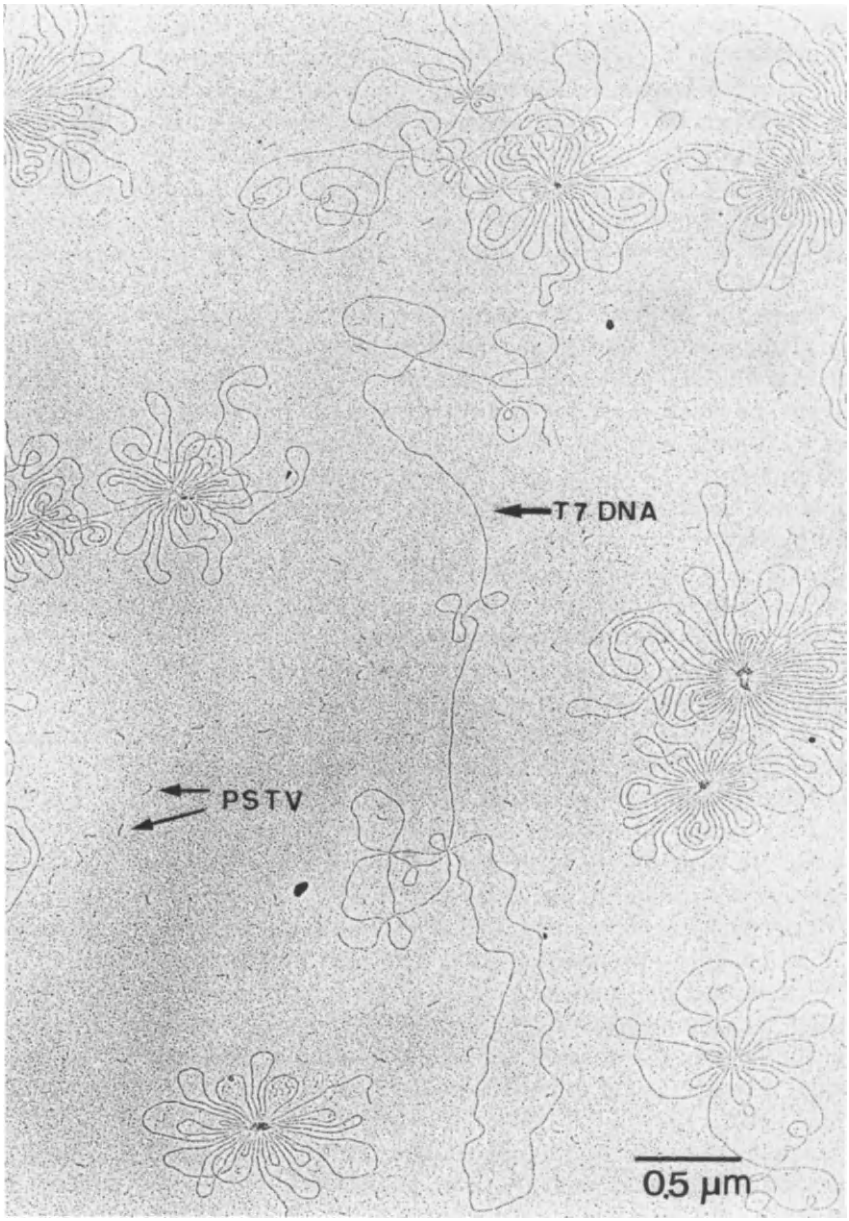

FIG. 11. Electron micrograph of PSTV mixed with a double-stranded DNA, coliphage T7 DNA. Native T7 DNA (0.8 μg/ml) was mixed with PSTV (0.4 μg/ml) previously heated for 10 minutes at 63°C in the presence of 8 *M* urea, followed by quenching in ice water. Note that double-stranded T7 DNA and PSTV have similar widths. (Courtesy of T. Koller and J. M. Sogo.)

weight, which were added to PSTV preparations and which were thus treated identically.

