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Studies on shope fibroma virus: A tumorigenic poxvirus

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STUDIES ON SHOPE FIBROMA VIRUS:

A TUMORIGENIC POXVIRUS

by

Kristina M. Obom

A dissertation presented 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

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Abstract

SHOPE FIBROMA VIRUS: A TUMORIGENIC POXVIRUS

by

Kristina M. Obom

Adviser: Dr. Beatriz G.-T. Pogo

Shope fibroma virus (SFV) is a leporipoxvirus which induces benign tumors in its natural host but fibrosarcomas in newborns and immunocompromised adults. The ability of SFV to induce oncogenesis *in vitro* was investigated by inoculating an immortalized rabbit cell line (SIRC) with ultraviolet-irradiated virus. The resulting cell transformants displayed the characteristic properties of the malignant phenotype: lack of infectious particles, low serum requirement, high efficiency of cloning, resistance to superinfection, presence of viral DNA sequences in the nucleus, expression of viral proteins and induction of tumors in rabbits. This transformation was not stable since in all cell lines studied, a loss of the malignant phenotype was recorded close to the 50th passage.

To assess the oncogenic potential of SFV, NIH 3T3 cells were transfected with SFV DNA. The results indicated that SFV DNA could induce formation of *foci* in certain NIH 3T3 cell lines. *Focus*-derived cell lines were established to study the biological and molecular properties of the transformants. Results indicated that five of the six lines studied induced tumorigenesis in nude mice and that four lines were anchorage independent. At early passages all cell lines contained SFV DNA sequences and two of three lines tested expressed SFV DNA. It is concluded that SFV can induce the second stage of

carcinogenesis and that SFV DNA can induce transformation and tumorigenic conversion of NIH 3T3 cells.

To test which region(s) of the genome is (are) necessary for transformation, NIH 3T3 cells were transfected with cloned Bam HI fragments of SFV DNA containing terminal sequences of the molecule, either alone or in combination. Only fragment C which contains the SFV growth factor could induce focus formation but transformants failed to induce tumorigenesis in nude mice, suggesting that the growth factor alone is not responsible for tumorigenic conversion of NIH 3T3 cells.

Indiana virus is an orthopoxvirus which is cytolytic in vitro but induces benign tumors in rabbits. Comparative DNA studies of SFV, Indiana virus and vaccinia virus revealed the presence of mitochondria (mt) DNA in purified preparations of viral DNA. The viral and mt DNAs could be separated on the basis of their size by agarose gel electrophoresis but not by their buoyant density in isopycnic cesium chloride gradients. In addition, Southern hybridization using cloned SFV fragments as probes revealed a previously unrecognized region of homology between the SFV Bam HI fragment A and the orthopoxvirus Hind III fragments G, J and L. Taken together results from these studies are relevant to the understanding of the process of tumorigenesis by poxviruses and the genomic structure of different genera of poxviruses.

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I. The Poxviruses

A. Introduction

The vertebrate poxviruses infect a wide range of animal species inducing hyperplasia as part of their pathogenicity. Poxviruses were first in many respects. They were the first to be visualized microscopically, purified and chemically analyzed. The first example of the use of a virus as a vaccine to protect against a devastating disease was accomplished by Jenner in 1798 by the use of cowpox virus. The first virus ever to be completely eradicated was variola, the causative agent of smallpox. The first tumorigenic virus to be identified was myxoma in 1898 (Sanarelli, 1898). In recent years however, research in poxviruses has lagged behind that of other viruses so that many aspects of their mechanisms of replication, transcription, translation, maturation and transformation are not understood. The possibility of using vaccinia virus as a vector for foreign genes and as a vaccine has stimulated the study of this family of viruses again. The scope of this thesis is to investigate the phenomenon of transformation by poxviruses, in particular by Shope fibroma virus and gain an understanding about the mechanisms involved.

B. Classification

A classification scheme proposed by the International Committee on Taxonomy (Matthews, 1979) divides the family Poxviridae into two major subfamilies, the chordopoxviruses (vertebrate poxviruses) and the entomopoxviruses (insect poxviruses). The chordopoxviruses share a common group specific NP antigen (Takahashi et al., 1959, Woodroofe and Fenner, 1962) and the ability to rescue a

heat-inactivated poxvirus by the process of non-genetic reactivation (Hanafusa, H. et al., 1959, Hanafusa, T. et al., 1959). The vertebrate poxviruses are further divided into six genera on the basis of serologic cross-reactivity and similarities in morphology (Dales and Pogo, 1981). Shope fibroma virus is a member of the leporipox genus, while vaccinia the best studied of the poxviruses is a member of the orthopox genus.

