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MOLECULAR CHARACTERIZATION OF A RECOMBINANT POXVIRUS

City University of New York PH.D. 1986

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MOLECULAR CHARACTERIZATION OF A RECOMBINANT POXVIRUS

BY

ELCHONON MICHAEL BERKOWITZ

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1986

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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I. ABSTRACT

MOLECULAR CHARACTERIZATION OF
A RECOMBINANT POXVIRUS

by

ELCHONON MICHAEL BERKOWITZ

Adviser: Beatriz G.T. Pogo, M.D.

Vaccinia virus is a cytolytic orthopoxvirus and Shope fibroma virus is a tumorigenic leporipoxvirus. SFV-I, a fibroma isolate, is tumorigenic in vivo, but unlike other leporipoxviruses, is cytolytic rather than focus-forming in vitro. Antibodies raised against either vaccinia virus or SFV neutralize the infectivity of SPV-I. These observations suggested that SFV-I might contain genetic information from both orthopox and leporipox viruses.

SFV-I DNA was digested with various restriction enzymes and analyzed by comparison to patterns generated by the restriction endonuclease digestion of vaccinia virus and SFV-W DNAs. SFV-I DNA showed great similarity to vaccinia virus DNA with all enzymes used, but not to SFV-W DNA.

The genetic homology between vaccinia virus and SFV-

I DNAs was determined by the use of nick-translated probes to hybridize to restriction endonuclease digested DNA transferred to nitro-cellulose filters. These experiments showed that vaccinia virus and SFV-I were closely related; even fragments that migrated differently in agarose gels hybridized.

The genetic relatedness between the DNAs of orthopoxviruses, with vaccinia virus serving as the prototype, and leporipoxviruses, with SFV-W as the prototype, was also determined by Southern hybridizations. It was shown that orthopoxviruses and leporipoxviruses had no sequences in common.

When SFV-I DNA was probed with nick-translated SFV-W DNA, a subset of fragments were shown to cross-hybridize. This was also the case when the reverse experiment, in which SFV-I was the probe, was performed. These experiments showed that SFV-I, while an orthopox, also contained leporipoxvirus-related DNA.

Comparative studies of proteins of an orthopoxvirus, leporipoxvirus and SFV-I were also carried out. ^{35}S-methionine-labeled virion proteins and immunoprecipitation of virion proteins by heterologous antisera did not prove useful in identifying proteins specific for orthopoxviruses. ^{32}P-labeled

phophoproteins were in some instances shared by all three viruses: in others, they were genus-specific. Glucosamine-containing proteins of orthopoxviruses and leporipoxviruses, however, were different.

How manifold are Thy works, O Lord, in wisdom hast Thou made them all; the earth is full of Thy creatures.

Psalms 104,24

Natura nusquam magis est tota quam in minimis.

Nature is to be found in her entirety nowhere more than in her smallest creations.

Pliny the Elder
Natural History
Book 11, Section 1

II. ACKNOWLEDGEMENTS

It is my happy task, in this section, to thank the many people who encouraged, helped, and guided me during my studies at Mount Sinai. Undaunted by the prospect of sounding like a breathless Hollywood type on Oscar night, I now call the roll...

Many thanks to all the faculty of the Department of Microbiology for giving of their time and energy, and especially to the members of my Examining Committee. Kudos to Dr. Edwin D. Kilbourne, Chairman of the Department, for his interest, to Dr. Jerome Schulman for his support and advice through the (gasp!) years, to Dr. S. Mowshowitz (late of this institution) for his backing and for discussions both scientific and theologic, and to Dr. J.G. Wetmur. Thanks also to Dr. Charlotte Friend, Chairman, Department of Experimental Cell Biology, who gave me sanctuary in the Atran building.

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To Dr. R. Bablanian of Downstate Medical Center,

thanks for the words of encouragement at the poxvirus meetings (when I needed it most) and for serving on my Examining Committee.

Josh, Sharon and the kids-thanks for the banana cake and for not asking too many questions.

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Finally, as the Rabbis teach, achron achron choviv, most respected for last. I gratefully acknowledge my debt to my thesis advisor, Dr. Beatriz G.T. Pogo. From her I have learned what good science is all about, how to get the most out of every experiment, and that, though it grows increasingly harder to do so, one can still maintain one's virtue in the modern competitive world our discipline has become. Her support and encouragement will always be remembered.

III. DEDICATION

B'ezras Hashem

This work is dedicated, with all my love and respect, to my parents Emanuel Menachem and Chaya Berkowitz.

Dear Abba and Ima,

('till 120, together and in good health!)

In poor, poor recompense for all you have done for me, this is for you.

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VII. INTRODUCTION

Poxvirus is the most venerable of the virus families, having been studied microscopically and immunologically before the turn of the century. Even earlier, before the development of the germ theory of infectious disease, members of the family were being used in immune prophylaxis; variolation and vaccination were the first instances of artificial induction of immunity against infection (Fenner, 1979).

Many fundamental concepts of animal virology originated from the study of poxviruses. These include investigations on virus morphology, morphogenesis, and chemical composition, early evidence for intramolecular genetic recomination in animal viruses, and the first demonstration of the presence of virion-encoded enzymes encapsulated within the virion (Kates and McAuslan, 1967, Gold and Dales, 1968, Pogo and Dales, 1969).

A. Classification of Viruses

Early animal virology was concerned with the diseases of man and his domestic animals. This concern is reflected in the earliest virus classification schemes, which were based on symptoms caused by infection with a particular agent. The large size of poxvirus virions allowed them to be visualized in stained smears under the microscope, and they became,

in the 1930s, the first group of viruses to be classified on the basis of virion morphology (Dales and Pogo, 1981).

Animal viruses are now classified on the basis of their morphology, the physical and chemical nature of virion components, and genetic relatedness. Twenty animal virus families have been approved by the International Committee on Taxonomy of Viruses (Matthews, 1982). The division of families into genera is based on various criteria, such as antigenic relatedness, host range, or site of nucleocapsid assembly.

B. Poxvirus classification

In 1957, Fenner and Burnet gave a short description of the poxvirus group which remains the basis for classification. All members of the family Poxviridae share the following characteristics: a) a large oval-shaped (250 x 300 nm) virion, b) a genome that is a double-stranded linear molecule of DNA with a molecular weight of greater than 10^8 Daltons, and c) a cytoplasmic site of replication. The family is divided into six genera on the basis of host range and serologic relatedness (see Table 1). Within genera, species show a great deal of serologic cross-reactivity

TABLE 1.

Genera of Family Poxviridae

Orthopoxvirus	Avipoxvirus	Capripoxvirus	Leporipoxvirus	Parapoxvirus	Entomopoxvirus	Unallocated
Buffalo pox	Canary pox	Sheep pox	Lare fibroma	Bovine pustular stomatitis	Subgenus A: Melolontha	Molluscum contagiosum
Camel pox	Fowl pox	Goat pox	Myxoma			
Cow pox	Junco pox	Lumpy skin	Rabbit fibroma	Cheunois contagious ecthyma	Subgenus B: Amsacta	Swine pox
Ectromelia	Pigeon pox	disease	Squirrel fibroma			Tanci pox
Monkey pox	Quail pox			Milker's Node	Subgenus C: Chironomus	Yaba tumor virus
Rabbit pox	Sparrow pox					
Vaccinia	Starling pox					
Variola	Turkey pox					

and nucleic acid homology; the genera, as regarded by these criteria are quite distinct.

C. Poxvirus history

The large sizes of poxvirus virions and of structures associated with poxvirus replication made the family amenable to microscopic observation. The early observations predated not only the classification of poxviruses, but the differentiation between viruses and bacteria. Henderson and Patterson independantly published papers on molluscum contagiosum in 1841, describing intracellular structures that are known today as viral inclusion bodies. In 1886, Buist extracted a "clear lymph" from vaccinial or variolar pustules and was able to show that it contained tiny spherical particles. These he took to be "spores", but they are believed to have been the elementary bodies of vaccinia and smallpox viruses. In 1893, Guarnieri described inclusion bodies in the cytoplasm of cells from smallpox and vaccinia lesions, which he interpreted to be protozoa (Hughes, 1977).

Animal viral research, as an independent field of study, may be said to have begun at the Ninth International Congress for Hygiene and Demography, held in Madrid, 10-17 April, 1898. One month earlier, Loeffler and Frosch had published reports on obtaining

filterable, infectious, replicating virus from cattle with foot-and-mouth disease; they had suggested that "smallpox, cowpox, typhus, and cattle plague", among other diseases, might also be caused by such agents. Loeffler was at the Madrid meeting, and in attendance when Nocard presented his work with Roux on the discovery of the filterable mycoplasma of bovine pleuropneumonia. Also present at that historic Congress was Guiseppe Sanarelli, who described the infectious myxoma of rabbits and detailed experiments which showed the viral, and as it is now known, poxviral etiology of the disease (Hughes, 1977).

Poxviruses were at first propagated, for laboratory study, in rabbits. This remained a popular method for a time, even after Woodruff and Goodpasture (1931), working with fowlpox and vaccinia viruses, introduced the use of embryonated chicken eggs into virology. Growth of poxviruses on the chorioallantoic membrane (CAM) of embryonated eggs allowed Burnet (1936) to introduce pock counting as an assay of viral infectivity. Keogh (1936) subsequently reported on different and characteristic pock morphologies for different strains of vaccinia virus. These differences were later used to show intramolecular recombination and genetic linkage in an animal virus, using pairwise

crosses of white pock mutants of vaccinia virus to arrive at a linkage map for various "white" loci (Fenner, 1959; Gemmel and Cairns, 1959).

T.M. Rivers and his colleagues, working in the late 1930s and early 1940s purified vaccinia virus sufficiently to make accurate chemical analyses possible -ten to twenty years before similar studies could be carried out with other animal viruses. Poxviruses were the first animal viruses to be studied by electron microscopy, and the first studies of the morphogenesis of an animal virus were performed with vaccinia virus (Dales and Siminovich, 1961).

In 1967, Kates and McAuslan and Munyon et al. discovered the DNA-dependent RNA polymerase within vaccinia virus cores. This stimulated others to search for, and find, similar enzymes in the virions of other viruses, including the reverse transcriptase of retroviruses (Temin, 1971).

Finally, no review of the highlights of poxvirology would be complete without mention of the eradiction of smallpox, brought about by the interruption, through mass vaccination and intense surveillance, of human-to-human transmission of the smallpox virus.

D. Tumorigenic poxviruses

Cell proliferation is the initial result of an in vivo infection by any virus belonging to the family Poxviridae. In many instances, the pox or "microtumors" produced during infection regress quickly, due to either cell death or inflammatory response against the multiplying cells. In some cases, discussed below, cell proliferation is more pronounced. Fibromas or other tumors develop, which can persist for weeks or months before regressing, or lead to the death of the host.

Infectious myxoma, as mentioned earlier, was one of the first viral diseases to be described as such. The virus causes benign myxomas in its natural host, South American rabbits. Sanarelli observed the results of an infection by the virus of laboratory rabbits, descended from a European stock. As the disease progresses "there appear in various parts of the body small subcutaneous tumors" (Sanarelli, 1898). In addition, there is low grade fever, loss of appetite, edema of the mucous membranes, and difficult breathing (Smith, 1952). Death occurs five to fifteen days after infection. The history of the deliberate introduction of the myxoma virus, in Australia, to control the wild population of European rabbits that had become an

economic pest has been reviewed in Fenner and Ratcliffe (1965).

Shope fibroma virus (SFV) induces benign tumors in adult rabbits and malignant tumors in neonates and immunocompromised hosts. The development of lesions in adult rabbits has been described by a number of investigators (Duran-Reynals, 1940, Allison 1966, Allison and Friedman, 1966). Sell and Scott (1981) have described the immunohistological events associated with tumorigenesis. Within two days post infection, mononuclear cells markedly increase around the dermal cells. By days 7 - 10, much of the tumor is composed of atypical anaplastic fibroblasts in a lose myxomatous stroma. By day 14, necrosis of the upper dermis has occured, with a dense mononuclear infiltration adjacent to the necrotic layer. The fibroxanthosarcomatous tumor of the adult is self-limiting, most likely because of a delayed hypersensitivity response to SFV antigens; T-cell cytotoxicity to SFV-infected target cells is greatest on day 10, when the rejection reaction is at its greatest intensity.

Neonates infected with SFV show lesions much larger than those in adults (Duran-Reynals, 1940, 1945). Systemic changes are also observed; spleen and lymph nodes are enlarged, and macrophages of newborns

produce increasing numbers of viral particles. The reticuloendothelial system is thought to be flooded with particles, which due to its immaturity it cannot clear. Progressive lesions also occur in immunosuppressed adult animals (Ahlstrom and Andrewes, 1938; Harel and Constantin, 1954; Allison and Friedman, 1966).

Two other tumorigenic poxviruses are the virus of molluscum contagiosum, which causes benign growths in the skin of humans, and Yaba tumor virus, discovered in the 1960s, which causes benign tumors in Rhesus monkeys and humans (Postlethwaite, 1970; Niven et al., 1961).

The higher oncogenic potential of other virus families (Papovaviridae, Adenoviridae, Herpesviridae, and Oncornoviridae) have eclipsed the use of poxviruses as oncogenic agents; however, as the poxviruses are unique in being DNA-containing viruses which replicate in the cytoplasm of infected cells, interactions between host and virus which lead to tumorigenesis may be quite different for this family in comparison with other tumor viruses.

The study of poxvirus tumorigenicity has received added impetus by recent reports on the homology of a vaccinia virus 19 kilodalton early protein to transforming growth factor (TGF I) and epidermal growth

factor (EGF) (Blomquist, et al., 1984; Brown, et al., 1985). This protein is coded for by a gene at the left end of the genome and may be responsible for the cellular proliferation associated with poxvirus infection.

E. Poxvirus recombinants

a. Laboratory isolates

In 1958, Fenner described the biological characteristics of several strains of vaccinia virus. Pock morphology, hemagglutinin production, heat resistance, mouse neuropathology, and skin lesion production in rabbits were used to create a phenotypic profile for each isolate (Fenner, 1958). These strains were then used in co-infection experiments, which led to the demonstration of recombination between two strains of vaccinia virus (Fenner, 1959). Further advances in the study of poxvirus genetics and recombinants were made possible by the introduction of mutants and recombinants of rabbit poxvirus restricted in host range, and temperature-sensitive mutants (McClain and Greenland, 1965; Sambrook, et al, 1966).

In addition to these intra-species recombinants, there have been reports on the isolation of inter-species recombinants between various members of the orthopox genus. Woodroofe and Fenner (1960)

described hybrids between members of the vaccinia sub-group (vaccinia, cowpox, and variola viruses) and Dumbell and Bedson (1964) described a method of viral propagation which led to the isolation of hybrids between alastrim (variola minor) and rabbitpox viruses, and variola major and cowpox viruses (Bedson and Dumbell, 1964a,b). Chernos et al. (1985) recently reported on the characterization of a number of vaccinia-ectromelia virus recombinants, some of which bear the pathogenic markers of both parents (i.e., rabbit and mouse pathogenicity). In no instance has any laboratory reported on the isolation of experimentally-derived recombinants between two genera of poxviruses.

b. Natural isolates

As part of the World Health Organization campaign to erdicate smallpox, isolates of human and animal poxviruses from various locations were carefully screened. In 1972, Bourke and Dumbell reported on an isolate taken from a woman in Nigeria. They characterized this "Lenny" isolate, on the basis of its phenotypic profile, as a recombinant between vaccinia and variola viruses. Another poxvirus, taken from sentinel mice placed in Cotia county, near Sao Paulo Brazil, was isolated in 1965, but only fully

characterized in 1978 (Ueda, et al). Cotia virus is similar to myxoma and fibroma viruses on the basis of some serologic and protein comparisons, but with no obvious similarities in restriction endonuclease patterns to either the orthopox or leporipox virus groups (Esposito, 1980). While some investigators speculated on the possibility that Cotia virus might represent an orthopox-leporipox virus recombinant, further analyses were not carried out.

Racoonpoxvirus and malignant rabbit virus (MRV) are two other naturally occuring recombinant poxviruses. Racoonpoxvirus, though presently classified as an orthopoxvirus, has been shown, at the DNA level, to be only 60% homologous to other members of the genus; the source of the other 40% of the genome is unknown (Esposito and Knight, 1985). MRV is a newly-discovered poxvirus of rabbits first described by Strayer et al. in 1983. By clinical, pathological, and immunopathological criteria, the syndrome caused by MRV infection of rabbits was different from the disease induced by SFV or myxoma virus. It has now been shown that MRV is a recombinant poxvirus, with some 10 kilobasepairs (Kbp) of SFV DNA integrated into a myxoma virus genome.

Finally, a strain of SFV, designated SFV-I, has

been characterized as a possible recombinant between an orthopoxvirus and a leporipoxvirus. Studies on this isolate are discussed in the section on SFV below.

c. Marker rescue experiments

In addition to cocultivation, another technique has been developed which allows for the creation of recombinants in vitro. Marker rescue was first described for the rescue of vaccinia virus temperature sensitive mutants (Sam and Dumbell, 1981) and size variants (Nakano, Panicali, and Paoletti, 1982). In the latter case, 6.3 MDal of DNA were re-introduced into a variant that had lost the sequences during serial propagation. The rescue of these sequences was effected by infecting cells with the variant, and then adding calcium orthophosphate precipitated full length DNA. Even restriction endonuclease digested DNA could be used in the experiment, but only with enzymes that gave fragments that overlapped the deletion; thus a double recombinational event was thought to occur. These observations have led to the development of vaccinia recombinant vaccines (Mackett, Smith, and Moss, 1984), which contain for example, influenza and hepatitis B genes. My experiments with the rescue of the phenotype for plaque formation by a normally focus forming leporipoxvirus are described in the Results

section.

F. Poxvirus virion and genome

The most extensive studies on the structure and physical properties of poxvirus virions have been done with orthopoxviruses, and especially with vaccinia virus. The same general features delineating vaccinia virus characterize the family as a whole.

a. Virion

The virion of vaccinia virus is composed of proteins (88%), DNA (6%), and lipids (6%) (Dales and Pogo, 1981). The oval or brick-shaped virion is surrounded by a lipid bilayer. Superimposed onto the membrane are surface tubules, randomly arranged 5 nm diameter structures. These have been shown to consist of a single viral polypeptide with a molecular weight of 58,000 Daltons. Antibodies raised against the purified surface tubule elements (STE), a prominent polypeptide of the virion, neutralize infectivity (Stern and Dales, 1976). Certain strains of vaccinia virus are released from infected cells with an additional membrane, which is derived as a virus-modified Golgi complex membrane (Payne and Norby, 1978).