Figure 11 shows an electron micrograph of a mixture of a double-stranded DNA, namely, coliphage T7 DNA, and PSTV. Length measurements indicated that T7 DNA is about 280 times longer than PSTV. It is also apparent that the width of PSTV is similar to that of T7 DNA. Assuming a molecular weight of T7 DNA of 25×10^6 daltons (Lang, 1970), and assuming that PSTV in urea is formed by two more or less base-paired strands (either as a hairpin or a double helix), then one obtains a molecular weight estimate for PSTV of 8.9×10^4 daltons.

Figure 12 shows an electron micrograph of a mixture of heat-denatured, formylated CarMV RNA and PSTV. Evidently, PSTV is thicker than this single-stranded viral RNA. Since the longest filaments of formylated, heat-denatured PSTV were about 700 Å long, they were about $^1/_{17th}$ the length of denatured CarMV RNA. Assuming a molecular weight for CarMV RNA of 1.35×10^6 daltons (Kaper and Waterworth, 1973), one obtains a molecular weight estimate for PSTV of 7.9×10^4 daltons.

The molecular weight estimates obtained by electron microscopy are,

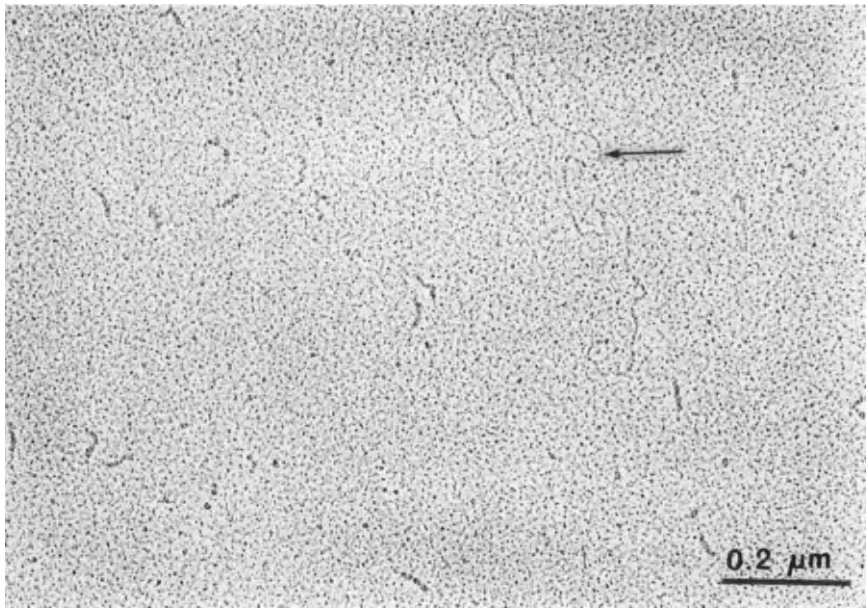

FIG. 12. Electron micrograph of PSTV mixed with a single-stranded viral RNA, carnation mottle virus RNA. Heat-denatured formylated CarMV RNA (0.5 μg/ml) was mixed with unheated PSTV (0.6 μg/ml). Note that PSTV is thicker than single-stranded CarMV RNA (arrow). Scale line, 200 nm. (From Sogo *et al.*, 1973).

therefore, in excellent agreement with the values obtained from analysis of heat-denatured, formylated PSTV in polyacrylamide gels.

5. *Structure of PSTV*

Although the low molecular weight of PSTV has been conclusively demonstrated, in light of present knowledge, a decision as to its exact structure cannot be made unambiguously. In some analytical systems, PSTV displays properties typical of double-stranded RNA, in others of single-stranded RNA.

Thus, the elution pattern of PSTV from columns of methylated serum albumin suggests double-strandedness (Diener and Raymer, 1969); whereas the elution pattern from CF-11 cellulose columns (Franklin, 1966) is consistent with both single- and double-stranded molecules (Diener and Raymer, 1969). A possible explanation for the elution properties of PSTV from cellulose columns, however, may be deduced from the work of Engelhardt (1972), who showed that the extent of secondary structure of an RNA has a profound influence on its elution pattern from such columns. He found that the greater the amount of secondary structure of an RNA at the time of addition to the column, the greater is the fraction that will elute in ethanol-free buffer, i.e., in the eluate that formerly was believed to consist solely of double-stranded RNA (Franklin, 1966). Judged by this criterion, native PSTV has an extensive secondary structure.