C. General Characteristics

Poxviruses are large brick-shaped viruses of approximately 220 nm surrounded by a lipid envelope. The internal structure of the virion as determined by electron microscopy is composed of two lateral bodies and a central core containing the viral DNA (Pogo and Dales, 1969). The viral genome consists of 160 to 180 kilobases (kb) of double standed DNA with covalently closed termini (Berns and Silverman, 1970, Geshelin and Berns, 1974). In addition, the termini of poxviruses are inverted terminal repeats or mirror images (Garon et al., 1978). The genome has a coding capacity for over 200 polypeptides and Essani and Dales (1979) have identified at least 110 proteins by two dimensional polyacrylamide gel electrophoresis. As the virus life cycle is entirely cytoplasmic, the enzymes required for transcription and replication are virus encoded (Dales and Pogo, 1981). Recent evidence suggests that the virus may package some host proteins and nucleic acids including the host thymidine kinase (tk) (Franke and Hruby, 1987) a host polymerase II subunit (Morrison and Moyer, 1984) and ribosomal RNA (Roening and Holowczak, 1974). The biological role of these cellular components remains to be determined.

D. Life Cycle

Most of the information gathered on the life cycle of poxviruses comes from extensive studies of vaccinia virus. Other poxviruses are assumed to replicate in a similar manner although the time required for viral morphogenesis varies.

Adsorption and Penetration

The pox virion is thought to bind to a specific cellular receptor possibly by its surface tubular elements, although neither a cell receptor or specific viral ligand has been identified. Entry into the cell is by the process of viropexis or by fusion of membranes (Dales and Siminovitch, 1961, Dales, 1973). After entry the lateral bodies and core are released into the cytoplasm.

Transcription

Within minutes of entering the cell transcription is initiated within the viral cores. Transcription occurs even in the presence of inhibitors of DNA or protein synthesis because the cores contain the transcriptional enzymes. There is a sequential appearance of messages with DNA replication dividing transcription into early and late phases. Both classes of mRNA contain a 7-methylguanosine cap (Wei and Moss, 1975) and are polyadenylated at the 3' end (Kates and Beeson 1970). However, there are many differences between early and late transcripts. Early messages are regulated by a promoter located 20-30 nucleotides upstream from the start of transcription. The promoter region is very AT-rich but varies significantly from eukaryotic and prokaryotic promoters (Weir and Moss, 1983). There is no evidence for splicing (Wittek et al., 1980) or 5' processing (Venkatesan and Moss,

1981). Early messages are precisely terminated, approximately 30 bases downstream from a termination signal (Rohrmann et al., 1986).

Three necessary events precede the transcription of late mRNAs; early protein synthesis, exposure of the genome by secondary uncoating and replication of the DNA. Late messages can be distinguished by their larger and more heterogeneous size (Oda and Joklik, 1967). The heterogenecity in length is due, in part, to imprecise termination of the messages (Moss, 1985). A large fraction of messages can self-hybridize to form double-stranded structures indicating extensive symmetrical transcription (Colby and Duesberg, 1969). Recent evidence from Bertholet et al. (1987) indicated that the 5' end of the messages have extreme length heterogenecity and that these additional 5' nucleotides come from other regions of the genome, upstream and downstream of the coding region. These sequences are attached to the coding region by a poly A sequence and are not translated in vivo. Finally the promoters for late transcription appear to be different than those for early transcription (Hanggi et al., 1986).

The regulation of poxvirus transcription has not been completely elucidated, however the data suggest several mechanisms. First, as previously stated, the promoters for early and late transcription are different, implicating a different or modified polymerase. One group of investigators has identified a subunit of host RNA pol II in the core of rabbitpox virions (Morrison and Moyer, 1984) and speculated that this enzyme subunit might play a role in transcriptional regulation (Moyer, 1987). Second, as the course of infection progresses, the stability of the transcripts decreases. Third,

regulation may occur at the translational level since at late times, early transcripts are present but not translated (Oda and Joklik, 1967).

Translation

To synthesize proteins the virus commandeers the host ribosomes, degrading host polysomes and forming viral polysomes (Metz et al., 1975), which preferentially translate viral messages. Viral translation is temporally regulated such that there are two major classes of proteins; the early proteins, which are synthesized before DNA replication and the late proteins which are made during and after DNA replication. There appear to be three subsets of early proteins with the following characteristics: first, polypeptides that are synthesized and immediately shut-off; second, polypeptides with a progressively increased synthesis and third, polypeptides which are synthesized for prolonged periods (Esteban and Metz, 1973).

Late proteins are made in a similar manner with some polypeptides being synthesized at the time of maximal DNA replication reaching a peak 1 hr. later and then shut-off. The production of other proteins begins during DNA replication and synthesis lasts longer and a third set of peptides begin to appear 1 hr. after maximal DNA replication and continue to be synthesized for a prolonged period (Pennington, 1974). Blocking DNA replication with hydroxyurea results in continuous synthesis of early proteins with no switchover to late protein production (Pennington, 1974).

A key element in the regulation of viral translation is the regulation of transcription. However other mechanisms may also be

important, since early transcripts are present at late times, as shown by hybridization competition, but are not translated *in vivo* (Oda and Joklik, 1967).