One dimensional polyacrylamide gel electrophoresis (PAGE) has resolved 50 or so polypeptide bands from the

vaccinia virion (Sterns and Dales, 1976), and two dimensional PAGE has given evidence for more than 100 polypeptides (Essani and Dales, 1979). Among virion polypeptides are a number of phosphoproteins, two glycoproteins, and a large number of proteins with enzymatic activities relating to transcription, replication, and protein synthesis. These include a DNA-dependent RNA polymerase, ssDNA exo- and endonucleases, a polyA polymerase, and a protein kinase and a protease (Dales and Pogo, 1981).

b. Viral DNA

The first estimates of the molecular weight (mol. wt.) of the DNA of poxviruses were based on the percent DNA content of the virion. This corresponded to a molecule of DNA with a mol. wt. of 160 x 10^6 daltons (Allison and Burke, 1962). Contour length measurements of vaccinia virus genomes in the electron microscope followed, leading to an estimate of 150-160 x 10^6 daltons (Hyde et al, 1967). Improvement of methods for extraction and purification of DNA led to a re-examination of the genome, with the conclusion that the mol. wt. is 120-130 x 10^6 daltons (Holowczak, 1982).

Poxvirus DNA is a linear double-stranded molecule. Two structural features which are common to all members

of the family thus far studied are 1) the presence of terminal crosslinks and 2) the presence of terminal inverted repeated sequences. The terminal cross-links were first posited during reannealing experiments with fowlpoxvirus DNA, and proven for vaccinia virus DNA through biochemical and electron microscope studies (Geshelin and Berns, 1974). The covalent linkage has been described as a continuous phophodiester backbone which connects the two strands in a hairpin conformation (Baroudy et al., 1982).

The evidence for inverted terminal repeats came from experiments in which cross-linked vaccinia virus DNA was cleaved with a single-strand specific endonuclease. The strands were then separated; allowed to reanneal, and the resulting structures were observed in the electron microscope (Geshelin and Berns, 1974). Single-stranded circular molecules with a duplex projection were seen; measurements of contour lengths showed that each molecule contained an inverted terminal repetition of approximately 10.5 Kbp. The size of the repetition varies among orthopoxvirus members; leporipoxviruses have inverted terminal repetitions of about 12 Kbp (Wills, et al., 1983).

G. Comparative studies

Comparative studies between genera of poxviruses, and between species, strains and isolates have taken

advantage of several techniques. These studies are of interest in understanding the biology of poxviruses, and for the purpose of classification as well.

Classification of a poxvirus into a particular genus, e.g. Orthopoxvirus, is relatively simple, making use of the close serologic cross-reactivity and nucleic acid homology of all the members of the genus. Identification to a particular species is less simple and relies on either serological tests with cross-adsorbed monospecific sera or phenotypic differences, such as pock morphology and host range.

As the W.H.O. program for the global eradication of smallpox progressed, two important questions were posed: 1) Does a non-human reservoir of variola virus exist in nature, and 2) can a poxvirus, which might on rare occasions infect humans, mutate into a virus with the same phenotype as variola virus. To answer both questions, methods were neccessary for the differentiation of isolates from various animal species and humans. Comparative analyses would allow both for the screening of field isolates for similarities to variola virus, and for the characterization of isolates that behaved phenotypically like variola. Two approaches that investigators pursued in their consideration of interrelationships among poxviruses

consideration of interrelationships among poxviruses were comparative studies on their proteins and DNA.

a. Proteins

One dimensional PAGE was first used to differentiate structural polypeptides and to identify polypeptides specific to subvirion structures, e.g. capsid polypeptides (Sarov and Joklik, 1972) and viral core polypeptides (Holowczak et. al, 1975). Obijeski et al. (1973) compared fowlpoxvirus (an avipoxvirus) and vaccinia virus proteins. PAGE was used by Arita and Tagaya (1977) to compare variola, vaccinia, cowpox and monkeypox viruses. Differences in the profile of polypeptide bands in the 30,000 to 40,000 (30 to 40 K) size range among these four species were seen. On the basis of these patterns, rabbitpoxvirus was placed by the authors into the vaccinia species. Ikuta et al. (1978a) compared the polypeptides of virions of vaccinia, cowpox, and Shope fibroma viruses. The two orthopoxviruses were similar, with some differences in the 40-50 K range, and in the concentration of various polypeptides, while SFV presented a considerably different profile. Turner and Baxby (1979) described experiments which showed that the polypeptides which enabled the orthopoxviruses to be differentiated were located in the surface and sub-surface layers, while

the cores of the viruses all gave the same complex polypeptide pattern.

Another approach has been to compare polypeptides induced in cells infected with various viruses. Ikuta et al. (1978b) compared the early and late virus-induced proteins of cells infected with vaccinia, cowpox, or Shope fibroma viruses. They found that the orthopoxvirus-infected cells gave similar profiles, which were different from the leporipoxvirus-infected cells. Harper et al. (1979) suggested the use of PAGE of late intracellular proteins to identify orthopoxviruses. These workers examined 24 isolates, and were able to discern four major groups of viruses on the basis of their polypeptide patterns. They used the patterns to classify buffalopoxvirus and the "Lenny" isolate as vaccinia-like viruses.

b. DNA

The development of techniques for purifying intact poxvirus DNA, the selection of appropriate restriction endonucleases, and improved procedures of agarose gel electrophoresis led to the use of restriction endonuclease methodology for the analysis of poxvirus genomes (Holowczak, 1982).

DeFilippes (1976) was the first to publish studies on poxvirus DNA with restriction endonucleases, but

reported difficulties in resolving the large number of fragments generated by Hpa I and Hpa II digestion. Gangemi and Sharp (1976) reported on the use of Hind III, in a comparison of the genomes of the CV-1 and WR strains of vaccinia virus; a small molecular weight difference in the B fragment of the DNAs was noted. Many investigators began using restriction endonucleases for comparative studies. Muller et al. (1977) compared one avipoxvirus and four orthopoxvirus genomes by analysis of the patterns generated by digestion with Hind II, Bam HI, and Eco RI, and suggested that rabbitpoxvirus was very closely related to vaccinia virus. Esposito's group at the Center for Disease Control (CDC) (1978) compared twelve species and strains (isolates within species) using DNA endonuclease electropherograms generated by digestion with Hind III, Sal I, and Bam HI.

With the utility of the technique, and the imprimatur of the CDC, restriction endonuclease patterns became, and continue to be, the method of choice for identification and classification of poxviruses. Esposito and Knight have very recently published (1985) the latest catalogue of restriction electropherograms, which compare 38 orthopoxviruses (species and strains) on the basis of their Hind III

digestion profiles.

In addition to their use in classification, restriction endonuclease analysis has been used to address questions of genome structure. Physical maps of cleavage sites for various enzymes have been generated for vaccinia virus DNA (DeFilippes, 1982) and other orthopoxvirus (Mackett and Archard, 1979) and lepripoxvirus (Delange et al, 1984) genomes. These studies have revealed certain shared structural features, as well as differences among poxviruses. Orthopoxviruses were shown to have a central region, comprising 50 - 60% of the genome, conserved among members of the genus. Variations among species were generally limited to the more plastic termini. Minor spontaneous deletions occur frequently in this region, as shown for example with vaccinia virus propagated serially in vitro (McFadden and Dales, 1979) or isolated from persistently-infected cells (Paez et al., 1985). Termini are also a locus for phenotypic changes; a white pock variant of red-pock-forming cowpoxvirus was shown to have lost 11% of its genome, all from the left end of the DNA (Archard and Mackett, 1979). A similar major terminal deletion occurs in the case of rabbitpoxvirus, also accompanied by a shift in phenotype from red pock to white pock (Moyer and Rothe,

1980).

The use of protein and nucleic acid comparisons in classification are illustrated by attempts to identify "whitepox" and Cotia virus.

Monkeypoxvirus can on occasion infect humans and give rise to a disease similar to smallpox, although human-to-human transmission is not observed. Four isolates of poxvirus taken from monkeys captured in Malaysia were found to produce white pocks when innoculated on the CAM of embryonated eggs, as does variola virus. These isolates were also reported to be variola-like by biological tests in the laboratory (Arita, 1979), and were designated as "whitepox" virus. At the same time Marennikova et al. (1978) reported on the isolation of white pock mutants of monkeypoxvirus, similar in laboratory phenotype to the "whitepox" virus. The implication was then that monkeypoxvirus might be able to mutate into a variola-like virus. Through protein PAGE (Harper et al., 1979) and nucleic acid electropherograms (Esposito and Knight, 1985), it has been established that almost all these monkeypox isolates are indistinguishable from true variola major virus. It is assumed that stocks of these isolates were contaminated by variola virus in the laboratories reporting on their isolation.

Cotia virus, as mentioned earlier in the section on recombinant poxviruses, was also subjected to both protein (Ueda et al., 1978) and DNA (Esposito et al., 1978) analysis. The results, however, were ambiguous. PAGE of the structural proteins of the virion suggested a similarity to leporipoxviruses, while the DNA electropherograms showed a unique DNA profile, unlike either orthopoxvirus or leporipoxvirus.

Comparative studies with the protein and DNA moieties of poxviruses have allowed for differentiation among isolates, providing data which show that there is no non-human reservoir of variola virus in nature, and no virus that has mutated into showing a variola virus-like phenotype. In addition, as the studies with pock variants show, these studies have been used to correlate specific genetic loci with particular phenotypes.

H. Shope fibroma virus

a. History

SFV was isolated in 1932, by Richard E. Shope, from a fibroma on the foot of a wild cottontail rabbit. Shope (1932a) described the transmissibility of the tumor-like condition to laboratory rabbits, and the clinical and pathological characteristics of the condition. In an accompanying paper (Shope, 1932b), he

presented evidence for the etiology of the condition - a filterable virus, immunologically related to the agent of myxoma. The virus has since been isolated on various occasions from tumors of rabbits trapped in the wild (Kilham and Woke, 1953; Dalmat, 1958; Kasza, 1974).

SFV was used in the 1930s and 1940s as a tumorigenic agent in various studies. Ahlstrom and Andrewes (1938) showed that rabbits injected with tar responded to SFV innoculation by producing lesions that regressed much slower than in normal rabbits, and which could grow progressively and invasively, leading to generalised fibromatosis and death. Clemmesen (1939) showed similar results with irradiated rabbits. Duran-Reynals (1940, 1945) compared the effects of SFV infection of adults versus newborns, finding the disease usually fatal in the neonate. Allison and Friedman later (1966) used these observations and other experiments (such as studies on the fatal consequence of SFV infection of immunosuppressed animals (Harel and Constantin, 1956)) to conclude that regression of fibromas was due to a cell-mediated immune response.

b. Virion and Genome

The first electron micrographs of SFV were published in 1955 (Lloyd and Kahler, 1955). These

revealed the typical brick-like structure of a poxvirus, with dimensions of 230 x 280 nm. Studies on the structural proteins of the virion were performed by PAGE; 35 - 40 polypeptide bands were separated by Ikuta's group (Ikuta et al, 1978a) and a similar number were visualised by Pogo et al. (1982a). No correlation between bands and structural features have been reported, and no two dimensional gels have been published.

The size of the SFV DNA was first estimated by Jacquemont et al. (1972) to be 153 megadaltons (230 Kbp). Summation of the molecular weights of fragments produced after digestion with various restriction endonucleases leads to a figure of 106.5 megadaltons (160 Kbp) (Wills et al., 1983). The genome is terminally cross-linked (Pogo et al, 1982a) and contains 12 - 13 Kbp of terminal inverted repeat sequences (Wills et al., 1983). The DNA of SFV has been cloned, and a physical map constructed (Delange et al., 1984; Cabirac et al., 1985).

c. In Vitro Studies

SFV was first grown in tissue culture by Kilham (1956). The development of a focus assay for the virus (Padgett et al., 1962) was an important step for quantitation, since the virus does not grow in the

chorioallantoic membrane (CAM). Hinze and Walker (1964) infected rabbit cells with SFV and observed a persistent viral infection in the cell cytoplasm, along with an altered cell morphology and cell multiplication with loss of contact inhibition. Cells made incapable of proliferation either by gamma irradiation or by cell crowding and serum deprivation still showed an altered cell morphology upon infection with SFV (Tompkins et al., 1969), indicating that the cytopathological effects of SFV infection were independent of cell division.

Studies of the infectious cycle of SFV in rabbit kidney cells (Chan and Hodes, 1973) have revealed a complex set of events. Very early after infection, a burst of host DNA synthesis in the nucleus is observed, followed by a progressive shut-off of nuclear functions that lasts for three to four days post infection. Afterwards, a sub-population of infected cells begin to divide at an increased rate. The progressive inhibition may be caused by endogenous DNAses brought in by the virus (Pogo et al, 1982b); these enzymes are known to digest newly-synthesied host DNA in vaccinia virus infected cells (Olgiati et al., 1976). Inhibition of host nuclear functions may also be due to the inhibition of host protein synthesis that commonly

follows infection with a poxvirus (Shatkin, 1963). While the work of Chan and Hodes was done with a SFV variant, the results parallel findings with other poxviruses (Bablanian, 1984).

The synthesis of viral mRNA and progeny DNA, which, as with other poxviruses, takes place in the cytoplasm, occurs mainly during the first twenty-four hours after infection (Ewton and Hodes, 1967).

d. Classification

SFV is classified as a leporipoxvirus on the basis of the following considerations: 1) Antigenic relatedness to myxoma virus, 2) Same host range and tumorigenic potential as myxoma virus, 3) Structural polypeptides similar to myxoma virus and 4) No nucleic acid homology to orthopoxvirus, but some homology to myxoma virus (Wills et al., 1983).

e. Cytolytic and non-cytolytic strains of SFV

Hinze and Walker (1971) reported on the characterization of a naturally-isolated strain of SFV which, instead of causing cell proliferation, was cytolytic in rabbit kidney cells. When this strain was compared to the original Patuxent strain, and to other fibroma strains, no differences in plaque type, or in reactivity to antibody were found. Tumors were induced in rabbits by the cytolytic strain slightly faster

(i.e., three weeks post infection) than tumors induced by other strains (four to five weeks post infection). The gross and microscopic appearances of the tumors induced by cytolytic and non-cytolytic strains were the same.

Two strains of SFV, one cytolytic, obtained from Dr. Hodes, U. of Indiana and named SFV-I, and one non-cytolytic, obtained from Dr. Hinze, U. of Wisconsin and named SFV-W have been studied by Pogo, et al. (1982a, b). Both strains were known to induce tumors in rabbits and to be sensitive to anti-SFV antiserum raised against the Patuxent strain (See Table 2). The synthesis of viral DNA was studied by following the incorporation of labeled thymidine into the cytoplasmic fraction of infected BSC-1 cells. The kinetics of incorporation were similar in both strains, though the amount incorporated was two- to three-fold greater in SFV-I infected cells.

Preliminary restriction enzyme digests of isolated DNA from the two strains showed differences in the

TABLE 2. EFFECTS OF ANTISERA ON VIRUS INFECTIVITY

ADDITIONS	VACCINIA	SFV-I	SFV-W
None	1×10^8 PFU/ml	5×10^8 PFU/ml	1.5×10^5 FFU/ml
Anti vaccinia (dil 1:20)	6×10^5 PFU/ml	2×10^5 PFU/ml	1.5×10^5 FFU/ml
Anti vaccinia (dil 1:10)	5×10^4 PFU/ml	1×10^5 PFU/ml	1.5×10^5 FFU/ml
Anti SFV-I (dil 1:5)	1×10^8 PFU/ml	1×10^5 PFU/ml	5.0×10^4 FFU/ml
Anti SFV-I (dil 1:2)	1×10^7 PFU/ml	1×10^5 PFU/ml	1.0×10^4 FFU/ml
Anti SFV-W (dil 1:5)	1×10^8 PFU/ml	1×10^5 PFU/ml	2.0×10^3 FFU/ml
Anti SFV-W (dil 1:2)	1×10^8 PFU/ml	5×10^4 PFU/ml	1.0×10^3 FFU/ml

Virus preparations were incubated with serial dilutions of the antisera for one hour at 37 C and then assayed for infectivity as described in Materials and Methods.

size, number, and molar amounts of fragments. One-dimensional polyacrylamide gel electrophoresis of the structural proteins of the two SFV strains also revealed differences in the polypeptide composition (Pogo et al., 1982a).

Some of the enzymatic activities present in the virions of the two strains were also studied. Both SFV strains showed equivalent amounts of the nucleotide phosphohydrolase, protein kinase, and DNA-dependent RNA polymerase activities. In contrast, SFV-W has less of the pH 4.5 DNAse activity, and an almost undetectable level of the pH 7.8 endonuclease activity as compared to SFV-I.

Another difference observed between the two strains was the fate of the parental DNA after infection. As mentioned above, the genome of SFV is cross-linked at each end of the linear molecule. Removal of these cross-links would seem to be essential for replicaton of viral DNA (Berns and Silverman, 1970). When both parental DNAs were extracted from cells three hours after infection and sedimented through alkaline sucrose gradients, it was found that only 25% of the SFV-I molecules remained cross-linked (faster sedimenting), while 75% of the SFV-W DNA still contained cross-links (Pogo et al, 1982b).

The differences in DNAse activities and in the efficiency of the removal of cross-links between the two strains may help to explain, in part, their distinctive behaviors in cell culture. Nucleases have been implicated in the early shut-off of host DNA synthesis and the removal of viral cross-links (Pogo and Dales, 1973, 1974, 1977). Thus, less DNAse activity may result in less detrimental effects to the host cell, and also in a dimunition in the number of viral templates available for replication.

I. Goals of Research

The aims of my research were to establish the basic, molecular differences between the cytolytic SFV-I and non-cytolytic SFV-W strains of Shope fibroma virus. The results are relevant to the study of poxviruses in general, the biology of tumorigenic poxviruses, and the elucidaton of mechanisms involved in tumorigensis and cytolysis.

Antibody studies have shown that vaccinia virus antiserum has no effect on the focus-forming ability of SFV-W, but diminishes the number of plaques produced by SFV-I. This latter result was unexpected, because there was thought to be no cross-neutralization between orthopox viruses and leporipox viruses. In addition, preliminary studies indicated that vaccinia virus and

SFV-I, but not SFV-W, share many restriction endonuclease sites and virion proteins. It is important to emphasize that SFV-I is still able to induce tumors when innoculated into rabbits, and that SFV-W antiserum inhibits the production of plaques by SFV-I, indicating that both SFV strains share common antigenic determinants.

Taken together, these results suggested that SFV-I might be a recombinant between an orthopox virus (e.g., vaccinia) and SFV. An important aspect of my work was thus to establish the origin of SFV-I and to provide new data on the relatedness of the two SFV strains, the degree of homology between leporipoxvirus and orthopoxvirus, and the possible recombinant provenance of the SFV-I genome.

In addition to clarifying the relationship among various poxviruses, the results I obtained would allow for the identification of sites in the DNA and specific polypeptides that might be related to the different biological activities of these viruses. The methods I employed (and which are described in the following section) allowed me to carry out comparative studies on the genes and some gene products of orthopox and leporipox viruses. My results revealed, to various degrees, molecular similarities and differences which

might account for these viruses' distinctive behaviors.