From hydroxyapatite, on the other hand, PSTV elutes mostly at a phosphate buffer concentration of about 0.125 *M*, apparently indicating that the molecule is single-stranded. Some PSTV, however, elutes at higher buffer concentration (Diener, 1971c).

The thermal denaturation properties of PSTV (see Section II,D,2) clearly rule out a regularly base-paired double helix; yet, in electron micrographs, PSTV appears definitely to be thicker than single-stranded RNA and to be of about equal thickness as double-stranded DNA.

Furthermore, immunological tests made with antisera that react specifically with double-stranded RNA (Schwartz and Stollar, 1969) gave no evidence for the presence of double-stranded RNA in highly infectious RNA preparations (Stollar and Diener, 1971).

Heat-denatured, formylated PSTV, however, was shown to consist of two components with somewhat different electrophoretic mobilities (Diener and Smith, 1973).

Two models are compatible with these properties of PSTV: (1) The RNA may be a single-stranded molecule with some sort of hairpin structure, involving extensive base-pairing, or (2) the RNA may be a double-stranded, but incompletely base-paired molecule.

E. Biological Properties

1. Split-Genome Model

The low molecular weight of PSTV raises the question of how such an RNA can contain sufficient genetic information to induce its replication in a susceptible host. The molecular weight of PSTV is sufficient only to code for 70 to 80 amino acids; i.e., it is barely sufficient to code for a very small protein, but not for a specific RNA-dependent RNA polymerase (replicase) of a size comparable to those of known replicases.

It is conceivable, however, that PSTV is not a single molecular species, but a population of several RNA molecules of similar length, but different nucleotide sequence, which together may comprise a viral genome of more or less conventional size. As discussed previously (Diener, 1972b, 1973b), existing knowledge does not support this model, and the high resistance of PSTV to UV irradiation (see Section II,D,3) may be interpreted as further evidence for the smallness of the PSTV genome.

2. Question of Helper Virus

In view of the very small genome of PSTV, it is tempting to speculate that PSTV is analogous to a satellite RNA and that it requires a helper virus for its own replication. Efforts to demonstrate the presence of such a helper virus in uninoculated tomato plants were fruitless (Diener, 1971b), and, since PSTV replicates in a number of solanaceous plant species other than potato and tomato (O'Brien and Raymer, 1964), hypothetical helper viruses would have to be involved in PSTV replication in these species also. In O'Brien and Raymer's experiments, such viruses could have been introduced into the plants concurrently with PSTV during inoculation with crude sap from infected plants, but Diener *et al.* (1972) showed that a number of species that can be infected with PSTV-containing crude sap can also be infected with low molecular weight RNA eluted from polyacrylamide gels. Thus, if helper viruses are involved in the replication of PSTV, such viruses must be universally present in apparently normal plants of a number of solanaceous plant species.

In view of these and other results (Diener, 1971b, 1972b, 1973b), it appears unlikely that helper viruses are involved. Hence PSTV, in spite of its small size, appears to be able to replicate autonomously in susceptible plants. So far, nothing is known about the biochemical mechanisms of this process, but theoretically possible schemes will be discussed below (see Section IV).

III. Other Viroids and Suspected Viroids

A. The Agent of the Citrus Exocortis Disease

Semancik and Weathers (1968) showed that, in plant extracts, the agent of the citrus exocortis disease has properties similar to those described for PSTV by Diener and Raymer (1967). As a result of later studies, however, Semancik and Weathers (1970) concluded that nucleic acid preparations from infected tissue contain two infectious entities, a major species that sediments with a rate of 10 S to 15 S and a minor form, that sediments with rates larger than 25 S. Since the infectious nucleic acid eluted from methylated albumin-Kieselguhr columns in the region of DNA and from CF-11 cellulose columns in 0% ethanol, the authors suspected that the RNA is double-stranded. They were unable, however, to melt the RNA or to observe any resistance of the RNA to ribonuclease in a high ionic strength medium.