Poxvirus Replication

Replication of the viral genome begins within 90 minutes of virus penetration. While the exact mechanism is not known and it is not yet possible to synthesize a full-length genome *in vitro*, the data suggest a replication scheme similar to that employed by parvoviruses. The viral DNA is replicated by the viral DNA polymerase, a 110 KD protein which is made early in infection and has polymerase and 5' to 3' exonuclease activity, (Chalberg and Englund, 1979). DNA replication is semi-conservative and bi-directional. The terminal cross-links are removed and replication begins at the termini (Pogo et al., 1981). Small fragments of DNA (12 S) are synthesized onto RNA primers. The 12 S fragments are ligated to make intermediate fragments (34 S) which are joined to make a full length genome (Esteban and Holowczak, 1977, Pogo and O'Shea, 1978). Concatemeric intermediates were suggested by mirror-image deletions within the inverted terminal repeats of vaccinia (McFadden and Dales, 1979) and by restriction enzyme analysis of replicative intermediates which revealed terminal fragments twice the size of the mature virus length (Moss et al., 1983). The hairpin loops appear to be added after replication (Esteban and Holowczak, 1977). Additional experimental evidence from the study of rabbitpox mutants (Moyer and Graves, 1981) and vaccinia mutants (Pogo et al., 1984) strongly suggested that replication proceeds by a mechanism similar to parvoviruses which have

hairpin termini and concatameric replication intermediates.

Assembly and Release

Even before replication of the genomic DNA is complete, assembly of new virions begins in factories. The lipid membrane is assembled and the rigidity of the membrane is provided by the spicules (Dales and Mossbach, 1968). The newly synthesized DNA and proteins are inserted into the membrane for assembly of the core and lateral bodies. The viral envelope is further modified by the replacement of the spicule layer with surface tubular elements (Stern and Dales, 1976).

The majority of the virions remain host cell associated, however a small number are released by budding. These particles obtain a second lipid envelope from the Golgi apparatus and this new envelope contains an additional eight virus specific proteins and glycopeptides, none of which are found elsewhere in the virion (Appleyard et al., 1971, Payne, 1978). Released virus is more efficiently taken up by the cell, than the cell associated-virus.

Effect on the host cell

Poxviruses have a very profound effect on the host cell. The majority of poxviruses are highly cytolytic in vitro, resulting in the death of the cell. The demise of the cell can be attributed to a number of viral induced processes which affect host macromolecular syntheses. Very early in the infectious cycle and in absence of virus replication, host protein and DNA syntheses are inhibited. Two mechanisms have been suggested to explain the shut-off of host protein synthesis. One mechanism involved the direct involvement of virion

proteins. Thus, it has been shown that viral cores inhibit host translation directly (Cooper and Moss, 1978). On closer examination of partially purified core proteins, Ben-Hamida et al., (1983) suggested that the core phosphoproteins inhibited translation by preventing 40 S initiation complex formation. A second mechanism implicates small viral transcripts, specifically the viral poly (A), as selective inhibitors of translation (Bablanian and Banerjee, 1986).

Host DNA replication is also inhibited early in infection and does not require viral replication for shut-down. A mechanism has been proposed whereby a virus-associated nuclease may be released from the incoming virions and enter the nuclei where it can cleave DNA (Pogo and Dales, 1973). This enzyme is known to digest exclusively single stranded newly synthesized host DNA (Olgiati et al., 1976).

Host ribosomal RNA is affected by 2 hrs. post-infection, the cleavage of the 45 S precursor is blocked and by 3 hr. post-infection transport of ribosomal RNA to the cytoplasm is inhibited (Jefferts and Holowczak, 1971). The synthesis of host heterogeneous mRNA is also affected late in infection (Dales and Pogo, 1981).

E. Tumorigenic poxviruses

All poxviruses initially induce the proliferation of cells *in vivo*. For example, the pox produced during smallpox infection can be regarded as a small tumor that quickly regresses. However, a number of these viruses have been shown to induce persistent benign and malignant tumors *in vivo* and the transformation of cell *in vitro*.

In vivo

Based on their oncogenic potential, the tumorigenic poxviruses

can be classified in two categories, viruses which induce self-limiting neoplasia and viruses which induce malignant growth. The first category includes Shope fibroma virus (SFV), squirrel fibroma and hare fibroma, all members of the leporipox genus, which induce fibroblastic hyperplasia in their natural hosts (Dales and Pogo, 1981). Two unclassified poxviruses are members of this category, Molluscum contagiosum which induces benign tumors of epithelial origin in humans (Postlewaite, 1970), and Yaba monkey tumor virus that causes subcutaneous tumors in monkeys (Nivens, 1961). Indiana virus, classified as an orthopox virus (Berkowitz and Pogo, 1985) has been shown to induce tumors in rabbits but is highly cytolytic in vitro (Pogo et al., 1982a).