VIII. MATERIALS AND METHODS

A. Cells and Media

L cells (mouse, fibroblast, similar to ATCC CCL #1.2) and SIRC cells (Rabbit cornea, fibroblast-like, ATCC CCL #60) were used in the studies described in this work. The cells were maintained at 37°C, in a 5% CO_2 atmosphere, in tissue culture media. The composition of the culture media was Modified Eagle's Media (MEM) and Earle's salts (Flow Laboratories), 2 mM Glutamine (Grand Island Biologicals Company (GIBCO)), 0.19% Sodium bicarbonate (M.A. Bioproducts), and 10% heat-inactivated (56°C for 30 minutes) fetal bovine serum (FBS) (M.A. Bioproducts).

B. Viruses

The following plaque- or focus-purified viruses were used:

1) Vaccinia virus - strain IHD-W (similar to ATCC VR 156); 2) Shope fibroma virus - strain Wisconsin - gift of Dr. Hinze, University of Wisconsin - a Patuxent (ATCC VR 364) strain; 3) Shope fibroma virus - strain Kasza -gift of Dr. McFadden, University of Alberta - (ATCC VR 113); and 4) Shope fibroma virus - strain Indiana - gift of Dr. Hodes, University of Indiana.

C. Virus propagation

Viruses were propagated in the appropriate cell lines (vaccinia virus in L cells, SFV in SIRC cells) by infecting 80-100% confluent monolayers at a multiplicity of infection (moi) of 1 - 5 plaque forming units (PFU) or focus forming units (FFU) per cell, and harvesting after either 36 - 48 hours (vaccinia and SFV-I) or 4 - 5 days (SFV-W and SFV-K). To obtain radioactively labeled virions, the following media were used:

For ^{35}S-methionine labeling: MEM and Earles salts, without methionine (Selectamine Kit, Gibco) with 2% FBS and 1 - 2 uci/ml L-(^{35}S) - Methionine (100 Ci/mmol) (NEN); For ^{3}H-glucosamine labeling: MEM and Earle's salts, without glucose (gift from Dr. M. Esteban, Downstate Medical School, New York) with 2% FBS, 100 mg/L glucose (i.e., 10% of normal level) and 2 - 5 uci/ml D - (1,6 - ^{3}H (N)) - Glucosamine hydrochloride (38.9 Ci/mmol) (NEN); For ^{32}P-phosphate labeling: MEM and Hank's salts with 2% FBS and 3 - 5 uci/ml ^{32}P-orthophosphate (NEN).

D. Virus purification

Virus purification is conveniently considered as a two-step process: making a crude virus preparation, and then further processing the preparation to obtain pure virus. As most of the virus produced during

infection remains associated with cell membranes and debris, the purification procedures have been designed to allow for the production of virus free from such contaminants.

Crude virus preparation:

Virus-infected cells and media are centrifuged, in a Sorvall GSA rotor, at 7000 x g for 30 min. The pellets are resuspended in a small aliquot (2-6 ml) of cell media without FBS, and sonicated in a Raytheon Oscillator, at 10K cycles/second, for one min. The resulting cell suspension is centrifuged at 1000 x g for 10 min. The supernatant, usually cloudy, is saved, and the pellet resuspended and resonicated. This procedure is repeated until the supernatant is clear. All the supernatants are combined, and treated for 20 min. with 1/10 volume of trypsin (.125%; Cooper Biomedical) at 37°C. The trypsin is then inactivated by the addition of 1/10 volume of FBS. This is now a crude preparation of pox-virus, which can be used as a stock for infection, after determination of infectivity (see plaque and focus assay below) or further processed to obtain purified virus.

Pure virus preparation:

The crude preparation is centrifuged for 30 min. at 23,000 (23 K) rpm in a SW 50.1 rotor. Pellets are

resuspended in 1.2 ml aliquots of 10 mM Tris pH 7.2 and 0.3 ml of 40% sucrose (w/v) in 10 mM Tris pH 7.2. The number of 1.5 ml aliquots used are determined by the size of the pellets. The aliquots are homogenized by means of a Dounce homogenizer and loaded onto a discontinous sucrose gradient consisting of 2 ml 20% (w/v) sucrose in 10 mM Tris pH 7.2 over 2 ml 40% (w/v) sucrose in 10 mM Tris pH 7.2, and centrifuged at 18 K rpm for 90 min. Pellets are resuspended in 0.8 ml 10 mM Tris pH 8.5 plus 0.2 ml 50% potassium tartrate (w/v) in 10 mM Tris pH 8.5, homogenized with a Dounce homogenizer, loaded onto a continuous 20 - 50% potassium tartrate (w/v) in 10 mM Tris pH 8.5 gradient, and centrifuged for one hour at 18K. The viral particles, banding at a density of 1.5 gm/ml are combined, resuspended in 10 mM Tris pH 7.2, and centrifuged for 30 min. at 23 K. The pellet, comprising the pure virus, is resuspended in a small volume (200 - 400 ul) of 10 mM Tris pH 7.2.

E. Plaque assay for vaccinia virus and SFV-I

Aliquots of 0.1 ml of crude virus preparations are diluted with 1.9 ml tissue culture media, and serial 10-fold dilutions are prepared. 0.2 ml aliquots of the dilutions are adsorbed onto confluent 60 x 15 mm dishes (Falcon) of L or SIRC cells for one hour at 4°C. The

virus suspension is removed, the monolayer washed once with cell media, and then covered with an overlay composed of MEM plus 5% FBS plus 0.9% (w/v) agar (Difco). The plates are incubated, at 37°C, for 3 days, then fixed with 37% formaldehyde (Fisher) for 30 min. Afterwards, the formaldehyde is decanted, the agar overlay removed, and the cells stained with hematoxylin for 30 min. The hematoxylin is removed, the stained cells washed with H_2O, and the plaques counted.

F. Focus assay for SFV-W and SFV-K

Virus dilution and adsorption are performed as for plaque assay (see above) using SIRC cells. The virus suspension is removed, and the monolayer is washed with cell media. Plates are incubated for 4 - 5 days, at 37° C, with 5 - 6 ml tissue culture media, to allow foci to develop. Afterwards the media is decanted, the cells fixed with methanol for 15 min., and then stained with Giemsa - Wright (21:3:1 of H_2O: Giemsa stain: Wright stain) for 30 min. The stain is washed off and the foci are counted in an Integrid tissue culture dish (Falcon) as described by Pogo et al. (1982a).

G. DNA Extraction

Preparations of purified poxivrus are digested overnight, at 37°C, with 1-1.5 mg/ml proteinase K (E.M.

Laboratories) and 0.5% Sarkosyl. Phenol extraction is carried out by additon of an equal volume of phenol (equilibrated with buffer - 10 mM Tris pH 8.0 plus 0.2% 2-mercaptoethanol), gentle mixing, and centrifugation in an Eppendorf microfuge for 3 - 5 min. The upper phase is re-extracted with phenol, and then treated with an equal volume of water-saturated ether. The ether is removed and the tube is placed in a chemical hood to allow ether fumes to evaporate from the lower phase. Precipitation of the DNA is achieved by addition of sodium acetate to 150 mM, and 2 volumes of cold ethanol. After gentle mixing, fibers of DNA are usually visible at this point. The DNA is spun down in the microfuge for 15 min. and the ethanol removed with a syringe. The pellet is washed once with 70% ethanol, and resuspended in H_2O or TE (10 mM Tris pH 7.6 plus 1 mM EDTA).

H. Quantitation of DNA

The amount of DNA present was determined by measuring the absorbance of aliquots of resuspended DNA at wavelengths of 260 and 280 nm in a Gilford spectrophotometer (Model 2400).

I. Restriction endonuclease digestions

Bam HI, Eco RI, Hind III, Sal I, and Sst I were used according to the manufacturers (Bethesda Research

Laboratories (BRL), International Biotechnologies, Inc. (IBI)) recommendation. Enzymes were always added in excess, while keeping the final concentration of glycerol (present in the enzyme solutions) below 5%. Digestions were carried out for 3 hours, with half the final volume of enzyme added 1.5 hours after the start of the incubation.

Restriction endonuclease reactions were terminated by addition of 1/10 volume of a solution containing 60% sucrose, 100 mM EDTA, and 0.15% Bromphenol blue.

J. Agarose gel electrophoresis

Agarose (BRL) gels were run in a 22 x 14 cm horizontal apparatus using a buffer (TBE) containing 89 mM Tris base, 89 mM boric acid, 2 mM EDTA and 0.3 ug/ml ethidium bromide. Bands were visualized on an ultraviolet light box (UV Light Products, Model TM-36), and photographed through a Wratten 23A filter with Polaroid type 084 film.

Gel concentration was generally 0.5% agarose (1.5 g/ 300 ml TBE). This concentration gave the best spread of bands while giving enough rigidity for further handling of the gels. There was no difference in pattern observed when using TBE or a Tris-acetate buffer. After running the samples out of the wells and into the matrix at 120V for 20 min., the gels were run

at 50 V for 16 - 18 hours.

K. Southern procedures

a. Southern transfer

Transfer of the restriction fragments from agarose gels to nitrocellulose paper can be performed immediately after being run, or after storage at 4°C. Gels are first treated for 20 min. with 250 mM HCl to depurinate the DNA. This is followed by treatment with several volumes of 1.5 M NaCl plus 0.5 M NaOH for 1 hour at room temperature, with constant shaking. Further neutralization was carried out by soaking the gel in a solution containing 1.5 M NaCl plus 1M Tris pH 8.0 for 1 hour with constant shaking.

The transfer is performed as follows: the two chambers of a horizontal gel apparatus are filled with 10X SSC (20X SSC is composed of 175.3 g NaCl and 88.2 g Na Citrate in 1 liter H_2O). A strip of Whatman 3MM paper long enough to bridge the distance between the two chambers, and wide enough to accomodate the gel, is placed in the apparatus and wetted with 10X SSC. The gel is inverted so that its bottom side is now on top, and placed onto the 3MM paper. A piece of nitrocellulose paper (Schleicher and Schuell BA 85), cut to the gel's size, and thoroughly wetted with 2X SSC, is placed on the gel. (The upper left corner of the

filter is cut for orientation.) Two pieces of 2X SSC-wetted Whatman 3MM paper, cut to size, are placed on the nitrocellulose paper, and atop these a 5 cm stack of cut-to-size paper towels are placed. Finally, weights are added to the stack, to insure an efficient transfer across the entire area of the gel. The transfer is allowed to proceed overnight. The paper towels and 3MM paper are removed and discarded; the filter is removed from the dehydrated gel, and then washed in 6X SSC for 5 min. The filter is allowed to air dry and then is baked in a vacuum oven (National Appliance Company) for 2 hours at 79°C. The blot can be stored at this stage.

b. Southern hybridization

Two methods were utilized in hybridization experiments (see Results section). One method, described in the Molecular Cloning Manual, (Maniatis et al., 1982), is designated CSH, and the other, taken from Wahl et al. (1979) is designated Formamide. Both methods consist of two steps: prehybridization (to saturate non-specific sites of DNA binding on the filters) and hybridization.

1. CSH method

The filter is wetted with 6X SSC and placed in a heat-sealable pouch (Kapak Company). 0.2 ml of

prehybridization fluid per square cm of filter, warmed to 68°C, is then poured into the bag. Prehybridization fluid consists of 6X SSC plus 0.5% SDS, with 5X Denhardt's solution (50X Denhardt's solution is made by combining 5 g Ficoll, 5 g polyvinyl pyrolidine (PVP), and 5 g BSA (Pentax fraction V) in 500 ml H_2O) and 100 ug/ml denatured calf thymus DNA (Worthington) (The DNA, in a concentration of 10 mg/ml H_2O is sheared by passing the solution a number of times through a 18 guage needle, and denatured by boiling for 10 min.).

The bag is sealed in a heat sealer (Krups Electronic) and submerged in a water bath, at 68°C, for three hours. Afterwards, the bag is cut at one corner, the prehybridization fluid is removed and replaced with 50 ul/cm^2 of filter hybridization buffer. The hybridization solution consists of 6X SSC, 10mM EDTA, ^{35}S-labeled, denatured probe DNA, 5X Denhardt's solution, 0.5% SDS, and 100 ug/ml denatured calf thymus DNA.

The bag is resealed and submerged in the water bath, at 68°C, overnight. Afterward, the hybridization buffer is removed and the bag is cut along three sides and the filter removed. The filter is washed in the following solutions: Solution 1 - 2X SSC plus 0.5% SDS at room temperature, 5 min.; Solution 2 - 2X SSC plus

0.1% SDS at room temperature, 15 min.; and Solution 3 - 0.1X SSC plus 0.5% SDS at 68°C, for 2 1/2 hours. The filter is then air dried, placed in a cassette with X-ray film, and exposed for an appropriate time, at -70°C.

2. Formamide method

The procedures and times of prehybridization, hybridization, and washings are similar to those used in the CSH method. The prehybridization solution consists of 5 ml containing 50% formamide, 0.1% SDS, 2X SSC, 5% dextran sulfate (Pharmacia), 1X Denhardt's solution, and 250 ug/ml denatured DNA. The hybridization solution consists of 5 ml containing 50% formamide, 0.1% SDS, 2.4X SSC, 10% dextran sulfate, 100 ug/ml DNA and 5mM EDTA.

L. Preparing DNA probes

The nick-translation method of Rigby, et al. (1979) was used, employing the BRL nick translation kit and 150 pmoles deoxyadenosine 5' (α-thio) triphosphate, (^{35}S) (NEN) per reaction.

The reaction mixture is made up of five elements: Buffer, enzymatic mixture, DNA, H_2O, and radioactive nucleotide. The buffer contains 0.02 mM dCTP, DGTP, and dTTP; 50 mM Tris-Cl, pH 7.8; 5 mM $MgCl_2$; 10 mM 2-mercaptoethanol; and 10 ug/ml BSA. The enzymatic mixture consists of 2 U of DNA Pol I, 200 pg DNAse I, 5

mM Trics-Cl, pH 7.5, 0.5 mM MgAceate, 0.1 mM 2-mercaptoethanol, 0.01 mM PMSF, 5% glycerol, and 10 ug/ml BSA.

An aliquot of DNA (0.5 - 1 ug), resuspended in H_2O, is added to an Eppendorf tube in which the radioactive nucleotide has been previously lyophilized (with a Speedvac apparatus). Sterile water is added to bring the final volume to 50 ul. The tube is placed on ice while the other components of the reaction are added. The tube is then incubated in a 15°C water bath for 70 min., a time at which I had established that the nick translation reaction had reached a plateau. The reaction is terminated by the addition of EDTA to a final concentration of 30 mM. The nick-translated DNA is precipitated by the addition of 1/2 volume of 7.5 M $(NH_4)_2$ OAc and 2 volumes of ethanol, and kept at -20°C overnight. The DNA is centrifuged in the cold in a microfuge for 20 min. The supernatant is aspirated by syringe, and the precipitate resuspended in 50 ul of H_2O.

M. Quantitation of incorporation

A 1 ul sample of the reaction mixture plus 20 ug carrier DNA is mixed with cold 10% TCA in a test tube, at 4°C for 15 min. The precipitate is then collected on a Millipore filter. The filter is dried, and

counted in a mixture of Toluene plus Omnifluor, in a Beckman LS 6800 scintillation counter. Incorporation of $5 \times 10^7 - 1 \times 10^8$ cpm/μg DNA are routinely achieved.

N. Autoradiography

Filters and gels are placed in Kodak X-ray film cassettes with Kodak XOMat (XR-5) film, and with Dupont Cronex Lightning Plus B intensifying screens for ^{32}P-Labeling experiments, and allowed to expose at $-70^{o}C$. At appropriate times, the films are developed in an automatic X-ray film developing machine (Mount Sinai Dept. of Radiology).

O. Polyacrylamide gel electrophoresis

A Biorad vertical slab gel apparatus was used. All reagents were purchased from BRL. The procedure was based on Laemli, et al. (1970).

The following stock solutions are prepared: Acrylamide: bisacrylamide 30% : 0/8% (w/v): Tris-HCl, 3M, pH 8.9: Tris-HCl, 1.25 M, pH 6.8, 20% SDS, 10% ammonium persulfate, and TEMED.

After the apparatus is assembled, the gel is set up in two stages - Running gel and stacking gel. The running gel consists of 12.6% acrylamide and is prepared by combining 14.4 ml H_2O plus 13.5 ml Acryl:bis, with 4 ml Tris, 3 M, 0.2 ml SDS, 20 ul TEMED and 70 ul ammonium persulfate. The stacking gel

consists of 4.5% acrylamide and is prepared by combining 7 ml H_2O, 1.5 ml acryl:bis, 1.5 ml Tris, 1.25 M, 50 ul SDS, 8 ul TEMED, and 50 ul ammonium persulfate. The ammonium persulfate must be a fresh solution (less then two weeks old).

The solution containing the 12.6% acrylamide is applied up to 8.5 cm from the bottom of the gel plate. A layer of water (1 - 2 ml) is then added on top of the gel solution, which is then allowed to polymerize for 30 min. The water layer is then decanted and the solution containing the 4.5% acrylamide is applied. This stacking gel, with well-forming comb in place, is allowed to polymerize for 20 min. The comb is removed, and the top and bottom chambers of the gel apparatus are filled with running buffer, which consists of 28.8 g glycine, 6 g Tris base, and 7.5 ml 20% SDS per 1.5 liters H_2O. Aliquots containing protein samples are loaded, and run through the stacking gel at 150 V. When the dye front (see below) has moved into the running gel, the voltage is turned down to 100 V. The dye front is run off the gel; the run is completed in 3 to 4 hours.

The samples are prepared before loading by adding an equal volume of dye mixture and boiling for 5 min. Dye mixture contains, per ml, 100 ul of 1.25 M Tris pH

6.8, 50 ul of 20% SDS, 100 ul of glycerol, 20 ul of 2-mercaptoethanol, 20 ul of bromphenol blue, and 710 ul H_2O.

The amount of proteins and radioactivity in the samples are determined as described in sections P and Q, below.

After the run, the gel is dried down on Whatman 3MM paper in a gel drier (BioRad, Model 224) for 2 hours, and then autoradiographed for the appropriate time.

P. Labeling virion proteins

The virus is propagated in the presence of radioactive media (see virus propagation, section C above) to produce labeled virus proteins. For this purpose, thirty 100 x 20 mm plates (Falcon) are infected with 1 - 5 pfu or ffu per cell. After the appropriate time (24 - 48 hours for vaccinia virus and SFV-I; 4 days for SFV-W), the infected cells are scraped off the plates with a rubber policeman and processed as described in section D, virus purification. Protein concentrations are determined by the Lowry assay (1951) on an aliquot from the viral preparation; another sample is taken to be TCA precipitated to determine the radioactivity.

The Lowry assay is performed by first preparing

the following three reagents: A1 - 2% sodium tartrate; A2 - 1% cupric sulfate; A3 - 2% sodium carbonate, in 0.1 N NaOH. These are combined in a ration of 1 A1: 1 A2: 100 A3 and designated solution A. Solution B is Folin reagent, diluted 1:1 with H_2O. 400 ul of sample are mixed with 2 ml of solution A and then allowed to stand for 10 min. 200 ul of solution B are added, the mixture is allowed to stand for 30 min. and then the absorbance of the solution is read at 750 nm.