On the basis of its low buoyant density in cesium sulfate equilibrium density gradients, Semancik and Weathers (1972a) suggested that the infectious agent is either a low molecular weight, tRNA-like RNA or an RNA–DNA hybrid molecule, but showed later (Semancik and Weathers, 1972b) that the duration of sedimentation used was insufficient to achieve equilibrium of low molecular weight RNA's. Electrophoresis of infectious preparations in polyacrylamide gels indicated that the RNA has a molecular weight of 1.25×10^5 daltons and suggested that a UV-absorbing component was correlated with infectivity distribution in the gels (Semancik and Weathers, 1972b).

Sänger (1972) similarly examined the agent of citrus exocortis disease in polyacrylamide gels. His results, however, indicate that the RNA has a molecular weight of 5 to 6×10^4 daltons.

Recent evidence indicates that PSTV and the agent of the citrus exocortis disease are identical or at least closely related (Semancik and Weathers, 1972c).

B. Chrysanthemum Stunt Viroid

Lawson (1968) showed that the agent of chrysanthemum stunt disease, which was formerly believed to be a virus (Dimock, 1947), has properties somewhat similar to those of PSTV. With this agent, however, the larger portion of the infectious entity was pelleted during high-speed centrifugation. Treatment of infectious extracts with phenol yielded preparations with infectivity levels comparable to those of untreated preparations (Lawson, 1968).

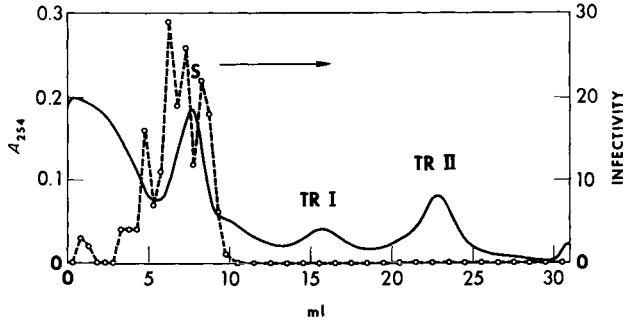

FIG. 13. UV-absorption profile (—) and infectivity distribution (--O--) of a sucrose density gradient containing partially purified CSV, tobacco ringspot satellite virus RNA (S), and the two RNA's of tobacco ringspot virus (TR I and TR II). Linear log gradient, centrifugation for 26 hours at 24,000 rpm. Centrifugation is from left to right. A_{254}, absorbance at 254 nm; infectivity, average number of lesions on 5 half-leaves. (From Diener and Lawson, 1973.)

Partial purification of the infectious RNA by methods developed for the purification of PSTV, followed by sedimentation and electrophoretic analyses demonstrated that the chrysanthemum stunt agent is a low molecular weight RNA (Diener and Lawson, 1973). As shown in Fig. 13, the infectious RNA sediments at low rates, corresponding to about 5 S to 7.5 S. Coelectrophoresis of the stunt RNA and of PSTV in 20% polyacrylamide gels showed that the RNA migrates into such gels and moves through them as a well-defined, homogeneous band with a migration rate somewhat greater than that of PSTV (Fig. 14). These results demonstrate that the chrysanthemum stunt disease is a viroid disease incited by a low molecular weight RNA similar to, but distinct from, PSTV.

C. Question of Viroids Affecting Animals

Although viroids so far identified cause diseases of higher plants, similar agents may exist in other forms of life, and it appears reasonable to search for viroids in the many instances where viral etiology of an infectious disease has been assumed, but where no causative agent has ever been identified.

One case in point is the agent of the scrapie disease of sheep. Based on comparisons of known properties of PSTV with those of the scrapie agent, the hypothesis has been advanced that the latter may also be a viroid (Diener, 1972c).