Members of the second category, either independently or in association with chemical agents induce malignancy. Although SFV has a relatively benign course in the adult rabbit it induces highly malignant fibrosarcomas in the newborn (Allison, 1966) and rabbits immunosuppressed with coal tar or cortisone (Andrewes and Ahlstrom, 1938; Allison and Friedman, 1966). The first tumorigenic poxvirus to be described was myxoma virus in 1898 (Sanarelli, 1898). This agent is both highly malignant and immunosuppressive, and the host (rabbit) dies rapidly after infection. A recently identified virus, malignant rabbit fibroma appears to be a recombinant between myxoma and SFV and expresses characteristics of both (Strayer et al., 1983).

The cocarcinogenic potential of vaccinia virus was demonstrated by Duran-Reynals (1957) who showed that mice painted with methylcholanthrene and then inoculated at that site with vaccinia

virus by scarification, developed malignant tumors. In addition, there have been several reports in the literature indicating that malignant tumors developed at the site of smallpox vaccination (Marmelzat et al., 1964, Marmelzat, 1968).

In vitro

Three poxviruses have been shown to transform cells in culture: vaccinia virus, Molluscum contagiosum and Yaba monkey tumor virus. Vaccinia virus was shown to transform mouse embryo cells (Koziorowska, 1971) for more than 90 passages. Molluscum contagiosum abortively infects cells in culture expressing only early functions (Neva, 1962). An abortive infection of human embryonic fibroblasts resulted in a transient transformation characterized by loss of contact inhibition and growth in soft agar (Barbanti-Brodano et al., 1974). Rouhandeh and Vafai (1982) demonstrated the transforming ability of Yaba monkey tumor virus by infecting JINET cells with ultraviolet (uv) irradiated virus. The transformants were anchorage independent, grew in reduced serum concentration and contained Yaba DNA sequences in the nucleus.

F. Shope fibroma virus

Biological characteristics

In vivo

SFV was first isolated in 1932 from a tumor on the hind foot of a wild cotton-tailed rabbit shot near Princeton, N.J. (Shope, 1932). The virus induces a fibroma in the adult experimental rabbit which reaches its maximum size of approximately 4 cm by day 9 post-infection and then spontaneously regresses. In the wild

cotton-tailed rabbit the tumor persists for up to 77 days (Shope, 1932). The virus has a very limited host range replicating only in the rabbit but not in mice, guinea pigs, rats, chickens (Shope, 1932) or nude mice (Obom and Pogo, unpublished results). As mentioned previously, SFV induces a highly malignant fibrosarcoma in the newborn which often results in the death of the animal (Allison, 1966). The lethality of SFV in the neonate is due to a weak cell mediated immune response as demonstrated by weak delayed-type hypersensitivity reaction and macrophage migration inhibition tests (Tompkins et al., 1973). The immunosuppressed adult suffers a similar fate as generalized fibromata and lethal fibrosarcomas develop (Andrewes and Ahlstrom, 1938, Allison and Friedman, 1966).

In vitro

Unlike other poxviruses which are highly cytolytic, when susceptible cells in culture are infected with SFV, foci develop three to five days post-infection. The cells lose contact inhibition, piling up on each other and change morphologically to a more rounded shape (Padgett et al., 1962, Hinze and Walker, 1964). The change in cell morphology appears to be an early function since uv-inactivated virus induces cell rounding (Crouch and Hinze, 1977). The number of foci can be increased two-fold by the addition of 12-0-tetradecanoyl-phorbol-13-acetate (TPA) to the media (Pogo, 1983).

The virus has a very limited host range replicating only in cells of rabbit or monkey origin but not in cells of human or mouse origin (Pogo et al., 1982a).

SFV has also been shown to induce a persistent infection. The

virus can persist in rabbit cells after a few passages, altering the cell morphology and increasing cell multiplication with loss of contact inhibition (Hinze and Walker, 1964).

Effect on the host cell

SFV has a profound effect on host DNA synthesis. Initially, a burst of host DNA synthesis is observed followed by a gradual shut-off (Chan and Hodes, 1973). The inhibition of incorporation of ^{3}H-thymidine is first noted at 8 hrs. post-infection (PI) and a maximum inhibition of 60% as compared to control levels is attained by 16 hrs. PI. At 48 hrs. the cell's DNA synthesis returns to control levels and by 72 hrs. increases two to three-fold (Pogo et al., 1982a). The incomplete shut-off of host DNA synthesis by SFV as compared to vaccinia has been related to the very small amount of virus specific endonucleases present in the infected cell (Pogo et al., 1982b). The effect of SFV infection on other host processes has not been determined.

Molecular characteristics

Viral DNA

The size of the SFV genome has been estimated to be 160 kb by restriction enzyme analysis (Wills et al., 1983). The ends of the genome have covalently closed termini (Pogo et al., 1982a) and inverted terminal repeats of 12.2 kb (Delange et al., 1984). There are nine open reading frames in the termini (Macauley et al., 1987).