Q. TCA precipitation

The reaction mixture consists of an aliquot of sample plus 5 ml of cold 10% TCA, which is placed on ice for 15 min. The precipitate is collected on a millipore filter, which is dried and counted.

R. Method for immunoprecipitation

The following reagents are prepared. TES, consisting of 10 mM Tris pH 8.0, 100 mM NaCl, and 1 mM EDTA; 5% Triton in TES, 100 mM PMSF in ethanol; and 1% deoxycholate, 0.5% Triton in TES.

To reduce the background due to nonspecific precipitation, the virus protein samples (50 ul) are incubated with 10 ul pre-immune sera, plus 50 ul Triton-TES solution and 1 ul PMSF solution for 1 hour on ice. This is followed by the addition of 50 ul of a protein A -Sepharose bead (Pharmacia) suspension, 10%

in Tris pH 7.8, and incubation for 1 hour at 4°C. The sample is clarified by centrifugation in a microfuge for 15 min.

Immune precipitation is then carried out by the addition of 10 ul of antisera, plus 50 ul Triton-TES solution and 1 ul PMSF solution, and incubation for 1 hour at 4°C. Antigen - antibody complexes are isolated by incubation with 50 ul protein A -Sepharose bead suspension for 1 hour at 4°C. Reaction tubes are spun in a microfuge for 15 min., and the pellets are washed three times in with deoxycholate-Triton solution. The pellets are then resuspended and boiled for 5 min. in electrophoresis sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.001% bromphenol blue) in preparation for SDS PAGE.

S. Calcium chloride precipitation and transfection

The procedure used is based on Wigler et al. (1979) and Perucho, et al. (1981). The DNA to be transfected, at a concentration of 30 ug/ml is precipitated in a solution of 250 mM $CaCl_2$, 1 x HBS (2 x HBS : 280 mM NaCl, 50 mM Hepes, 1.5 mM sodium phosphate pH 7.1) at room temperature for 30 min. Precipitated DNA (60 ug total) are added to a 60 mm dish containing 1 x 10^6 cells. Cells are incubated at 37°C in the presence of the precipitate for 48 hours.

The cells are then harvested, homogenized, and tested for the production of plaques in SIRC cell monolayers under agar.

IX. RESULTS

A. Isolation and restriction endonuclease analysis of poxvirus DNA

Experiments with vaccinia virus showed that the procedure chosen for isolation of DNA resulted in material suitable for subsequent analysis. Figure 1 shows the restriction patterns obtained when vaccinia virus, strain IHD-W, was digested with Bam HI, Hind III, or Sal I and separated by agarose gel electrophoresis. Table 3 shows the molecular weights of the fragments observed in Figure 1, and compares the Hind III and Sal I digests of strain IHD-W to those of the more extensively studied strain WR (DeFillipes, 1982). Differences between the strains were slight and negligible. This result was not unexpected, given the high degree of conservation that exists among orthopoxviruses (Mackett and Archard, 1979).

Comparisons among genomes of viruses grown in different host cells are valid if no differences that are found are attributable to host-related factors. Vaccinia virus grows to a high titer in L cells, and is thus the cell line of choice for its propagation. SFV, however, does not grow in L cells, but in SIRC cells.

Figure 1. Restriction endonuclease patterns of vaccinia virus DNA. 1 ug of DNA was digested with the indicated enzyme, and elctrophoresed in a 0.5% agarose gel. Lanes: 1 - Bam HI; B - Hind III; C - Sal I; D- λ DNA digested with Hind III. The numbers indicated are the mol wts, in kilobases, of the DNA restriction fragments.

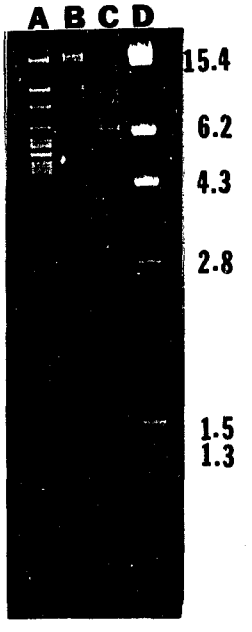

A B C D
15.4
6.2
4.3
2.8
1.5
1.3

TABLE 3. COMPARISON OF RESTRICTION FRAGMENTS OF VACCINIA VIRUS DNA STRAIN IHD-W AND STRAIN WR

Hind III, mol wt x 10^{-6}				Sal I, mol wt x 10^{-6}			
	WR		IHD-W		WR		IHD-W
A	32.8	A	30.0	A	17.0	A	17.0
B	18.8	B	18.0	B	14.9	B	14.0
C	13.8	C	15.0	C	9.8	C	13.0
D	10.7	D	11.0	D	9.25	D	10.0
E	10.2	E	10.5	E	9.25	E	9.9
F	9.0	F	9.0	F	8.65	F	9.5
G	5.7	G	5.0	G	8.08	G	9.0
H	5.5	H	5.5	H	6.7	H	8.0
I	4.0	I	4.0	I	6.3	I	7.0
J	2.98	J	3.3	J	6.05	J	7.0
K	2.62	K	3.0	K	5.78	K	4.0
L	2.40	L	2.5	L	3.75	L	2.0
M	1.35	M	1.4	M	3.55	M	2.0
N	1.0	N	1.0	N	2.8	N	1.5
O	0.9	O	0.9	O	2.02	O	1.4
				P	2.02		
				Q	1.76		
				R	1.45		
				S-W	3.0		
	121.75		118.0		122.08		115.0

It was therefore important to determine if differences in the genome of vaccinia virus could be detected after it had been grown in different cell lines. In Figure 2, the Sal I restriction pattern of vaccinia virus DNA which was grown in L cells or SIRC cells is shown. No host specific alterations of the genome can be seen. The blur seen above the slowest-migrating band in lane A may represent some uncut DNA, or some other non-specific residue in the preparation.

Because of the biological characteristics of vaccinia virus and SFV-I (Pogo et al., 1982a, b), the DNA of these viruses were compared. Figures 3 and 4 show a comparison of the two genomes after digestion with various enzymes. Table 4 is a tabulation of the molecular weights of the restriction fragments observed. Both similarities and differences in patterns can be detected. The Bam HI pattern (Figure 3, lane A and B; Figure 4, lane A, B) showed a difference in the top three bands, a similar concentration though different distribution in the 6 - 3.5 Kb region, and similar fragments from 3.1 Kb down. The Hind III pattern (3, C, D), used by CDC to classify orthopoxviruses (Esposito and Knight, 1985), are quite similar. The Sal I lanes show an overall similarity of

Figure 2. Comparison of the DNA of vaccinia virus grown in two different cell lines. Lanes: A - 1 ug DNA, Vaccinia virus grown in L cells; B - 1 ug DNA, virus grown in SIRC cells. Both DNAs were digested with Sal I.

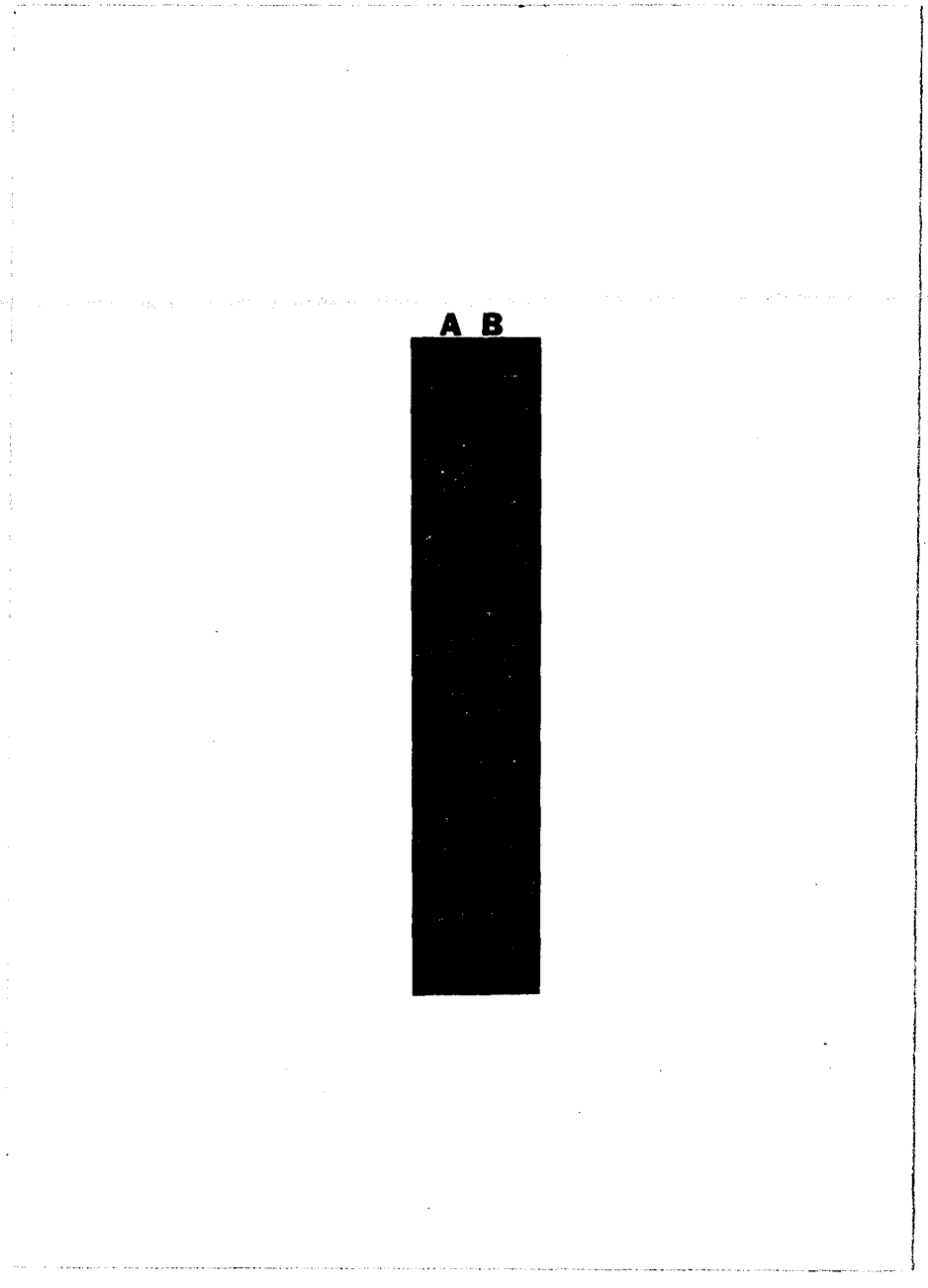

A B

Figure 3. Comparison of Vaccinia and SFV-I DNA.

Lanes:

A - vaccinia, B - SFV-I, digested with Bam HI;

C - vaccinia, D - SFV-I, digested with Hind III;

E - vaccinia, F - SFV-I, digested with Sal I.

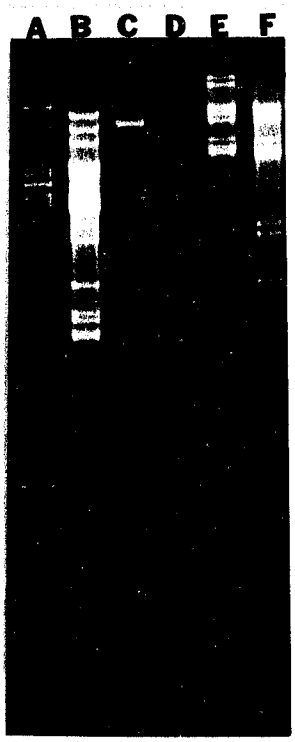

A B C D E F

Figure 4. Comparison of vaccinia virus and SFV-I DNA. Lanes:
A - vaccinia, B - SFV-I DNA digested with Bam HI;
C - vaccinia, D - SFV-I DNA digested with Sst I;
E - vaccinia, F - SFV-I DNA digested with Sal I;
G - λDNA digested with Eco RI.

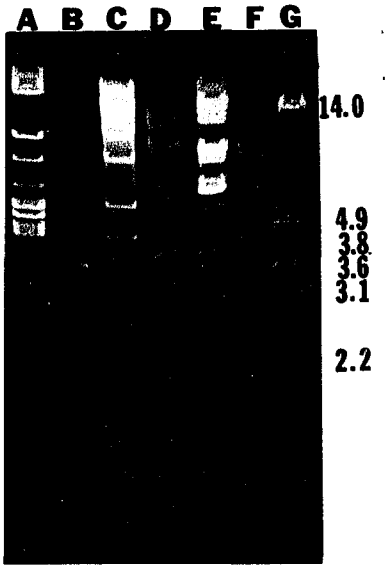

A B C D E F G
14.0
4.9
3.3
3.1
2.2

TABLE 4. COMPARISON OF RESTRICTION FRAGMENTS OF VACCINIA AND SFV-I DNAs

Bam HI Fragments, mol wt x 10^{-6}

VAC		IND	
A	24	A	9.0
B	10	B	8.5
C	9.0	C	7.8
D	7.2	D	5.45
E	5.5	E	5.45
F	5.45	F	5.3
G	5.45	G	5.2
H	5.2	H	5.0
I	5.0	I	4.9
J	4.9	J	4.8
K	4.8	K	4.7
L	4.7	L	3.6
M	3.6	M	3.0
N	3.5	N	2.6
O	3.0	O	2.5
P	2.6	P	2.4
Q	2.5	Q	2.2
R	2.4	R	1.85
S	2.2	S	10.8
T	1.85	T	1.65
U	1.8	U	1.60
V	1.65	V	1.56
W	1.6	W	1.36
X	1.56		
Y	1.36		

Hind III Fragments, mol wt x 10^{-6}

VAC		IND	
A	30	A	30
B	18.0	B	15.5
C	15.0	C	11.0
D	11.0	D	11.0
E	10.5	E	9.0
F	9.0	F	6.0
G	6.0	G	5.5
H	5.5	H	4.0
I	4.0	I	3.3
J	3.3	J	3.0
K	3.0	K	2.5
L	2.5	L	1.4
M	1.4	M	1.0
N	1.0	N	0.9
O	0.9		

SAL I Fragments, mol wt x 10^{-6}

A	17.0	A	17.0
B	14.0	B	14.0
C	13.0	C	10.0
D	10.0	D	9.9
E	9.9	E	9.5
F	9.5	F	9.0
G	9.0	G	8.0
H	8.0	H	7.0
I	7.0	I	7.0
J	7.0	J	6.8
K	4.0	K	4.0
L	2.0	L	4.0
M	2.0	M	3.0
N	1.5	N	2.0
O	1.4	O	2.0
		P	1.5
	115		115

pattern - a tri-modal distribution between 14 and 6 Kb, with other fragments of lower molecular weights. Even within the high molecular weight areas, however, some differences can be detected. Likewise, the Sst I pattern reveals some similarities and differences in enzyme recognition sites between the viruses.

In some instances, the totals for the molecular weights do not equal the expected, and in the case of vaccinia virus DNA, known molecular weights of the total genome. This may be due to a number of factors including: a) more than one band of the same molecular weight that comigrates the same distance in the lane, b) miscalculation of the molecular weight of the higher bands, since accurate measurements in 0.5% agarose of bands above 12 Kbp are difficult, and c) low molecular weight bands that have run off the gel, or are in too low a concentration to be visualized by ethidium bromide staining. These problems were circumvented by: 1) taking note of increased intensity of staining of certain bands within a pattern, which would indicate more than a single band at a particular position; b) running lower concentration agarose gels to separate and measure higher molecular weight bands; and c) running higher concentration gels, with higher concentrations of DNA.

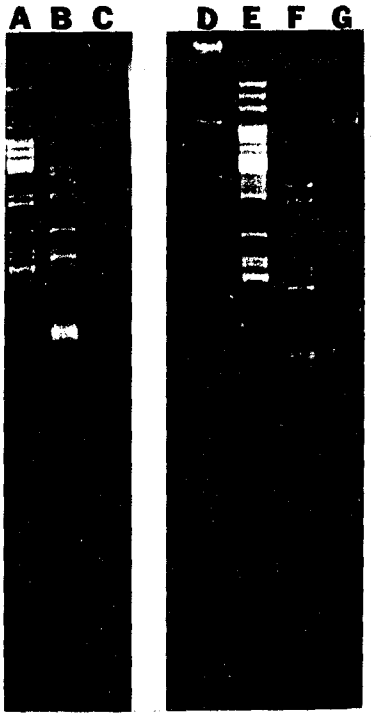
The DNAs of vaccinia virus and SFV-I were also subjected to double digestion in an attempt to differentiate between the two genomes. Bam HI and Hind III and Bam HI and Sal I were two enzyme pairs used (Figures 5 and 6). The Bam HI - Hind III digest, as in the case of the Bam HI pattern showed identical patterns for SFV-I and vaccinia virus DNAs in the lower portion of the lane, with differences apparent in the higher molecular weight fragments. The Bam HI - Sal I digest results in a large number of fragments. The similarities noted between the two DNAs on the basis of single enzyme digests with Bam HI and Sal I become less evident as the number of fragments are increased. Gaps in the patterns follow a common pattern, so that no bands are visible in the 10 - 7.2 mdal, 5.2 mdal, 2.4 - 2.2 mdal regions of either the vaccinia virus or SFV-I lanes. On the other hand, it is difficult to ascertain the homology between specific bands in the patterns.

In contrast to vaccinia virus and SFV-I, SFV-W grew with low efficiency. Even after infection with a high m.o.i. yields for SFV-W were comparatively lower than for other viruses. The DNA of the virus was more difficult to extract and more fragile; producing clean

Figure 5. Comparison of Vaccinia virus and SFV-I DNA. Lanes: A - vaccinia virus DNA and B - SFV-I DNA digested with Bam HI and Hind III.


A
B

Figure 6. Comparison of Vaccinia virus and SFV-I DNA. Lanes: A - Vaccinia DNA digested with Bam HI; B - Vaccinia DNA digested with Bam HI and Sal I; C - Vaccinia DNA digested with Sal I. D - λDNA digested with Hind III. E - SFV-I DNA digested with Bam HI; F - SFV-I DNA digested with Bam HI and Sal I; G - SFV-I DNA digested with Sal I.


A B C
D E F G

digestible SFV-W DNA proved most challenging. Increasing the number of cells for infection (e.g., roller bottles instead of flat bottles) and limiting exposure of the virus preparation to sonication allowed the production of usable SFV-W DNA.

A comparison of the restriction endonuclease patterns of the DNAs of vaccinia virus, SFV-I and SFV-W is shown in Figures 7, 8, and 9. The similarities between vaccinia virus and SFV-I DNA are again seen, as are the differnces between these and SFV-W DNA. This holds true for all three enzymes used, including the reference enzyme, Hind III (Figure 8). Table 5 shows the molecular weight of BAM HI restriction fragment of SFV-W DNA. The patterns for SFV-W DNA, a Patuxent-derived strain, are similar to the patterns obtained by restriction enzyme digestion of SFV strain Kasza, an independent isolate of the virus (Kasza, 1974); Kasza has two bands in the Hind III digest, at 7.0 and 3.4 mdal that SFV-W does not, and SFV-W has a 20 mdal band in the Eco RI pattern that Kasza DNA does not (Figure 10, Table 6).