From the dose of ionizing radiation required to give an average of one

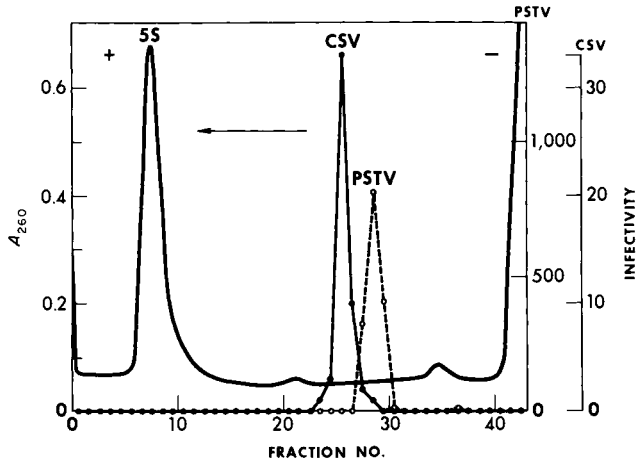

FIG. 14. Electrophoretic mobilities of 5 S RNA (5 S), of CSV, and of PSTV in a 20% polyacrylamide gel. Ultraviolet absorbance profile (—), infectivity distribution of CSV (●—●), and infectivity distribution of PSTV (○--○) determined after electrophoresis for 7.5 hours at 4°C (5 mA per tube, constant current). A_{260}, absorbance at 260 nm. Infectivity of PSTV is infectivity index; of CSV, average number of lesions on 5 half-leaves. Electrophoretic movement is from right to left. (From Diener and Lawson, 1973.)

inactivating event per infective unit of scrapie, Alper *et al.* (1966) calculated a target volume corresponding to a molecular weight of 1.5×10^5 daltons and concluded that this target size "is implausibly small as a nucleic acid code." This value is, however, compatible with the proposition that the agent is a viroid.

The scrapie agent is known to be highly resistant to UV irradiation (Haig *et al.*, 1969; Latarjet *et al.*, 1970), if exposed in homogenates from infected brains, and this high resistance has led some investigators to suggest that scrapie does not depend on a nucleic acid replicating system (Alper *et al.*, 1967).

Other investigators believe, however, that the high resistance of the scrapie agent to UV irradiation does not necessarily rule out nucleic acid as the genetic material (Adams, 1970).

Since the scrapie agent so far has not been separated from cellular constituents (Hunter and Millson, 1967), all experiments with scrapie were made with relatively crude extracts from infected tissue. Hence, energy-transfer processes, interactions between parts of a complex (Latarjet *et al.*, 1970), or shielding from UV radiation by surrounding structures (Adams, 1970) may all contribute to the apparent high resistance of the scrapie agent to UV irradiation.

Although purified PSTV does not appear to be as resistant to UV irradiation as the scrapie agent, definitive comparisons await purification of the scrapie agent. Our results (Diener *et al.*, 1973) suggest, however, that low molecular weight nucleic acids are far more resistant to UV irradiation than high molecular weight nucleic acids. Indeed, trace amounts of a small, single-stranded DNA have been identified in extracts from scrapie-infected, but not in extracts from healthy brains (Adams, 1972). No evidence exists, however, as to whether or not this DNA is specific scrapie agent DNA (Adams, 1972).

Evidently, only future work will determine whether or not the scrapie agent is a viroid and whether viroids are responsible for certain animal (including human) diseases with obscure etiology.

IV. Viroid Replication

The question of the origin of viroids can only be resolved once their mode of replication has been elucidated. Although, at present, nothing is known of these mechanisms, two basic schemes can be envisaged on the basis of known mechanisms of cellular and viral RNA biosynthesis.

A. DNA-Dependent Replication

The first scheme assumes that viroids are synthesized by the normal RNA synthesizing machinery of the cell, i.e., that they are transcribed from DNA templates.

In this case, we must assume that one or more DNA segments are present in the genomes of all host species in which a given viroid is able to replicate or, alternatively, that a novel DNA is produced in infected cells as a consequence of viroid infection, with the viroid RNA serving as a template.

If DNA segments that code for viroid RNA's are present in the genomes of all host cells, this genetic information must be completely repressed in uninoculated organisms, and the introduced viroid RNA (or a polypeptide translated therefrom) must act as a trigger to derepress these DNA segments.

This scheme invites the question of why a completely repressed DNA segment, which contains genetic information detrimental to the organism, should be maintained during evolution. Although this genetic information might normally be expressed only in another stage of development (for example, during embryogeny), one would expect that

"spontaneous" derepression of the DNA segment(s) should occasionally occur. With PSTV, large-scale commercial production of tomato plants, without reported outbreaks of the disease incited by the RNA, indicates that this is not the case.

More likely is the assumption that a novel DNA is produced as a consequence of infection and that the viroid RNA then is synthesized from this DNA through normal transcription.