Proteins

One dimensional polyacrylamide gel electrophoresis has identified at least 42 proteins in the purified virions (Pogo et al.,

1982a). In the future, two dimensional gels will give a far clearer estimate of the exact number of proteins. Only two proteins have been mapped the viral thymidine kinase and a putative growth factor by hybridization of cloned fragments of the SFV genome to degenerate oligonucleotide probes based on the vaccinia sequences for these genes.

The thymidine kinase gene is located in the left portion of the Bam HI fragment A (Upton and McFadden, 1986b). The region around this gene shares sequence homology with the region surrounding the vaccinia thymidine kinase gene. The putative growth factor gene is located in the Bam HI fragment C and based on the deduced amino acid code shares structural homology to epidermal growth factor (EGF), transformation growth factor-α (TGF-α) and the vaccinia growth factor (VGF) (Chang et al., 1987). The SFV growth factor has not been isolated yet, either from infected cells or from the culture media.

The vaccinia growth factor has been shown to be important in vivo for pox formation. A mutant vaccinia virus with deletions inactivating the VGF has been constructed. The mutant virus formed plaques and grew to normal titers in cell culture but had a markedly lower cell proliferative effect in rabbit skin and chick chorioallantoic membrane (Moss, 1987). Whether the SFV growth factor plays a similar role in tumor formation has not been established.

G. pSIC-9

An endogenous replicating plasmid has been identified in an established rabbit cell line and in primary rabbit cells (Upton and McFadden, 1986a), a portion of this 4.2 kb plasmid designated pSIC-9,

has been cloned and sequenced. It shares sequence homology with part of the inverted terminal repeat of SFV and with the rabbit genome. It has two open reading frames, one of SFV origin and the other which has homology with the serine protease inhibitor superfamily.

II. Cell Transformation

The change in a cell from a normal to a malignant state is a multistage process requiring the expression of several genes (Land et al., 1983). The oncogenic process can be divided into two major steps, immortalization or loss of senescence and tumorigenic conversion. The second step is characterized by major phenotypic changes in the cell such as loss of contact inhibition, anchorage independence, growth in low serum, ability to induce tumors in nude mice, morphologic changes and changes in karyotype.

The study of DNA and RNA tumor viruses has significantly contributed to our understanding of the mechanisms of oncogenesis. Another important contributor to the elucidation of the transformation mechanism and identification of transforming genes has been the use of the NIH 3T3 cell for transfection assays. Transfection of NIH 3T3 cells with DNA containing an oncogene often results in expression of the transformed phenotype or <u>focus</u> formation. This technique has allowed investigators to screen a large number of DNAs for their oncogenic potential.

Among the DNA viruses, studies on adenovirus, polyoma virus, SV40, papillomaviruses and herpesviruses have revealed two different mechanisms for transformation. The majority of these viruses encode one or more genes, whose expression is necessary for transformation.

For example the proteins encoded within the El region of the adenovirus genome are required for complete transformation of primary cells. The Ela gene cluster encodes the functions necessary for immortalization and the Elb region, those required for the second stage of transformation (Grand, 1987). Polyomavirus encodes three transforming proteins, all of which play a role in transformation. The large T antigen is required for immortalization and growth in low serum, middle T plays a key role in the second stage of transformation and small t increases the efficiency of transformation (Rassoulzadegan et al., 1983, Cuzin, 1984). In contrast it has been established that the transforming region of HSV-2 does not code for any polypeptides but does contain a sequence which might form a stem-loop structure (Galloway et al. 1984). This insertion-like element might act as an enhancer for an endogenous proto-oncogene.

Another characteristic of the transformation by certain DNA tumor viruses is that even after the transformed cells lose the viral sequences, they maintain the transformed state. Such is the case of herpesvirus (Galloway, 1982) and of adenovirus (Kuhlmann et al., 1982). A hit and run type phenomenon has been implicated.

Retroviruses, the only RNA viruses which are transforming, induce oncogenesis by two general mechanisms. The non-acute retroviruses transform cells by a mechanism called "insertional mutagenesis" by damaging the cellular gene directly or by bringing a cellular proto-oncogene under the control of viral regulatory elements (Varmus, 1982). In the second mechanism, recombination between retroviral and cellular genomes can implant cellular genes into the viral genome and

in this new setting the cellular genes may become oncogenic. This is referred to as transduction (Bishop, 1983). Transduced cellular genes include growth factors, growth factor receptors, tyrosine kinases and nuclear proteins (Land et al., 1983b).

A. Growth factors

Growth factors are polypeptides that function as extracellular agents which modulate cell functions or activate normally quiescent cells to enter a cycle of proliferation. Growth factors can interact either with the cell from which they are secreted (autocrine) or with cells at a distance (endocrine or paracrine stimulation). The autocrine mechanism may play an important role in malignant transformation (Sporn and Todaro, 1980; Sporn and Roberts, 1985).