In summary, after extensive endonuclease analysis, I could report that : a) tumorigenic SFV-I and

Figure 7. Comparison of vaccinia virus, SFV-I, and SFV-W DNAs after digestion with Bam HI. Lanes: A - Vaccinia virus DNA; B - SFV-I DNA; C - SFV-W DNA.


A B C

Figure 8. Comparison of Vaccinia virus, SFV-I, and SFV-W DNA. Hind III digest: Lane A - Vaccinia, B - SFV-I, C - SFV-W.


A B C

Figure 9. Comparison of Vaccinia virus, SFV-I, and SFV-W DNA. Sal I digest: Lane B - Vaccinia, C - SFV-I, D - SFV-W. Lane A -λDNA, digested with Hind III.

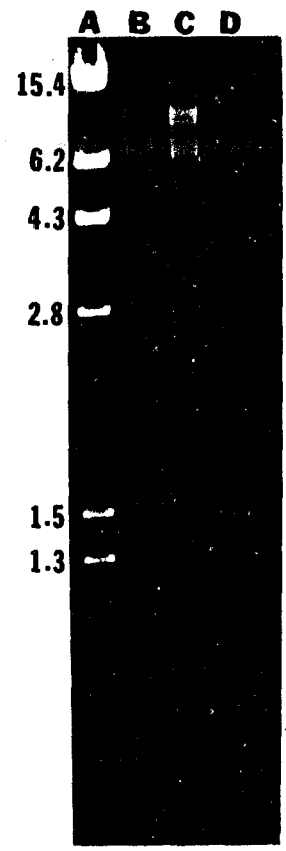

A B C D
15.4
6.2
4.3
2.8
1.5
1.3

TABLE 5. Bam HI RESTRICTION FRAGMENTS OF SFV-W DNA

	mol wt x 10^{-6}
A	30.0
B	20.0
C	19.0
D	17.0
E	12.5
F (x2)	8.0
G	6.0
H	5.0
I	3.8
J (x3)	3.5
K (x2)	3.0
L (x2)	2.8
M	2.4
N	1.9
O	0.4
	156.10

Figure 10. Comparison of SFV-I, SFV-W, and SFV-Kasza DNA. Lanes: A - SFV-I DNA; B - SFV-W DNA; C - SFV-Kasza DNA; all digested with Bam HI. D - SFV-I DNA; E - SFV-W DNA; F - SFV-Kasza DNA; all digested with Eco RI.

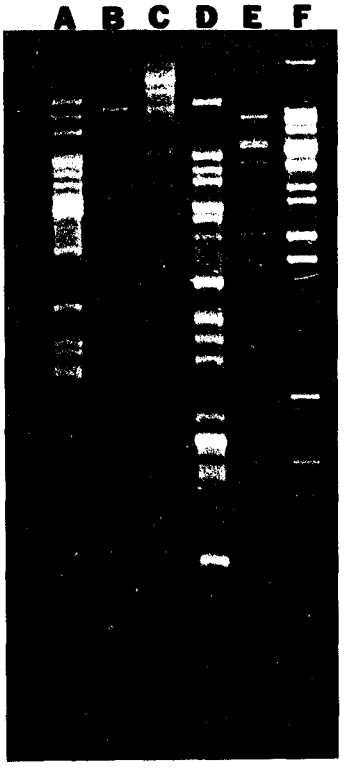

A B C D E F

TABLE 6. COMPARISON OF RESTRICTION FRAGMENTS OF SHOPE FIBROMA VIRUS STRAIN KASZA AND STRAIN W

Bam HI, mol wt x 10^{-6}

W		Kasza	
A	30	A	28
B	20	B	21
C	19	C	19
D	17	D	15.5
E	12.5	E	13.0
F(x2)	9.0	F(x2)	9.0
G(x2)	6.0	G	7.0
H	3.9	H(x2)	6.0
I	3.5	I	3.9
J	3.0	J	3.5
K	2.8	K	3.4
L	2.4	L	3.0
		M	2.8
		N	2.4

ECO RI mol wt x 10^{-6}

W		Kasza	
A	30	A	30
B	20	B	9
C	9	C	8
D(x2)	8	D	7
E	7	E	6
F	6	F	6
G	6	G	5
H	5	H	4
I	4	I	- 3.8
J	3.8	J	3.5
K(x2)	3.5	K	3.4
L	3.4	L	2.8
M	2.8	M	2.0
N	2.0	N	1.9
O	1.9	O	1.5
P	1.5	P	1.4
Q	1.4	Q	1.3
R	1.3		

cytolytic vaccinia virus showed great similarities in their DNAs, with all enzymes used, and b) that SFV-W DNA differed from SFV-I and vaccinia virus DNA. I also estimated that there was at most a 10% difference in DNA sequences between vaccinia virus and SFV-I DNA (Berkowitz and Pogo, 1982). These analyses could not show whether or not SFV-I shared any DNA sequences with SFV-W which might explain their common in vivo tumorigenicity.

B. Southern hybridizations

To address the question of homology among various poxviruses I turned to the transfer and hybridization technique of Southern (1975). These techniques had recently been introduced to poxvirology (Holowczak, 1982). I first found conditions under which transfer of all fragments from the gel were effected, and experimental protocols which allowed for the best hybridization and washing results. It was also necessary to find conditions for efficient nick translation of various probes.

I considered two methods for the hybridization reaction. One, described in the Cold Spring Harbor Manual (1982), is carried out at 68^{o}C, while the formamide technique, as described by Wahl et al. (1979), uses 50% formamide and 5% dextran sulfate and is performed

at 45°C. As shown in Figure 11, the CSH method resulted in autoradiograms with less background and so it was chosen for the rest of the hybridizations.

a. Hybridization of vaccinia and SFV-I DNA

The usefulness of the hybridization technique was seen when I compared the DNAs of vaccinia virus and SFV-I (Figure 12). The Bam A, B, and C fragments of SFV-I, despite their differences in migration, hybridized when probed with ^{35}S-labeled vaccinia virus DNA. In fact, all bands of SFV-I hybridized, emphasizing the close relationship between the genomes of vaccinia and SFV-I. The Eco RI pattern shows the same results - despite the numerous differences in the migration of fragments, hybridization was complete.

As expected, the reverse experiment, in which vaccinia virus DNA was hybridized with ^{35}S-labeled SFV-I DNA showed the same results (Figure 13). This was the case when the DNA was digested with one enzyme, such as Hind III (Figure 13, lane b) or Sal I (lane d) or even with double digests. The Bam HI and Hind III pattern (lane a) showed that even bands that migrated differently in double digests (i.e., the two top bands, compare in Figure 3) also hybridized to the heterologous DNA. The Hind III and Sal I and Bam HI and Sal I patterns of vaccinia virus DNA (c,e) also

Figure 11. Comparison of the CSH and Formamide methods of hybridization. SFV-W DNA, digested with Hind III, was transferred to nitrocellulose after agarose gel electrophoresis. The blots were hybridized with ^{35}S-labeled SFV-W DNA (4-5 x 10^8 cpm/ug). Lanes: A - CSH method; B - Formamide method. Autoradiograms were exposed for similar times, after being washed under similar conditions.

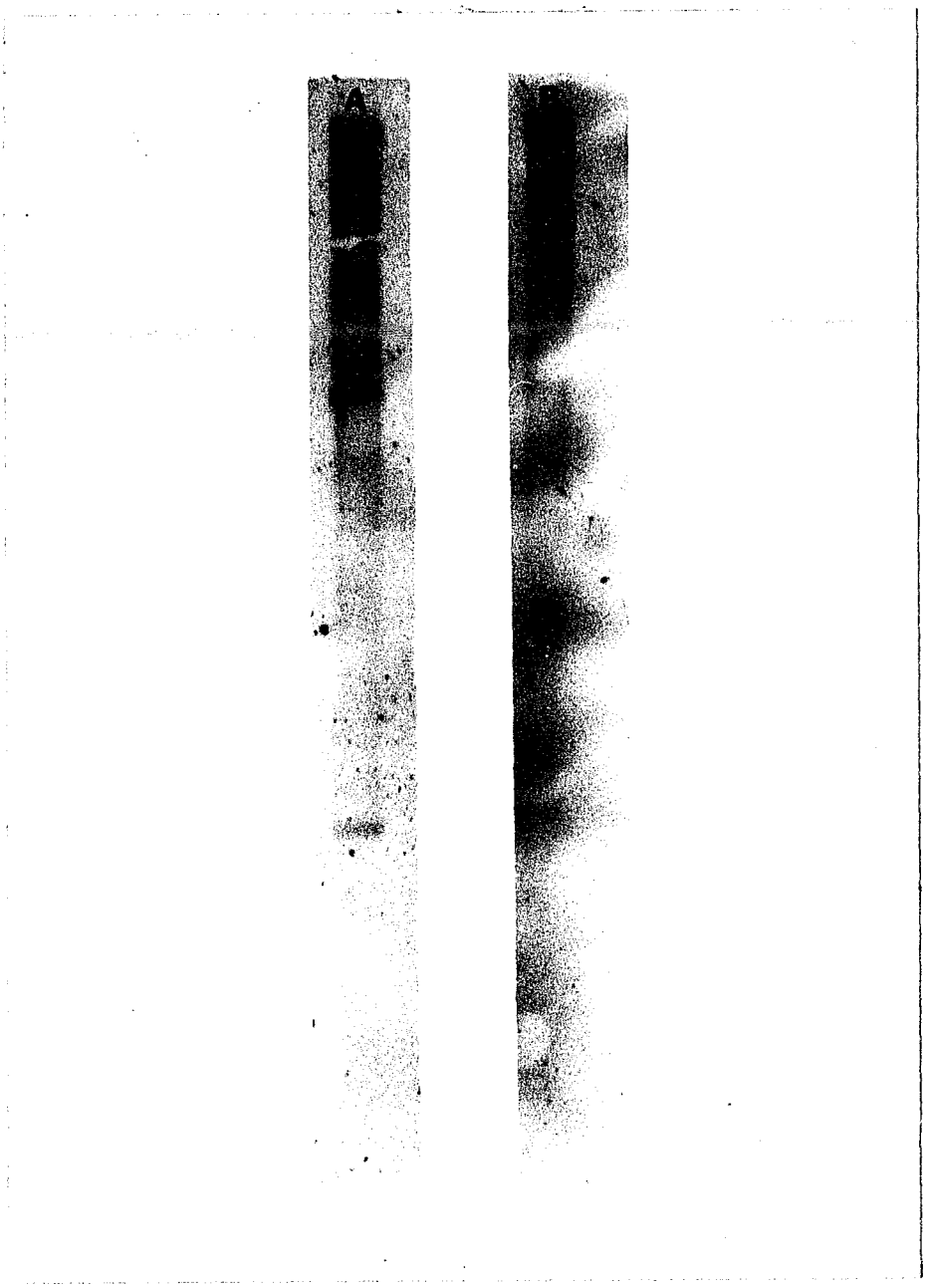


Figure 12. Comparison of Vaccinia virus and SFV-I DNA by gel electrophoresis, Southern blotting, and hybridization. Lanes: a - vaccinia virus DNA digested with Bam HI; b - vaccinia virus DNA digested with Eco RI; c - SFV-I DNA digested with Bam HI; d - SFV-I DNA digested with Eco RI. The DNA was separated by electrophoresis in 0.5% agarose, transferred to nitrocellulose paper, and hybridized with ^{35}S-labeled vaccinia virus DNA. Left: Gel stained with ethidium bromide. Right: blot hybridization.

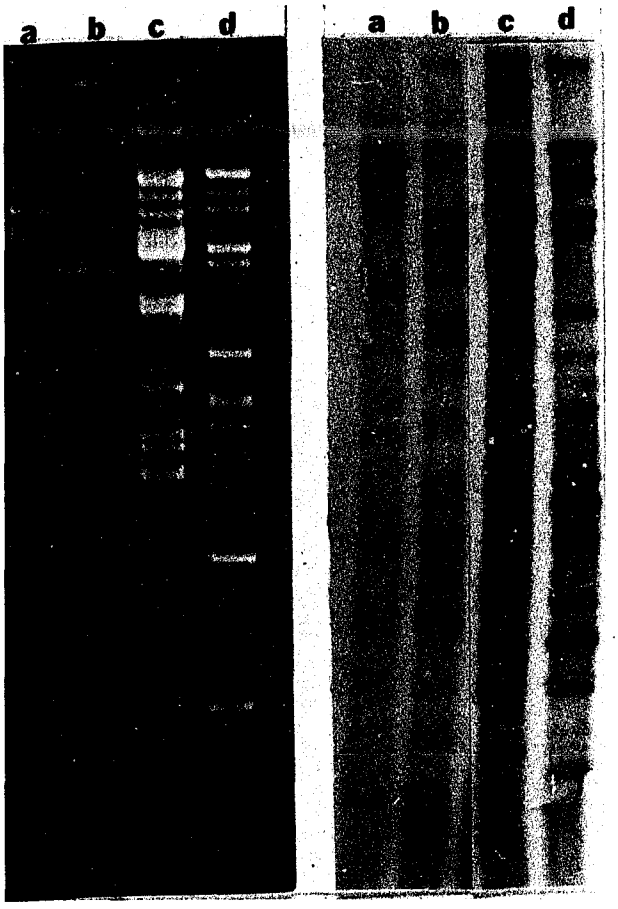
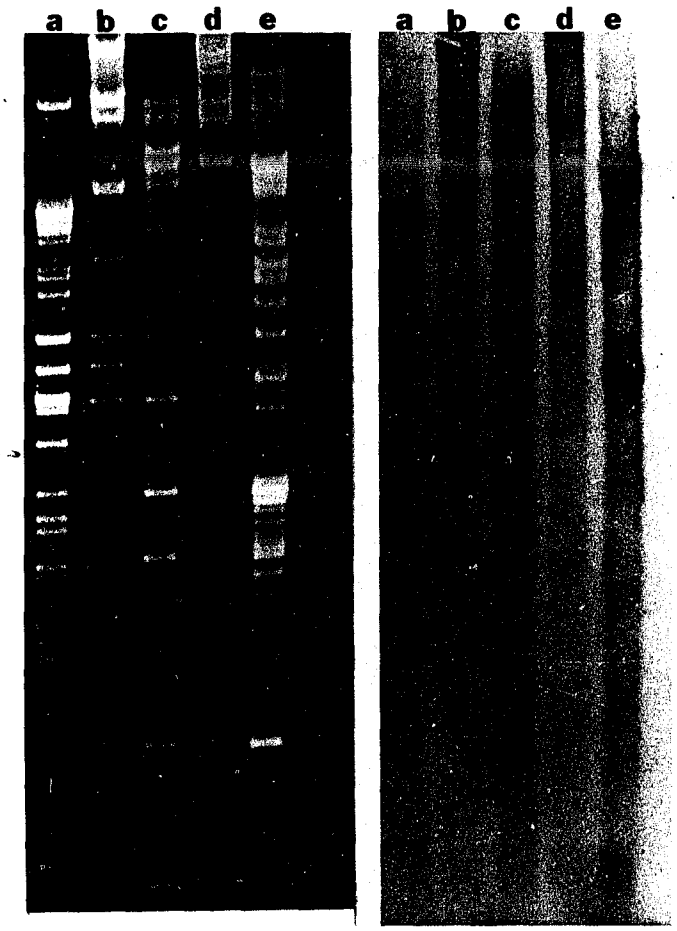

a b c d
a b c d

Figure 13. Vaccinia virus DNA probed with SFV-I DNA. Lanes: A - Bam HI + Hind III; B - Hind III; C - Hind + Sal I; D - Sal I; E - Bam + Sal. Left: gel visualized with ethidium bromide. Right: blot hybridization.


a b c d e
a b c d e

showed complete hybridization with the ^{35}S-SFV-I probe.

b. Hybridization of vaccinia virus and SFV-W DNA

What, if any, relationship was there, at the level of the Southern hybridization technique, between an orthopoxvirus, vaccinia and a leporipoxvirus, SFV-W? This question had not yet been addressed when I began this project, and was an important aspect in the analysis of the SFV-I genome. Under the stringency conditions of the CSH method, I showed that there was no DNA homology between the DNA of the two genera (Figures 14, 15). While a ^{35}S-labeled vaccinia virus DNA probe could recognize homologous DNA in very low concentration (Figure 14, lane c), no hybridization to SFV-W was detected. The reverse experiment, in which SFV-W DNA was used to probe vaccinia virus DNA (Figure 15) also showed no hybridization between the DNA of the the two genera (lanes b,c,d). As this work was in progress, Wills et al. (1983) published their observations on the lack of homology by Southern blot analysis between orthopoxvirus and leporipoxvirus DNA.

c. Hybridization of SFV-I and SFV-W DNA

When SFV-I DNA was digested with various enzymes and hybridized with ^{35}S-labeled SFV-W DNA, autoradiograms showed a subset of fragments in the

Figure 14. Comparison of vaccinia virus and SFV-W DNA by gel electrophoresis, Southern blotting, and hybridization. DNAs from vaccinia (a=1.5 ug, b=0.7 ug, c=0.3 ug), λ(d) and SFV-W (e, f, g) were digested with Bam HI (e), Hind III (a-d, f), or Sal I (g), separated by electrophoresis in 0.5% agarose, transferred to nitrocellulose paper, and hybridized with ^{35}S-labelled vaccinia DNA. Left: stained gel. Right: blot hybridization.