Implicit in this scheme is the assumption that RNA-dependent DNA synthesis occurs in normal cells, i.e., that RNA-directed DNA polymerases (reverse transcriptases) are part of the cell's normal enzymatic complement or that such enzymes are formed by the combination of normal host proteins with a polypeptide translated from the viroid RNA.

Recent evidence indicates that ribonuclease-sensitive DNA polymerase activity may occur in normal cells (Scolnick *et al.*, 1971; Coffin and Temin, 1971), but it is not clear whether this enzymatic activity in uninfected cells is due to chance association between a cell DNA polymerase and cell RNA, or whether it is related to an RNA-directed DNA polymerase involved in normal development (Coffin and Temin, 1971).

B. RNA-Dependent Replication

The second scheme assumes that the synthesis of viroids is analogous to that of many viral RNA's, i.e., that it is DNA independent. Implicit in this scheme is the assumption that RNA-dependent RNA synthesis occurs in normal cells or that a small polypeptide translated from the viroid RNA, if combined with one or more proteins already present in the host cell, constitutes a functional enzyme that replicates the viroid RNA. The assumption that RNA-directed RNA synthesis occurs in normal cells contradicts apparently conclusive evidence that all cellular RNA species are synthesized by transcription from DNA templates (Allfrey and Mirsky, 1962). If so, no need exists in normal cells for RNA-directed RNA polymerases (Spiegelman and Haruna, 1966).

Recent evidence, nevertheless, indicates that RNA-directed RNA synthesis may occur to a limited extent in normal cells. As discussed previously (Diener, 1972b), certain bacterial RNA polymerases synthesize RNA with both RNA and DNA templates. If similar enzymes occur in higher organisms and accept a similarly wide range of RNA species as templates, such enzymes may be responsible for the replication of viroids. One report (Astier-Manifacier and Cornuet, 1971) suggests that RNA-directed RNA polymerases may occur in apparently

healthy plants; and Koch and Vollersten (1972) reported that an enzyme, which accepts viral RNA as a template for RNA synthesis, is present in *Escherichia coli.*

The alternative assumption, namely, that a functional RNA-directed RNA polymerase is produced in infected organisms by a combination of a polypeptide translated from the viroid RNA with preexisting host polypeptides, parallels, at least qualitatively, the results obtained with a small RNA bacteriophage, Qβ. Kamen (1970) showed that, in this case, the RNA-replicating enzyme, which is present only in infected cells, consists of four polypeptide chains, only one of which is virus-specific, the remainder being host-specific. Quantitatively, however, the two systems differ in that the polypeptide coded for by the Qβ RNA has a molecular weight of 60,000 daltons (Kamen, 1970), which is a value far in excess of that possible with presently known viroids.

In summary, none of the theoretically possible mechanisms of viroid replication has an exact counterpart in known replication mechanisms of cellular or viral RNA's. According to classical doctrines of molecular biology, autonomous replication of viroids should not occur. The fact that viroids do replicate in susceptible hosts indicates that mechanisms different from generally accepted ones must be operative in certain, if not all, cells.

V. Possible Origin of Viroids

Evidently, in light of present knowledge, discussion of the possible origin(s) of viroids is somewhat premature. *A priori,* viroids may be extremely degenerate or extremely primitive viruses, or they may be cellular RNA's that are unrelated to viruses.

Possibly the most fruitful approach to this discussion is to examine whether cellular or virus-related RNA species with properties resembling those of viroids are known to exist. Although such similarities among RNA's obviously do not establish phylogenetic relationships, they nevertheless may serve as a starting point for the elucidation of viroid origin.

A. Are Viroids Degenerate Viruses?

In recent years, low molecular weight RNA's have been found in association with a number of virus infections. Reich *et al.* (1966) showed

that a new low molecular weight RNA appears in the soluble fraction of the cytoplasm at the late stage of the replicative cycle of adenoviruses in KB cells. This RNA is found only in infected cells. Its nucleotide sequence has been determined (Ohe and Weissman, 1970), and the authors concluded that it is a unique species of RNA with large regions of intramolecular base pairs (Ohe and Weissman, 1971). Ohe (1972) showed that the RNA is coded for by the viral DNA. Its function is unknown, but Ohe (1972) suggested a specific participation of this low molecular weight RNA in the process of adenovirus multiplication.