B. Role of EGF and TGF-α in transformation

TGF-α was first demonstrated in the culture media of cells transformed by murine sarcoma virus (Delarco and Todaro, 1978). The conditioned media was able to confer the transformed phenotype to non-transformed cells.

The actual role of EGF and TGF-α as direct acting oncogenes has only recently been studied. Two groups of investigators have transfected immortalized cells in culture with TGF-α under the control of strong promoters. Finzi et al. (1987) demonstrated that TGF-α could not transform NIH 3T3 cells although they did observe an increase in saturation density. On the other hand, Rosenthal et al. (1986) found that TGF-α could induce anchorage independence and tumorigenesis of Rat-1 cells. However, the tumors remained small in size and it appeared TGF-α only served to enhance the tumorigenic

effect of the already tumorigenic Rat-1 cell line.

A construct containing EGF under the control of MLV-LTR was also transfected into Rat-1 cells and FR 3T3 fibroblasts resulting in the induction of foci (Stern et al., 1987). None of the isolated clones were anchorage independent and only those which expressed high levels of EGF were tumorigenic in nude mice. The data suggested that the EGF family of growth factors may enhance tumorigenesis but they are not strong oncogenes by themselves.

Goals of research

The purpose of this study was to determine if Shope fibroma virus could transform cells in vitro and to gain some understanding about the mechanisms of poxvirus-induced tumorigenesis. In addition, the genetic relatedness of SFV to other poxviruses was studied.

The tumorigenic potential of SFV has intrigued investigators since its isolation in 1932, however, the oncogenic mechanism is still not known. A first step in unravelling SFV oncogenesis was to determine if SFV was transforming in vitro. First, immortalized rabbit corneal cells, SIRC cells, were infected with uv-irradiated virus and monitored for 70 passages for characteristics of transformation. Second, NIH 3T3 cells were transfected with calcium-phosphate precipitated SFV DNA and monitored for the transformed phenotype; focus formation, induction of tumors in nude mice, and anchorage independent growth.

Once established that SFV transformed cells in vitro, it was important to determine which region(s) of the genome were necessary for transformation. Transfection of calcium-phosphate precipitated

cloned SFV fragments into NIH 3T3 cells provided a way to screen sub-genomic fragments for focus formation, i.e. transformation.

My results revealed that Shope fibroma virus induced a transient transformation of an immortalized cell line, SIRC cells, and that the entire SFV genome elicited complete transformation of NIH 3T3 cells. The data also indicated that from the fragments employed the Bam HI fragment C, which contains the SFV growth factor, induced focus formation in NIH 3T3 cells. However this is not a complete transformation, since the cells from the foci were not tumorigenic.

Previous work in the lab (Berkowitz and Pogo, 1985) demonstrated the genetic relatedness between SFV, Indiana virus and vaccinia virus by Southern hybridization using entire genomic probes. The molecular relationship of these viruses was reexamined using cloned fragments of SFV DNA as probes. These studies led to the discovery that poxvirus cores contained discrete amounts of mitochondria DNA, that may be packaged during morphogenesis and that the genomes of leporipox and orthopoxviruses shared a previously unrecognized region of homology.

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Chapter 2

Characterization of the transformation properties of Shope fibroma virus

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Summary

To investigate if Shope fibroma virus (SFV), a leporipoxvirus that induces benign tumors in adult rabbits, can trigger the second step of carcinogenesis *in vitro* or malignant transformation, an already immortalized rabbit cell line (SIRC) was inoculated with ultraviolet-irradiated virus. The resulting cell transformants displayed the characteristic properties of the malignant phenotype: lack of infectious particles, low serum requirement, high efficiency of cloning, resistance to superinfection, presence of viral DNA sequences in the nucleus, expression of viral proteins and induction of tumors in rabbits. However, this transformation was not stable since in all cell lines studied, a loss of the malignant phenotype was recorded close to the 50th passage. To assess the oncogenic potential of SFV, NIH 3T3 cells were transfected with SFV DNA. The results of these experiments indicate that SFV DNA can induce the formation of *foci* in certain NIH 3T3 cell lines. Taken together these results support the notion that SFV can elicit the transformation of cells *in vitro*.

Key words: oncogenic poxvirus, cell transformation, 3T3 cells

Introduction

Transformation of cells *in vitro* by both RNA and DNA viruses has been well established. For poxviruses, a few studies have been published indicating that vaccinia virus, (Koziorowska et al., 1971), Yaba monkey virus (Rouhandeh and Vafai, 1982) and *Molluscum contagiosum* (Barbanti-Brodano et al., 1974) transformed cells *in vitro*. Moreover, recent reports have shown that cells infected with vaccinia virus secrete a growth factor that is encoded by the viral genome (Blomquist et al., 1984, Brown et al., 1985). However, the possible role of poxviruses as oncogenic agents is not well understood at the moment.