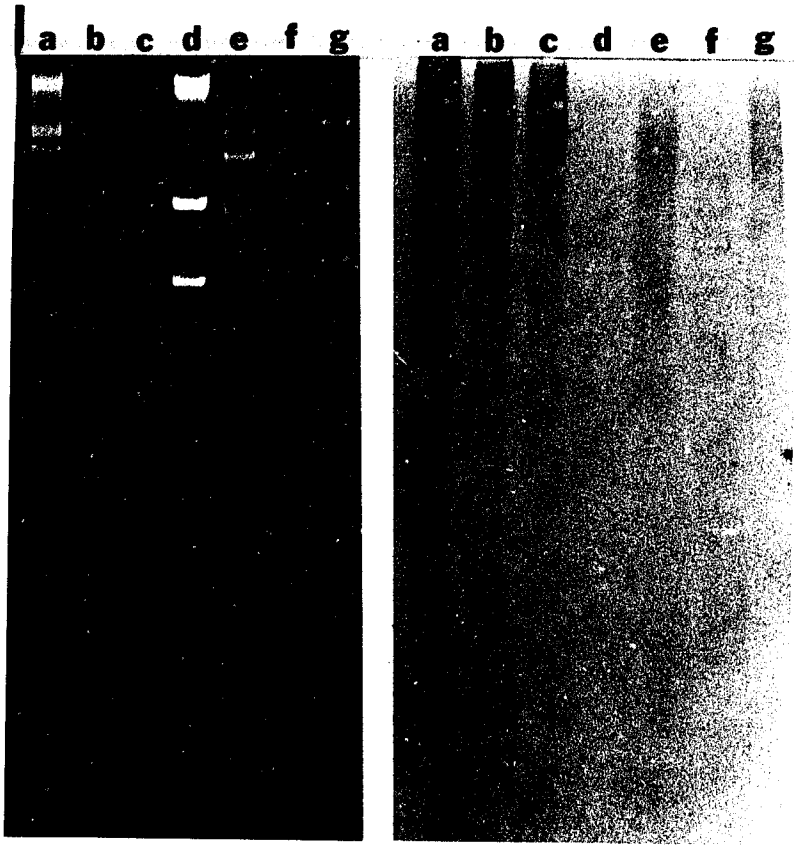
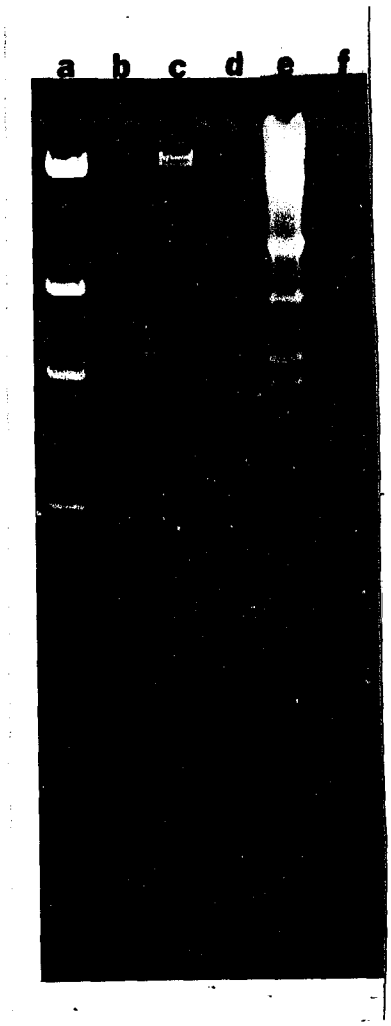

a b c d e f g
a b c d e f g

Figure 15. Comparison of vaccinia virus and SFV-W DNA. DNA from λ(a), vaccinia (b, c, d), SFV-I (e) and SFV-W (f) was digested with Bam HI (b), Hind III (a, c, e, f) or Sal I (d), separated by electrophoresis in 0.5% agarose gels, transferred to nitrocellulose, and hybridized with ^{35}S-labelled SFV-W DNA. Left: stained gel. Right: blot hybridization.


a b c d e f


a b c d e f

patterns reacting with the probe (Figures 16, 17). Bam HI, Eco RI, Sal I and Sst I- digested SFV-I DNA each had two fragments with homology to SFV-W DNA (Figure 16, lanes b, c; Figure 17, b,c) and Hind III- digested SFV-I DNA had four such fragments (Figure 15, lane e; 16, d). Thus, in addition to being quite homologous to the orthopoxvirus vaccinia, SFV-I had genomic sequences recognized by a leporipoxvirus, SFV. SFV-W DNA, when subjected to the same analysis, also showed a subset of restriction fragments that hybridized to ^{35}S-labeled SFV-I DNA. Thus two bands in the Bam HI and Eco RI digests (Figure 18) and one band each in the Hind III (Figure 19) and Sal I (Figure 20) digests hybridized to the probe. It is interesting to note that in the Bam HI and Eco RI digests, hybridizations were seen in bands that are more intensely staining, i.e., that have more than one fragment of the same size, such as Bam HI F and Eco RI K. That hybridization is specific can be argued from the fact that other bands present in higher concentration, such as Eco RI D, did not hybridize. In Table 7, a list of the molecular weights of the fragments recognized by heterologous DNA is shown.

The results described in this section indicate that: 1) SFV-I and vaccinia virus show extensive DNA

Figure 16. Cross hybridization between SFV-I and SFV-W DNA. DNA from λ (a) and SFV-I (b,c,d) were digested with Bam HI (b), Eco RI (c), or Hind III (a,d), separated by gel electrophoresis in 0.5% agarose, transferred to nitrocellulose, and hybridized with ^{35}S-labelled SFV-W DNA. Left: gel stained with ethidium bromide. Right: blot hybridization.

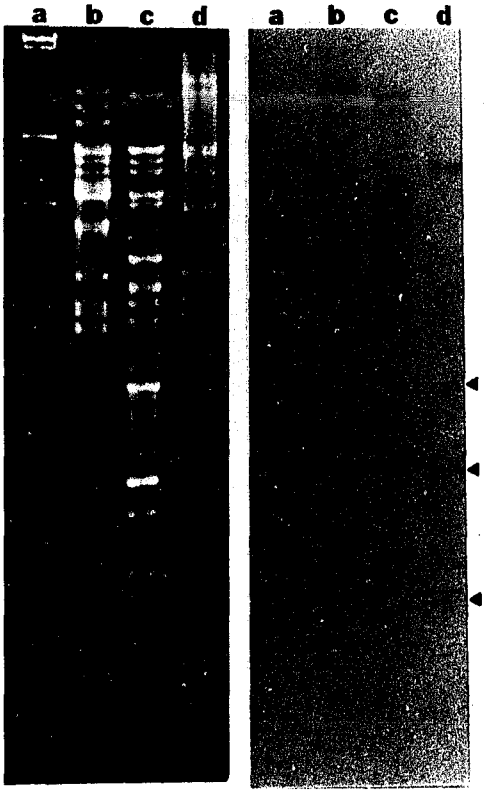

a b c d
a b c d

Figure 17. Cross hybridization between SFV-I and SFV-W. DNA from λ (a) and SFV-I (b,c) was digested with Hind III (a), Sal I (b), or Sst I (c), separated by gel electrophoresis in 0.5% agarose, transferred to nitrocellulose, and hybridized with ^{35}S-labelled SFV-W DNA. The autoradiogram has been enlarged.

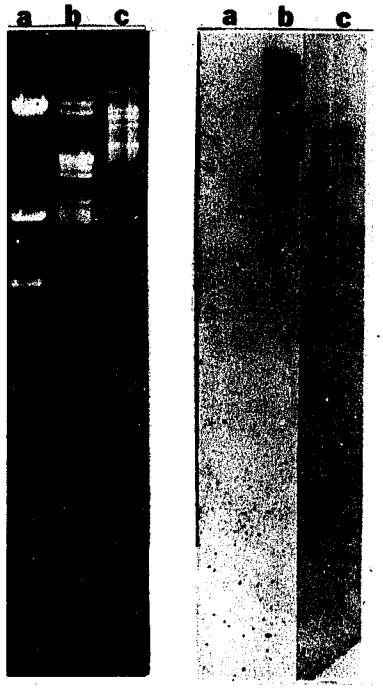

a b c
a b c

Figure 18. Cross hybridization between SFV-I and SFV-W DNA. DNAs from SFV-I (a,c) and SFV-W (b,d) were digested with Bam HI (a,b) or Eco RI (c,d), separated by gel electrophoresis in 0.5% agarose, transferred to nitrocellulose paper, and hybridized with ^{35}S-labelled SFV-I DNA. Left: gel stained with ethidium bromide. Right: blot hybridization.

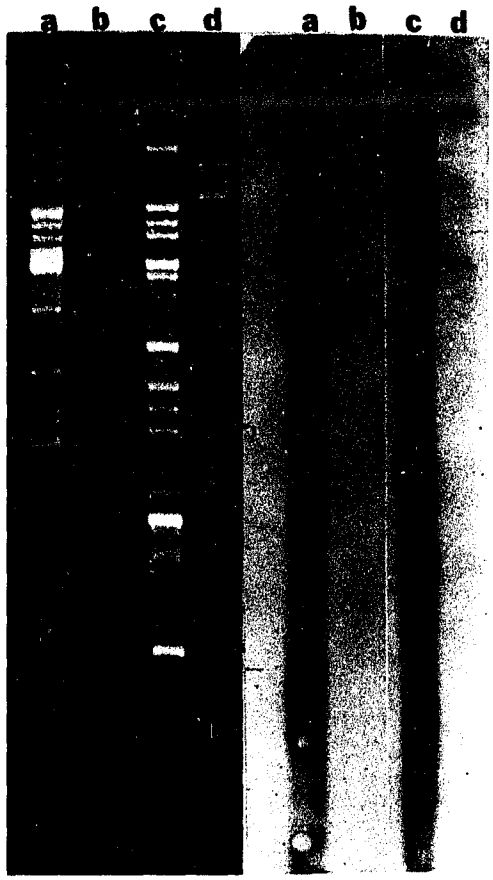

a b c d a b c d

Figure 19. Cross hybridization between SFV-I and SFV-W. Hind III digest of vaccinia (a), SFV-I (b), and SFV-W (c) visualized by ethidium bromide staining of 0.5% agarose gel (left), transferred to nitrocellulose, and hybridized with ^{35}S-labelled SFV-I DNA (right).

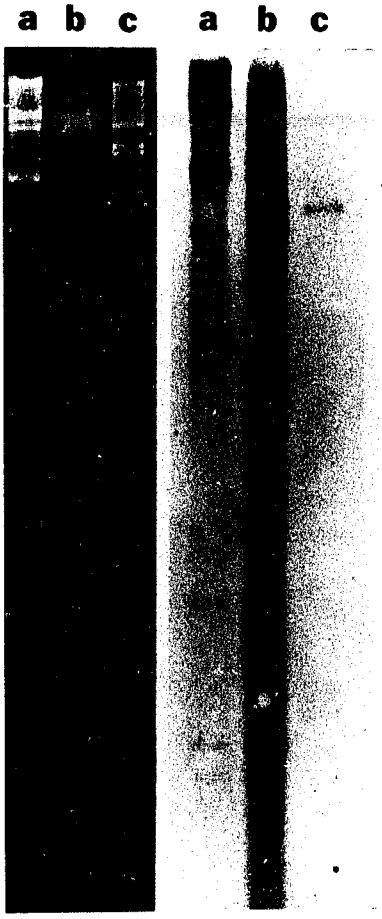

a b c a b c

Figure 20. Cross hybridization between SFV-I and SFV-W. Blot hybridization of Sal I digest of vaccinia (a), SFV-I (b), and SFV-W (c) transferred to nitrocellulose, and hybridized with ^{35}S-labelled SFV-I.

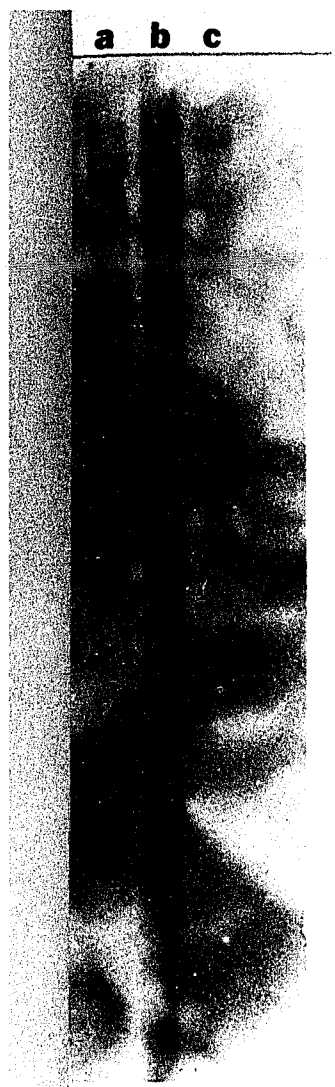

a b c

TABLE 7. Cross Hybridization Between SFV-I and SFV-W DNAs

ENZYME	NO. OF FRAGMENTS	SIZE ($\times 10^6$)
Restriction fragments of SFV-I DNA recognized by SFV-W DNA		
Bam HI	2	9 and 3.6
Eco RI	2	8.7 and 5.0
Hind III	4	5.6, 2.5, 2.0 and 1.5
Sal I	2	17 and 12
Sst I	2	11 and 10.5
Restriction fragments of SFV-W DNA recognized by SFV-I DNA		
Bam HI	2	9.0 and 3.9
Hind III	1	4.5
Eco RI	2	20 and 3.5
Sal I	1	8.0

homology; 2) there is no homology between vaccinia virus and SFV-W DNA; and 3) SFV-I and SFV-W share some common sequences. These findings suggest that SFV-I is a recombinant virus, an orthopoxvirus carrying some genetic informtion from a leporipoxvirus. Recombinants between genera of poxviruses have not previously been reported.

C. Marker rescue experiments

It is difficult, if not impossible, to ascertain whether SFV-I was generated in the field or arose as a laboratory recombinant. An important question was whether a recombinational event, such as is posited for the emergence of SFV-I, can occur between orthopoxviruses and leporipoxviruses. I addressed this question with a marker rescue experiment, in which the DNA from the cytocidal SFV-I was rescued by coinfection ith non-cytocidal SFV-W. Cells were infected with SFV-W at a moi of 1 ffu/cell, and then transfected with various amounts of uncut or restricted $CaCl_2$ precipitated SFV-I DNA. The DNA of poxviruses is non-infectious, and SFV-W provides the factors neccessary for replication. After forty-eight hours, the cells were harvested, homogenized, and tested for the production of plaques in SIRC cells under agar. Virus

progeny that gave rise to plaques should contain SFV-I DNA, since SFV-W, by itself, produces foci, not plaques.

The results of these experiments, summarized in Table 8, indicate that the phenotype of plaque formation can be rescued and that the number of plaques obtained as proportional to the amount of SFV-I DNA added. Digestion of the DNA by Hind III destroyed the ability of the DNA to express the plaque-forming phenotype at low DNA concentration, but did not affect the rescue of the marker at a higher DNA concentration. It is possi-ble that a small Hind III fragment is involved in the transfer of the phenotype, and that it is not present in sufficient concentration in the digested DNA preparation to be percipitated. Another possibility is that at the higher DNA concentration, some SFV-I DNA escaped digestion by the enzyme, and in its uncut form was able to rescue plaque formation. This second explanation is less likely, since the digestions were monitored with gel electrophoresis of aliquots of the DNA. Digestion with Bgl I and Bgl I and Sma I did not affect the rescue of the marker.

TABLE 8

RESCUE OF PFU WITH SFV-I DNA

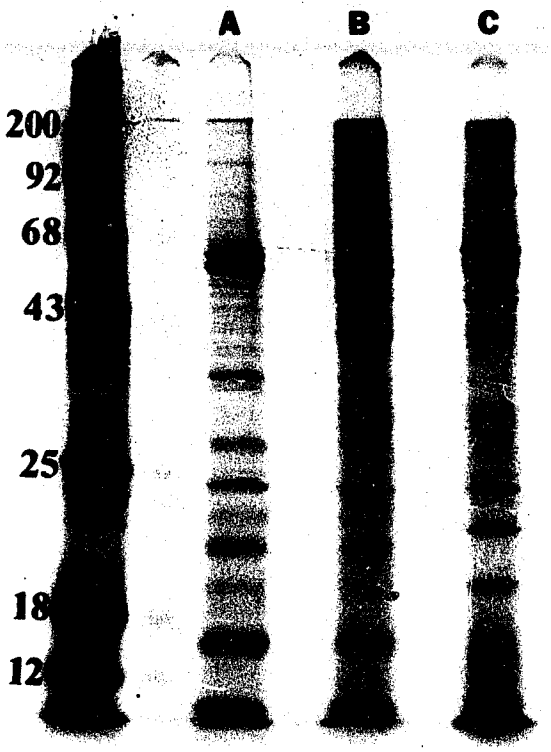
Calf Thymus DNA (ug)	SFV-I DNA (ug)	SFV-W FFU/cell	PFU/ml	PFU/ug DNA
50	10	1	200	20.0
40	20	1	380	19.0
30	30	1	700	23.3
20	40	1	900	22.5
50	10ug + Hind III	1	0	--
30	30ug + Hind III	1	400	13.3
30	30ug + BglI	1	400	13.3
30	30ug + BglI + SmaI	1	300	10.0
0	0	1	2	--
40	20	-	0	--

The experiments described here showed that rescue experiments could be performed between poxviruses of different homology. It is thus possible to imagine a recombinational event between SFV and an orthopoxvirus, taking place either in a cell of an infected rabbit or in cultured cells in vitro, and giving rise to a SFV-I-like isolate. It has also become possible to design experiments in which the leporipoxvirus phenotype of focus formation is rescued by coinfection with orthopoxvirus.

D. Comparative studies with poxvirus proteins

As discussed in the introduction, another approach to comparing the poxviruses has been to study the proteins of the virion. I extended the methods used with orthopoxviruses to the leporipoxvirus genus, which, in some instances, had not yet been reported on. I compared the ^{35}S-methionine-labeled virion proteins of vaccinia virus, SFV-I and SFV-W (Figure 21). The autoradiogram reveals the complex picture of the many proteins coded for by the large genome of the Poxviridae. A number of vaccinia virion polypeptide bands have been correlated with particular structures or functions, and it is tempting to suggest that similarly-migrating bands in SFV-I and SFV-W might

Figure 21. Polyacrylamide gel electrophoresis of structural polypeptides of poxviruses. Vaccinia virus, SFV-I and SFV-W were grown in the presence of ^{35}S-methionine, purified and run on a discontinuous PAG. Lanes: A - Vaccinia, B - SFV-I, C - SFV-W.


A
B
C
200
92
68
43
25
18
12

represent proteins with known functions. For exam ple, a 16 Kdal polypeptide has been identified as a possible RNA polymerase subunit; vaccinia virus seems to share this band with SFV-I, but not with SFV-W. A 23 Kdal band, representing a DNA nicking closing enzyme component is shared among all three viruses, while a poly A polymerase subunit at 35 Kdal seems to be unique to vaccinia virus. Some specific bands, and the number of these that co-migrate between two or among all three viruses are enumerated in Tables 9 and 10.