Low molecular weight RNA has also been identified in *Escherichia coli* infected with Qβ bacteriophage (Banerjee *et al.*, 1969) and after induction of prophage λ (Marcaud *et al.*, 1971). Similarly, infection of tobacco leaves with tobacco mosaic virus (Babos and Shearer, 1969; Jackson *et al.*, 1972), and of broadbean leaves infected with broadbean mottle virus (Romero, 1972) lead to the appearance of low molecular weight RNA's that are not present in uninfected leaves.

In purified virions of Rous sarcoma virus (Bishop *et al.*, 1970) and of other oncogenic RNA viruses (Harter *et al.*, 1971), low molecular weight RNA's have been shown to occur.

The function of none of these RNA's is known, and it is not clear whether they are related to one another. If, however, the synthesis of unique species of low molecular weight RNA's is a common consequence of virus infection, one might speculate that viroids developed from low molecular weight RNA's that were originally virus-induced, but which have become autonomous. They would thus represent degenerate viral entities.

B. Are Viroids Primitive Viruses?

RNA's with some properties of double-stranded RNA have been identified in a variety of apparently uninfected animal cells (Montagnier, 1968a; Duesberg and Colby, 1969; Stern and Friedman, 1970; Stollar and Stollar, 1970) as well as in tomato leaves (Lewandowski *et al.*, 1971). Significantly, synthesis of some of these RNA's is less sensitive to treatment with actinomycin D than the major cellular RNA species (Montagnier, 1968a; Stern and Friedman, 1970).

Although other interpretations are possible, Montagnier (1968b) suggested that "some self-replicating RNA's may have become part of cell genetic information," and Stern and Friedman (1970) concluded that "the assumption that the synthesis of all informational RNA in normal mammalian cells is directed by DNA templates must be held in abeyance until the origin of the present RNA has been determined."

Some of the RNA's share certain properties with viroids. Stern and Friedman (1970) pointed out that, although the RNA studied by them has many properties of double-stranded RNA, it is not a simple base-pair structure. Its sedimentation coefficient; its sensitivity to ribonuclease in media of low and high ionic strength; its elution from Sephadex G-100 columns; its properties after heating to 100°C, followed by quick cooling; and its properties after treatment with dimethyl sulfoxide all resemble properties of PSTV.

In view of these results, it is tempting to speculate that, as discussed previously (Diener, 1971b), RNA's which are synthesized on RNA templates may occur in most, if not all, seemingly healthy cells. If so, viroids may have originated from these cellular RNA's. They might thus be regarded as primitive viral entities that have achieved neither the genetic sophistication to induce in susceptible hosts novel biosynthetic machinery for their own replication nor to code for specific coat proteins.

C. Are Viroids Abnormal Host RNA's?

Low molecular weight RNA's, aside from tRNA and 5 S ribosomal RNA, occur in normal cells, particularly in nuclei and nucleoli (Weinberg and Penman, 1968; Prestayko *et al.*, 1970) and associated with chromatin (Benjamin *et al.*, 1966). Although the function of these RNA's is unknown, it has been suggested that certain nuclear RNA's may have a role in regulating some nuclear functions, and that they may be involved in the regulation of RNA transcription from the genome (Frenster, 1965). Kanehisa *et al.* (1971, 1972) added credence to such concepts by demonstrating that RNA synthesis in a cell-free system of *E. coli* polymerase with chromatin as template was stimulated by the addition of a low molecular weight chromatin RNA, whereas, with DNA as template, RNA synthesis was inhibited by addition of this RNA.

Conceivably PSTV, which is associated with chromatin (Diener, 1971a) and which is in the same molecular weight range, may be related to certain normal nuclear RNA's.

One might speculate that PSTV, and presumably other viroids, originated from nuclear RNA's that are normal constituents of the organism and that are essential for its development. The change from a normal constituent to a pathogenic RNA might have occurred either by mutation or by chance introduction into a foreign species, in which the RNA is replicated. In either case, the disease would be a consequence of interference with the functions of normally occurring nuclear RNA's.

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