Shope fibroma virus (SFV) is a leporipoxvirus that causes self-limiting neoplasia in adult rabbits (Shope, 1932) and malignant tumors in newborn and immunosuppressed animals (Smith et al., 1973, Allison and Friedman, 1966). Although SFV has been shown to stimulate the growth of cells *in vitro* and to establish persistent infections (Hinze and Walker, 1964, Padgett and Walker, 1970), information is lacking about the properties of these cells and whether SFV can elicit cell transformation *in vitro*.

A large body of evidence indicates that carcinogenesis *in vitro* is a two stage process involving first cell immortalization and second, malignant transformation (Land et al., 1983). In this communication, we explore the possibility that SFV may trigger the second step of carcinogenesis *in vitro* or malignant transformation. To assess the oncogenic potential of SFV, a spontaneously immortalized rabbit cornea cell line (SIRC), (ATCC CCL 60) was infected with

ultraviolet (uv) irradiated virus. The resulting cell transformants were shown to temporarily display, the characteristic properties of the malignant phenotype. In addition, transfection of SFV DNA into NIH 3T3 cells brought about the production of foci. These results support the notion that SFV can elicit the transformation of cells in vitro.

Materials and Methods

Cells

Rabbit cornea fibroblasts (SIRC cells) obtained from the American Type Culture Collection were cultured as monolayers in minimal essential medium (MEM, Flow Labs) supplemented with 10% fetal calf serum and dissociated with trypsin:EDTA (GIBCO).

NIH 3T3 cells were obtained from Dr. C. Friend, Mount Sinai School of Medicine, (originally from Dr. G. M. Cooper, Harvard Medical School) and cultured as monolayers in Dulbecco's modified Eagle's medium (Flow Lab) with 10% fetal calf serum, following the protocol used in Dr. Cooper's laboratory (personal communication). The monolayers were dissociated with trypsin:EDTA. Another line of NIH 3T3 cells was obtained from Dr. C. Tackney, Mount Sinai School of Medicine, (originally from Dr. W. Wigler, Cold Spring Harbor Lab) and was maintained according to the conditions described by Wigler et al. (1979).

Virus

The Patuxent strain of Shope fibroma virus obtained from Dr. Hinze, Univ. of Wisconsin was used in all the experiments. It was propagated, assayed and purified as previously described (Pogo et al.,

1982 and Berkowitz and Pogo, 1985).

Suspensions of SFV of known titer were spread in 30 mm plates and exposed to uv-light (GE-IS) at a 30 cm distance with constant agitation at 4°C for different lengths of time. Thereafter, the titer of the virus was determined by focus assay in SIRC cells as previously described (Pogo et al. 1982). A decrease of 3 logs in the virus titer was recorded after 60 sec.

Monolayers of SIRC cells were infected with virus that had been uv-irradiated for 60 sec, at a m.o.i. of 1 to 5 FFU/cell. After one hour of adsorption at 4°C, the cells were overlaid with MEM supplemented with 10% fetal calf serum. Cells were incubated at 37°C in the presence of 5% CO_2 and screened for transformed foci. The monolayers were dissociated with trypsin:EDTA and cells passaged twice a week.

To determine virus replication in the cells infected with uv-irradiated virus at different passages, the cells were disrupted by sonication, centrifuged at 1,000 rpm for 10 min at 4°C and the supernatant inoculated into 60 mm plates containing SIRC cell monolayers. These cultures were screened for the presence of foci after 1 week.

To measure the capacity of the transformed cells to sustain virus replication, they were infected with different m.o.i. of native virus. After 5-7 days of incubation at 37°C, the cells were harvested, sonicated and the amount of virus determined by focus assay in SIRC cell monolayers.

Soft agar cloning

The efficiency of cloning of normal and transformed cells was assayed using the procedure described by Rhim (1977). Aliquots of trypsinized cells containing either 1 x 10^4, 1 x 10^5 or 1 x 10^6 per ml were resuspended in 0.375% agar and were overlaid onto a base of 0.5% agar in MEM and 20% fetal calf serum in 60 mm plates. Triplicate plates of each cell concentration were incubated at 37°C for 2 to 3 weeks and the resulting colonies counted. The efficiency of plating was expressed as the number of colonies relative to the total number of cells plated.

Determination of cellular DNA synthesis

To measure cell DNA synthesis, 1 µCi per ml of [Methyl-^{3}H]-thymidine (TdR) was added to the culture medium at 24 h intervals. Incorporation was terminated after 1 h by removal of the medium and repeated washings with unlabeled medium. Thereafter, the cells were scraped off the plates and washed with phosphate-buffered saline solution and the cell pellet resuspended in 10% cold trichloroacetic acid (TCA) and maintained at 4°Cfor 20 min. The precipitate was spun down, washed once with 10% TCA and once with 95% ethanol and then extracted with 0.5 N perchloric acid (PCA) for 20 min at 70°C. The amount of DNA in the extract was measured by the Burton's procedure (1956), and the radioactivity determined by scintillation counting.