The use of immunoprecipitation to identify the serologically cross-reactive polypeptides in orthopoxviruses and leporipoxviruses had been previously reported by Ikuta et al. (1978a). I attempted similar experiments, using antisera raised in rabbits against the various viruses used in this study. I hoped that by reacting SFV-I virions with anti-SFV-W, and vice versa, I would be able to identify a subset of polypeptides that cross-reacted between two generally non-homlogous genera. The results of these experiments (Figure 22) did not prove helpful in identifying such polypeptides. There were no obvious differences when SFV-I was precipitated with either anti-vaccinia, anti-SFV-I or anti-SFV-W sera, and no obvious

TABLE 9

STRUCTURAL POLYPEPTIDES OF VACCINIA, SFV-I, AND SFV-W

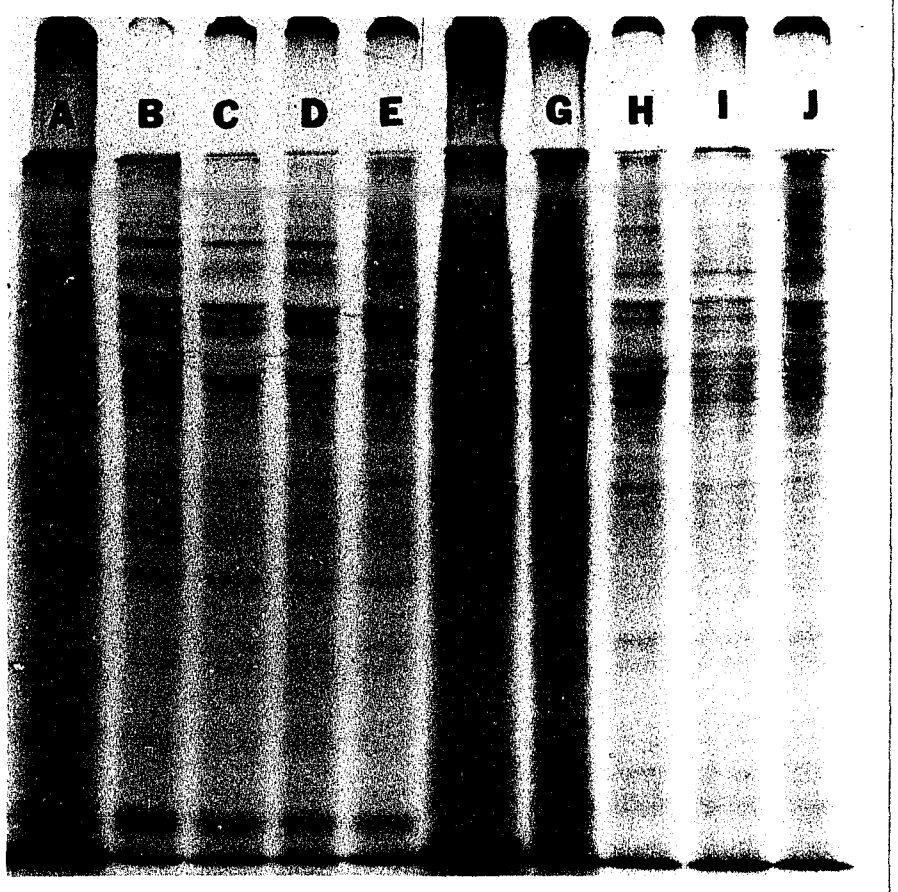
VACCINIA	SFV-I	SFV-W
	150	
100	100	100
	90	
		87
	85	
84		
	78	
74		74
	70	70
64		64
58	58	58
56	56	
50		
48	48	48
46	46	
		44
42		
41	41	41
40	40	
	39	
38	38	38
	37	
36		
	35	
34		
	33	
		32
	31	
		30
	28	
17		
24	24	24
		23
	22	22
21		
	20	
19		
18	18	18
16	16	16
		12
10	10	10

TABLE 10

POLYPEPTIDES CO-MIGRATING WITH SFV-I BANDS

FOR VACCINIA:	2 (56, 46)
FOR SFV-W :	3 (70, 40, 22)
FOR VACCINIA AND SFV-W :	7 (100, 58, 48, 41, 16, 10)

Figure 22. Immunopercipitation. Samples treated as described in Materials and Methods, were loaded on a 15% polyacrylamide gel. SFV-I Lanes: A - purified virions; B - after incubation with preimmune serum; C - after treatment with anti-I antisera; D - after treatment with anti-vaccinia virus antisera; E - after treatment with anti-W antisera. SFV-W Lanes: F - purified virions; G - after incubation with pre-immune serum; H - after treatment with anti-I antisera; I - after treatment with anti-vaccinia virus antisera; J - after treatment with anti-w antisera.


A
B
C
D
E
F
G
H
I
J

differences when the reciprocal experiment, using SFV-W virions, was performed.

The results obtained with immunoprecipitation implied that poxviruses of different genera still had enough polypeptides in common to cross-react extensively in this procedure, limiting its usefulness in identifying SFV-specific polypeptides in the SFV-I virion.

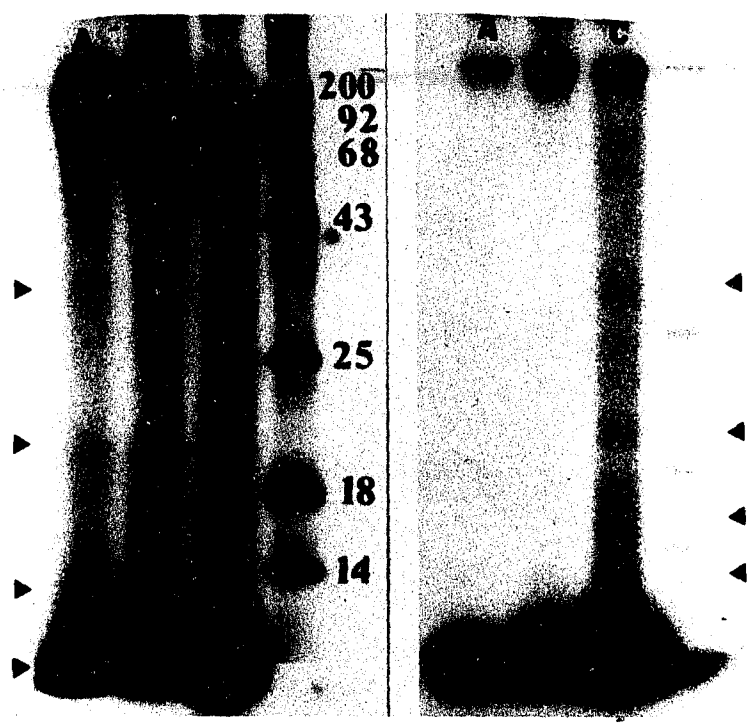
As the total virion polypeptide profiles of vaccinia virus, SFV-I, and SFV-W were too complex for meaningful analysis, I undertook the study of particular subsets of virion proteins. This would provide me with a more manageable number of variables. I examined the phosphoproteins and glycoproteins of the three viruses, as these had been reported, at least for orthopoxviruses to be limited in number. To identify phosphoproteins, viruses were grown in the presence of ^{32}P-orthophosphate, purified and the proteins electrophoresed in polyacrylamide gels. As indicated in Figure 23, the three viruses showed two ^{32}P-labeled polypeptides of mol. wt. 38 and 20 Kdal (top two arrows in Figure 23, left and right), in addition to the 11 Kdal basic protein, reported for vaccinia virus (Pogo et al, 1975), which ran at the bottom of the gel (bottom arrow at left). Both SFV-I and SFV-W had a 12

Kdal band, which was not present in the vaccinia virus lane. A unique characteristic of SFV-W was the presence of phosphopolypeptides in the 18 - 14 Kdal range.

Another subset of virion proteins that I examined were the glycoproteins. One or two such proteins had been described for vaccinia virus (Holowczak, 1970; Garon and Moss, 1971). They are thought to be localized at or near the surface of the virion, and therefore any differences found in them might relate to antigenic differences among the viruses.

Attempts to visualize ^{3}H-glucosamine labeled bands in autoradiograms were unavailing, despite long exposure times and the use of enhancer. I then chose to run cylindrical gels, and to determine the radioactivity in 1 mm slices of the gels. The results of these experiments are plotted in Figure 24. Virions of SFV-W showed only one strongly labeling peak at 70 Kdal, while SFV-I and vaccinia virus showed a similar pattern of two peaks, running closely together. For vaccinia virus, these peaks were at 45 and 35 Kdal; for SFV-I, 42 and 38 Kdal. The differences between the mol. wt. of the glycoproteins of vaccinia and SFV-I may be due to differences in the degree of glycosylation. The possibility of host-related differences in the

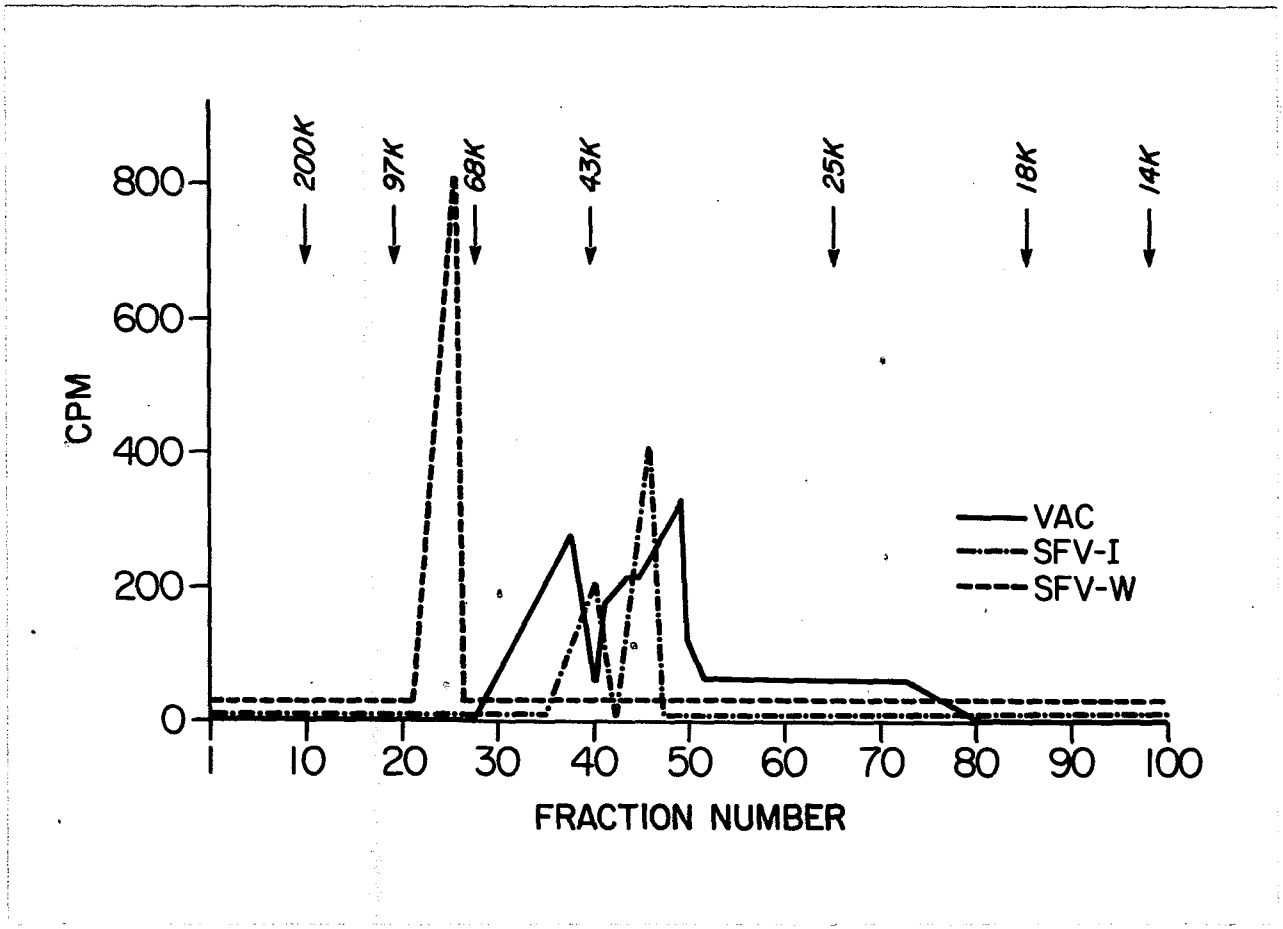
Figure 23. Phosphoproteins of the virions of poxviruses. Vaccinia virus, SFV-I, and SFV-W were grown in the presence of ^{32}P-orthophosphate, purified and run on a discontinuous PAG. Lanes: A - Vaccinia, B - SFV-I, C - SFV-W. Size markers are ^{14}C standards (Biorad).


A
C
200
92
68
43
25
18
14
A
C

glycosylation pattern was ruled out by growing the viruses, including vaccinia, in the same SIRC cell line.

The results of these experiments indicate, as in the case of the DNA hybridization studies, the similarity between SFV-I and vaccinia virus, and the uniqueness of SFV-W.

Figure 24. Glucosamine-containing polypeptides of poxvirus virions. Vaccinia virus, SFV-I and SFV-W were grown in the presence of ^{3}H-glucosamine, purified, and run in a cylindrical polyacrylamide gel. The gels were sliced into 1 mm discs, dissolved in NCS, and counted, in Biofluor scintillation fluid, in a scintillation counter.


200K
97K
68K
43K
25K
18K
14K
800
600
400
200
0
CPM
1
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER
VAC
SFV-I
SFV-W

X. DISCUSSION

The genome of orthopoxviruses, and of vaccinia virus in particular, has been extensively studied by many groups of investigators. Experiments that related to questions of replication, genome organization, and gene identification have all been published. In addition, the use of recombinant vaccinia virus carrying foreign genes as a vaccine has been proposed (Nakano, et al., 1982; Mackett et al., 1984). In contrast, studies on the tumorigenic poxviruses remain sparse. The DNA of one strain of SFV has only recently been cloned and physically mapped (Delange, 1984; Cabirac et al., 1985). The molecular mechanisms underlying tumorigenesis by the leporipoxviruses, as well as other host-virus interactions, remain unknown, and the proteins of this genus have not been subjected to a systematic analysis.

SFV-I provided a way in which to approach various aspects of the molecular biology of poxviruses. Through comparison of SFV-I, SFV-W, and vaccinia virus it might be possible to identify specific regions of DNA and specific polypeptides that might relate to the different biological activities of these viruses. Similarities between the genes and gene products of the two SFV strains would suggest involvement in

oncogenicity while genes and gene products shared by SFV-I and vaccinia virus would be implicated in cytolysis.

The studies of SFV-I DNA described here have established its identification as an orthopoxvirus, and therefore its reclassification as an orthopoxvirus-SFV recombinant (Berkowitz and Pogo, 1985). These findings emphasize the use of restriction endonuclease electropherograms, a well-established poxvirus diagnostic tool. It is important to note, though, that some enzymes are better than others in this regard. Hind III and Sal I patterns for vaccinia virus and SFV-I DNA are quite similar; however, the patterns observed with Eco RI, an enzyme that cleaves the poxvirus genome at many sites, shows differences that overwhelm the similarities. Likewise, the patterns obtained after digestion with two enzymes, as for example in the Bam HI and Sal I digest, are more complex then single-enzyme generated patterns. It is therefore impossible to reach a precise conclusion by the sole use of restriction enzyme profiles.

Hybridization with nick-translated probes have added a new dimension to studies of DNA homology. This technique has allowed an analysis of the relationship between orthopoxviruses and leporipoxviruses. It is

known that the orthopoxviruses DNA, despite their wide distribution among many species, is conserved (Mackett and Archard, 1979). The tumorigenic leporipoxviruses are members of the family Poxviridae by virtue of their morphology, DNA structure and site of replication, and a cross-reactive core protein believed to be common to all genera. The DNAs of these two genera show no DNA homology on the basis of Southern hybridizations, strengthening the findings of comparative restriction analysis.

The differences between the two genera may make it less certain that findings with orthopoxviruses can be extended to leporipoxviruses. On the other hand, it is possible, for example, that tumorigenic genes may be present in the vaccinia virus genome, but not expressed in vaccinia-infected cells and that genes for cytolysis may be present in SFV-W, but unexpressed under normal infectious conditions.

The use of recombinants to map genes and gene functions is one of the basic methods of virology. The size of the poxvirus genome has limited the use of this approach; advances have awaited the use of cloned fragments to create physical maps and isolation of gene-specific mRNAs. Recombinants reported in the literature have mainly involved members of

orthopoxviruses. There have been two reports on myxoma-fibroma recombinants, one experimental (Woodroofe and Fenner, 1960), and one natural isolate (MRV, Strayer et al., 1984). But in no instance has there been a report on an orthopox-leporipox virus recombinant.

SFV-I presented a phenotypic pattern which hinted at a recombinational nature. Originally isolated as a fibroma virus, it was noticed that it was cytolytic in vitro. This observation was extended to a number of cell lines; in all cases the in vitro picture was that of an orthopoxvirus. However, on the basis of neutralization studies, SFV-I shared antigenic sites with both vaccinia virus and SFV.

Probed with SFV-W DNA, SFV-I DNA was shown to contain a subset of fragments that hybridized to leporipoxvirus sequences. The exact location of SFV sequences within the SFV-I genome is unknown (there may be integration at more than one site), as is the mechanism by which the recombinant arose. It is certainly the case that recombination can occur between homologous poxvirus sequences, a fact used in the construction of vaccinia virus vaccine virus vectors. As it was shown in the marker rescue experiments described here, such an event might occur between

nonhomologous orthopoxviruses and leporipoxviruses. It is not difficult to imagine a rabbit being coinfected with rabbitpoxvirus and Shope fibroma virus, and thus allowing the two viruses to replicate in the same cell.

Another aspect in the analysis of SFV-I is the study of its proteins. The resolution of many bands in polyacrylamide gels makes the task of comparison on the basis of polypeptide bands daunting. In spite of reports in the literature on genus and species-specific patterns, this approach did not seem promising. Immunoprecipitation was also found to be of limited value. In common with other investigators (Esposito, private communications), and perhaps because of poxvirus protein complexity, a clear picture of cross-reactive polypeptides between different poxviruses was not obtained.

Aside from total virion proteins (Ikuta et al., 1979) and some viral-associated enzymes (Pogo et al., 1982b), no studies on SFV have been reported. In comparing SFV-I to vaccinia virus and SFV-W, subsets of proteins with relevance to antigenic and functional differences among them were chosen. Analysis of the phosphoproteins showed some of these conserved between genera, with others genus-specific. Genus specificity is maintained in the glucosamine-containing proteins.

The work reported in this thesis is significant in a number of areas. It points out the possibility of recombination between genera of poxviruses by identifying just such an isolate. This has implications for the general molecular biology of poxviruses and for the possibility of such events re-occuring in nature. The identification of SFV-W sequences in a tumorigenic orthopoxvirus implies that these sequences might contain genes responsible for tumorigenicity. There is a need for a more detailed specification of the sequences; probing SFV-I DNA with cloned SFV-W DNA fragments, now available (Delange, et al., 1984), should localize these sequences to a particular locus and allow for precise mapping and eventual sequencing of the relevant genes. The work on leporipoxvirus proteins is only beginning. The results reported here have shown that there are certain unique SFV virion polypeptides. A number of experimental approaches suggest themselves as ways of furthering these observations. Two dimensional gels would clarify the precise number of unique SFV proteins. Identification of SFV-specific polypeptides within SFV-I may then be attempted by comparing 2-D gels for vaccinia virus, SFV-I and SFV-W. Western hybridizations, using antibodies against SFV-specific

proteins can also be done. Finally, mapping of polypeptides to specific DNA sequences can also be tried.

The data reported here establish for the first time the existence of a recombinant between two poxvirus genera. They also suggest further experiments that would exploit the opportunities provided by a recombinant between a cytolytic an a tumorigenic virus. New information on leporipoxvirus polypeptides has also been presented.

XI. REFERENCES

Ahlstrom, D.G., Andrews, C.H. (1938): Fibroma virus infection in tarred rabbits. J. Path. Bact. 47,65-86.

Allison, A.C. (1966): Immune responses to Shope fibroma virus in adult and newborn rabbits. J. Nat. Cancer Inst. 36,869-876.

Allison, A.C., Burke, D.C. (1962): The nucleic acid content of viruses. J. Gen. Microbiol. 27,181-194.

Allison, A.C., Friedman, R.M. (1966): Effect of immunosuppressants on Shope rabbit fibroma. J. Natl. Cancer Inst. 36,859-868.

Archard, L.C., Mackett, M. (1979): Restricton endonuclease analysis of red cowpox virus and its white pock variant. J. Gen. Virol. 45,51-63.

Arita, I. (1979): Virological evidence for the success of the smallpox eradication programme. Nature 279, 293-298.

Arita, M., Tagaya, I. (1977): Structural polypeptides of several strains of orthopoxvirus. Microbiol. Immunol. 21,343-346.

Bablanian, R. (1984): Poxvirus cytopathogenicity: Effects on cellular macvomolecular synthesis in: Comprehensive Virology, v. 19, chap. 8. Plenum Publishing Co., New York.

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

Bedson, H.S., Dumbell, K.R. (1964a): Hybrids derived from the viruses of alastrim and rabbitpox. J. Hygiene 62,141-146.

Bedson, H.S., Dumbell, K.R. (1964b): Hybrids derived from the viruses of variola major and cowpox. J. Hygiene 62,147-158.

Berkowitz, E.M., Pogo, B.G.T. (1982): Presentation at Poxvirus Meeting, Cold Spring Harbor, New York.

Berkowitz, E.M., Pogo, B.G.T. (1985): Molecular characterization of two strains of Shope fibroma virus. Virology 142,437-440.

Berns, K.I., Silverman, C. (1970): Natural occurrence of cross-linked vaccinia virus deoxyribonucleic acid. J. Virology 5,299-304.

Block, W., Upton, C. Mc Fadden, G. (1985): Tumorigenic Poxviruses: Genomic organization of malignant rabbit virus, a recombinant between Shope fibroma virus and myxoma virus. Virology 140,113-124.

Blomquist, M.C., Hunt, T.L., Barker, W.C. (1984): Vaccinia virus 19-kilodalton protein: Relationship to several mammalian proteins, including two growth factors. Proc. Natl. Acad. Sci USA, 81,7363-7367.

Bourke, A.T.C., Dumbell, K.R. (1972): An unusual poxvirus from Nigeria. Bulletin World Health Organization 46,621-623.

Brown, J.P., Twardzik, D.R., Marquardt, H., Todaro, G.J. (1985): Vaccinia virus encodes a polypeptide homologous to epidermal growth factor and transforming a growth factor. Nature 313,491-492.

Burnet, F.M. (1936): The use of the developing egg in virus research. Med. Res. Coun. Spec. Rep. Ser. No. 220.

Cabirac, G.F., Strayer, D.S., Sell, S., Leibowitz, J.L. (1985): Characterization, molecular cloning, and physical mapping of the Shope fibroma virus genome. Virology 143,663-670.

Chan, J.C., Hodes, M.E. (1973): Mechanism of Shope fibroma virus-induced suppression of host deoxyribonucleic acid synthesis. Infection and Immunity 7,532-538.

Chernos, V.I., Antonova, T.P., Senkevich, T.G. (1985): Recombinants between vaccinia and ectromelia viruses bearing the specific pathogenicity markers of both parents. J. Gen. Virology 66,621-626.

Clemmesen, J. (1939): The influence of Roentgen radiation on immunity to Shope fibroma virus. Am. J. Cancer 35,378-385.

Dales, S., Pogo, B.G.T. (1981): Biology of Poxviruses. Virology Monographs, 18.

Dales, S., Siminovitch, L. (1961): The development of vaccinia virus in Earle's L strain cells as examined by

electron microscopy. J. Biophys. Biochem Cytol. 10, 475-503.

Dalmat, H.J. (1958): Passage of Shope's rabbit fibroma virus through one-day-old mice. Proc. Soc. Exp. Biol. Med. 97,219-220.

DeFilippes, F.M. (1976): Restriction enzyme digests of rapidly renaturing fragments of vaccinia virus DNA. J. Virology 70,227-238.

DeFilippes, F.M. (1982): Restriction enzyme mapping of vaccinia virus DNA. J. Virology 43,136-949.

Delange, A.M., Macaulay, C., Black, .W., Mueller, T., Mc Fadden G. (1984): Tumorigenic poxviruses: Construction of the composite physical map of the Shope fibroma virus genome. J. Virology 50,408-416.

Dumbell, K.R., Bedson, H.S. (1964): The use of ceiling temperature and reactivation in the isolation of pox virus hybrids. J. Hygiene 62,133-140.

Duran-Reynals, F. (1940): Production of degenerative inflammatory or neoplastic effects in the newborn rabbit by the Shope fibroma virus. Yale J. Biol. Med. 13,99-110.

Duran-Reynals, F. (1945): Immunological factors that influence the neoplastic effects of rabbit fibroma virus. Cancer Res. 5,25-39.

Esposito, J.J., Knight, J.C. (1985): Orthopoxvirus DNA: A comparison of restriction profiles and maps. Virology 143,230-251.

Esposito, J.J., Obijeski, J.F., Nakano, J.H. (1978): Orthopoxvirus DNA: Strain differentiation by electrophoresis of restriction endonuclease fragmented virion DNA. Virology 89,53-66.

Esposito, J.J., Palmer, E.L., Borden, E.C., Harrison, A.K., Obijeski, J.F., Murphy, F.A. (1980): Studies on the poxvirus Cotia. J. Gen. Virol. 47,37-46.

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

Ewton, D., Hodes, M.E. (1967): Nucleic acid synthesis in Hela cells infected with Shope fibroma virus. Virology 33,77-83.

Fenner, F. (1958): The biological characteristics of several strains of vaccinia, cowpox and rabbitpox viruses. Virology 5,502-529.

Fenner, F. (1959): Genetic studies with mammalian poxviruses II. Recombination between two strains of vaccinia virus in single HeLa cells. Virology 8,499-507.

Fenner, F. (1979): Portraits of viruses: The poxviruses. Intervirology 11,137-157.

Fenner, F., Burnet, F.M. (1957): A short description of the poxvirus group (vaccinia and related viruses). Virology 4,305-314.

Fenner, F., Ratcliffe, F.N. (1965): Myxomatosis. Cambridge University Press, London.

Gangemi, J.D., Sharp, D.G. (1976): Use of a restriction endonuclease in analyzing the genomes of two different strains of vaccinia virus. J. Virology 20, 319-323.

Garon, C.F., Moss B. (1971): Glycoprotein synthesis in cells infected with vaccinia virus. II. A glycoprotein component of the virion. Virology 46,233-246.

Gemmel, A., Cairns, J. (1959): Linkage in the genome of an animal virus. Virology 8,381-383.

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

Gold, P., Dales, S. (1968): Localization of nucleotide phosphohydrolase activity within vaccinia. Proc. Nat. Acad. Sci. USA, 60,845-852.

Harel, J., Constantin, T. (1954): Shope-fibroma. Sur la malignite des trimeurs provaoquees par le virus fibromateux de Shope chez le lapin nouveau-ne et le lapin adulte traite par des doses massives de cortisone. Bull. Assoc. Franc. Cancer 41, 482-497.

Harper, L., Bedson, H.S., Buchan, A. (1979): Identification of Orthopoxviruses by polyacrylamide gel

electrophoresis of intracellular polypeptides. Virology 93,435-444.

Hinze, H.L., Walker, D.L. (1964): Response of cultured rabbit cells to infection with the Shope fibroma virus. I. Proliferation and morphological alteration of the infected cells. J. Bacteriology 88,1185-1194.

Hinze, H.L., Walker, D.L. (1971): Comparison of cytocidal and non-cytocidal strains of Shope rabbit fibroma virus. J. Virology 7,577-581.

Holowczak, J.A. (1970): Glycopeptides of vaccinia virus. I. Preliminary characterization and hexosamine content. Virology 42,87-99.

Holowczak, J.A. (1982): Poxvirus DNA. Current Topics in Microbiology and Immunology 97,28-79.

Holowczak, J.A., Joklik, W.K. (1967): Studies on the structural proteins of vaccinia virus. I. Structural proteins of virons and cores. Virology 33,717-725.

Holowczak, J.A., Thomas, V.L., Flores, L. (1975): Isolation and characterization of vaccinia virus "nucleoids". Virology 67,506-519.

Hughes, S.S. (1977): The Virus: A history of the concept. Science History Publications, New York.

Hyde, J.M., Gafford, L.G., Randall, C.C. (1967): Molecular weight determination of fowlpox DNA by electron microscopy. Virology 33,112-120.

Ikuta, K., Miyamoto, H., Kato, S. (1978a): Studies on the polypeptides of poxvirus. I. Comparison of structural polypeptides in vaccinia, cowpox and Shope fibroma viruses. Biken J. 21,51-61.

Ikuta, K., Miyamoto, H., Kato, S. (1978b): Studies on the polypeptides of poxvirus. II. Comparison of virus-induced polypeptides in cells infected with vaccinia, cowpox and Shope fibroma viruses. Biken J. 21,77-94.

Jacquemont, B. Grange, J., Gazzolo, L., Richard, M.H. (1972): Composition and size of Shope fibroma virus deoxyribonucleic acid. J. Virology 9,836-841.

Kasza, L. (1974): Isolation and characterization of a rabbit fibroma virus froma naturaaly occurring tumor. Am. J. Vet. Res. 35,87-89.

Kates, J.R., Mc Auslan, B.R. (1967): Poxvirus DNA dependant RNA polymerase. Proc. Nat. Acad. Sci. U.S.A. 58,134-141.

Keogh, E.V. (1936): The use of the developing egg in virus research. Med. Res. Coun. Spec. Ref. Ser. No. 220.

Kilham, L. (1956): Propagation of fibroma virus in tissue culturs of cottontail testes. Proc. Soc. Exp. Biol. Med. 92,739-742.

Kilham, L., Woke, P. (1953): Laboratory transmission of fibromas (Shope) in cottontail rabbits by means of fleas and mosquitoes. Proc. Soc. Exp. Biol. Med. 83,296-301.

Laemmeli, U.K. (1970); Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227,680-685.

Lloyd, B.J. Jr., Kahler, H. (1955): Electron microscopy of the virus of rabbit fibroma J. Natl. Cancer Inst. 15,991-999.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193,265-275.

Mackett, M., Archard, L.C. (1979): Conservation and variation in Orthopox genome structure. J. Gen. Virol. 45,683-701.

Mackett, M., Smith, G.L., Moss, B. (1984): General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. J. Virology 49,856-864.

Maniatis, T., Fritsch, E.F., Sambrook, J. (1982): Molecular cloning, a laboratory handbook. Cold Spring Harbor Laboratory, New York.

Marennikova, S.S., Shelukhina, E.M., Maltseva, N.N., Matsevich, G.R. (1979): Monkeypox virus as a source of whitepox viruses. Intervirology 11,333-340.

Matthews, R.E.F. (1982): Classification and nomenclature of viruses. Intervirology 17,42-46.

Mc Clain, M.E., Greenland, R.M. (1965): Recombination between rabbitpox virus mutants in permissive and nonpermissive cells. Virology 25,516-522.

McFadden, G., Dales, S. (1979): Biogenesis of poxviruses: mirror image deletions in vaccinia virus DNA. Cell 18,101-108.

Moss, B., Rosenblum, E.N., Garon, C.F. (1971): Glycoprotein synthesis in cells infected with vaccinia virus. I. Non-viron glycoproteins. Virology 46,221-232.

Moyer, R.W., Rothe, C.T. (1980): The white pock mutants of rabbit poxvirus. I. Spontaneous host range mutants contain deletions. Virology 102,119-132.

Muller, H.K., Wittek, R., Schaffner, R., Schumperli, D., Menna, A., Wyler, R. (1977): Comparison of five poxvirus genomes by analysis with restriction endonucleases Hind III, Bam I and Eco RI. J. Gen. Virol. 38,135-147.

Munyon, W.E., Paoletti, E., Grace, J.T. Jr. (1967): RNA polymerase activity in purified vaccinia virus. J. Virol. 2,167-172.

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

Niven, J.S.F., Armstrong, J.A., Andrewes, C.H., Pereina, H.G., Valentine, R.C. (1961): Subcutaneous "growths" in Monkeys produced by a poxvirus. J. Path. Bact. 81,1-14.

Obijeski, J.F., Palmer, E.L., Gafford, L.G., Randall, C.C. (1973): Polyacrylamide gel electrophoresis of fowlpox and vaccinia virus proteins. Virology 51,512-516.

O'Farrell, P.H. (1975): High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250,4007-4021.

Olgiati, D.D.G., Pogo, B.G.T., Dales, S. (1976): Biogenesis of vaccinia: Specifice inhibition of rapidly labeled host DNA in vaccinia innoculated cells. Virology 71,325-335.

Paez, E., Dallo, S., Esteban, M. (1985): Generation of a dominant 8-mDa deletion at the left terminus of vaccinia virus DNA. Proc. Nat. Acad. Sci. USA 82,3365-3369.

Padgett, B.L., Moore, M.S., Walker, D.L. (1962): Plaque assays for myxoma and fibroma viruses and differentiation of the viruses by plaque form. Virology 17,462-469.

Payne, L.G., Norby, E. (1978): Adsorbtion and penetration of enveloped and naked vaccinia virus particles. J. Virology 27,19-27.

Perucho, M., Hanahan, D., Wigler, M. (1981): Genetic and physical linkage of exagenous sequences in transformed cells. Cell 22, 309-317.

Pogo, B.G.T., Dales, S. (1969): Two deoxyribonuclease activities within purified vaccinia virus. Proc. Nat. Acad. Sci. USA 63,820-827.

Pogo, B.G.T., Dales, S. (1973): Biogenesis of poxviruses: inactivation of host DNA polymerase by a component of the invading inoculum particle. Proc. Nat. Acad. Sci. USA 70,1726-1729.

Pogo, B.G.T., Dales, S. (1974): Biogenesis of poxviruses: further evidence for inhibition of host and virus DNA synthesis by a component of the invading inoculum particle. Virology 58,377-386.

Pogo, B.G.T., Dales, S. (1977): Elimination of naturally occurring cross-links in vaccinia virus DNA after viral penetration into cells. Proc. Natl. Acad. Sci. USA, 74,1739-1742.

Pogo, B.G.T., Freimuth, P., Stein, A. (1982a): Shope fibroma virus. I. Biological and molecular properties of a cytocidal and a non-cytocidal strain. J. Virology 41,97-103.

Pogo, B.G.T., Katz, J.R., Dales, S. (1975): Biogenesis of poxviruses: synthesis and phosphopylation of a basic protein associated with the DNA. Virology 64,531-543.

Pogo, B.G.T., Stein, A., Freimuth, P. (1982b): Shope fibroma virus. II. Role of the viron-associated nucleases. J. Virology 41,104-109.

Postlethwaite, R. (1970): Molluscum contagiosum. A review. Archives of Environmental Health 21,432-452.

Rigby, P.W.J., Dieckmann, M., Rhodes, C., Berg, P. (1977): Labeling deoxyribonucleic acid to high specific activity in

vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113,237-251.

Sam, C.K., Dumbell, K.R. (1981). Ann. Virol. Inst. Pasteur, Paris. 132 E: 135-150.

Sambrook, J.F., Padgett, B.L., Tomkins, J.K.N. (1966): Conditional lethal mutants of rabbitpox virus. I. Isolation of host cell-dependent and temperature-dependent mutants. Virology 28,592-599.

Sanarelli, G. (1898): Das myxomatogene virus. Beitrag zum Stadium der krankheitserreger ausserhalb es Sichtbaren. Zentbl. Bakt. Parasitkde, Abt. 1, Orig. 23,865-873.

Sarov, I., Joklik, W.K. (1972): Studies on the nature and location of the capsid polypeptides of vaccinia virons. Virology 50,593-602.

Sell, S., Scott, C.B. (1981): An immunohistologic study of Shope fibroma virus in rabbits: Tumor rejection by cellular reaction in adults and progressive systemic reticuloendothelial infection in neonates. J.N.C.I. 66,363-373.

Shatkin, A.J. (1963): Actinomycin D and vaccinia virus infection of HeLa cells. Nature 199,357-358.

Shope, R.E. (1932a): A transmissible tumor-like condition in rabbits. J. Exp. Med. 56,793-802.

Shope, R.E. (1932b): A filterable virus causing a tumor-like condition in rabbits and its relationship to virus myxomatosum. J. Exp. Med. 56,803-822.

Smith, M.H.D. (1952): The Berry-Dedrick transformation of fibroma into myxoma in the rabbit. Ann New York Acad. Sci. 54,1141-1152.

Southern, E.M. (1975): Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517.

Stern, W., Dales, S. (1976): Biogenesis of vaccinia: isolation and characterization of a surface component that elicits antibody suppressing infectivity and cell-cell fusion. Virology 75,323-341.

Strayer, D.S., Skaletsky, E., Cabirac, G.F., Sharp, P.A., Corbeil, L.B., Sell, S., Leibowitz, J.L. (1983): Malignant

rabbit fibroma virus causes secondary immunosuppresion in rabbits. J. Immunol. 130,399-404.

Temin, H. (1971): Mechanism of cell transformation by RNA tumor viruses. Ann. Rev. Microbiol. 25,609-648.

Tompkins, W.A.F., Walker, D.L., Hinze, H.C. (1969): Cellular deoxyribonucleic acid synthesis and loss of contact inhabition in irradiated and contact inhibited cell cultures infected with fibroma virus. J. Virology 4,603-609.

Tooze, J. (1980): DNA Tumor viruses. Cold Spring Harbor Laboratory, New York.

Towbin, H., Staehelin, T., Gordon, J. (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76,4350-4354.

Turner, A., Baxby, D. (1979): Structural polypeptides of orthopoxvirus: Their distribution in various members and location within the virion. J. Gen. Virol. 45,537-545.

Ueda, Y., Dumbell, K.R., Tsuruhara, T., Tagaya, I. (1978): Studies on Cotia virus - and unclassified poxvirus. J. Gen. Virol. 40,263-276.

Wahl, G.M. Stern, M., Stark, G.R. (1979): Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA 76,3683-3687.

Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G., Chasin, L. (1979): DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA 76,1373-1376.

Wills, A., Delange, A.M., Gregson, C., Macaulay, C., Mc Fadden, G. (1983): Physical characterization and molecular cloning of the Shope fibrom virus genome. Virology 130,403-414.

Woodroofe, G.M., Fenner, F. (1960): Genetic studies with mammalian poxviruses. IV. Hybridization between several different poxviruses. Virology 12,272-282.

Woodruff, A.M., Goodpasture, E.W. (1931): The susceptibility of the chorioallantoic membrane of chick embryos to infection with the fowlpoxvirus. Am. J. Path. 7,209-222.