Cell fractionation and extraction of cellular DNA

To prepare nuclear fractions, 10^7 cells were washed with PBS (phosphate buffer solution), then resuspended in a solution containing

0.01 M Tris-HCl buffer pH 7.8, 0.005 M EDTA and 0.01 M KCl (TEK) with 0.5% Triton X-100. After 10 min at 4°C, the cells were disrupted by 20 strokes of a Dounce homogenizer and centrifuged at 800 x g for 3 min. The pellet containing the crude nuclear fraction was washed once with TEK-0.5% Triton and centrifuged again at 800 x g for 3 min. The resulting pellet, or purified nuclear fraction, was resuspended in a solution composed of 0.05 M Tris-HCl buffer pH 7.8, 0.15 M NaCl and 1 mM EDTA (TES) 0.5% sarkosyl and 1 mg/ml of proteinase K and incubated at 37°C for 18-20 h. Phenol extraction was carried out as previously described (Obom et al., 1986). The amount of DNA was measured by the Burton's reaction (1956).

Transfection experiments

SFV DNA was extracted from purified virions as previously described (Berkowitz and Pogo, 1985). Aliquots containing between 1 to 10 µg of SFV DNA were mixed with carrier calf thymus DNA to a final concentration of 20 µg DNA in 0.5 ml and precipitated with calcium-phosphate following the conditions recommended by Wigler et al. (1979). The DNA precipitated in 0.5 ml was added to a 60 mm culture plate which was seeded the previous day with 0.3 x 10^6 NIH 3T3 cells. The cells were incubated for 4 to 12 h at 37°C, then the precipitate was removed and fresh tissue culture medium added. After 24 h, the cells from one plate were trypsinized and seeded into five 60 mm plates and incubated with Dulbecco's medium plus 5% fetal calf serum. The medium was changed every 4-5 days, and the cultures screened for the presence of *foci* for 2 to 3 weeks. c-Ha-ras/plasmid DNA, derived from the T24 bladder carcinoma, (Santos et al. 1982) a gift from Dr. Tackney, was

used in the transfection experiments as a positive control. Transfections with the pSV2neo plasmid (also a gift from Dr. Tackney) were carried out as described by Southern and Berg (1982). Geneticin (G418, Sigma) was used at concentrations of 400 µg per ml.

Analysis of nuclear and viral DNA by restriction enzymes, Southern blotting and hybridization

Aliquots containing 50 µg of nuclear DNA or 100 ng of viral DNA were digested with restriction enzymes following the conditions recommended by the manufacturer. Restriction fragments were separated by electrophoresis in 0.6% agarose gels for 18 h at 50 volts in a buffer composed of 90 mM Tris-HCl pH 8.2, 90 mM boric acid and 2.5 mM EDTA. After denaturation, the DNA fragments were transferred to nitrocellulose paper by the Southern procedure (Southern, 1975). Prehybridization and hybridization reactions were carried out following the conditions described by Wahl et al. (1979). Nick-translation of the viral DNA was performed as described by Rigby et al. (1977). Restriction enzymes and the enzymes for nick-translation were purchased from Boehringer and BRL respectively; [^{35}S]-thio-dATP and [^{35}S]-methionine from New England Nuclear.

Reconstitution experiments, in which known amounts of SFV DNA were mixed with nuclear DNA and probed with nick-translated SFV DNA, revealed that it was possible to detect between 0.5-1 ng of viral DNA (4 to 8 fmoles) in the presence of 50 µg of nuclear DNA (approximately 1 x 10^7 cells). Similar results have been reported using vaccinia virus DNA (Obom et al. 1986).

Immunoprecipitation and slab gel electrophoresis

Labeled cytoplasmic fractions were prepared by incubating the SIRC cells with 10 µCi of [^{35}S]-methionine per ml in methionine-free medium for 30 min at 37°C and by disrupting the cells as described above. Aliquots of the cytoplasmic fraction in 100 µl were incubated with 10 µl of preimmune serum at 4°C for 16 hours followed by addition of 10 µl of 10% protein A suspension (Pharmacia) for 2 hours at 4°C and then centrifuged at 1,000 x g for 10 min. The resulting supernatant was incubated with 10 µl of antiserum to SFV (Pogo et al., 1982) at 4°C overnight followed by addition of 50 µl of 10% protein A suspension for 2 h and centrifugation of the precipitate. The immunoprecipitates were washed three times with a solution containing 0.1 M Tris-HCl buffer pH 7.6, 1% Triton X-100, 0.5% Na deoxycholate and 0.1% sodium dodecyl sulphate (SDS) and finally resuspended in 50 µl of 0.1 M Tris-HCl pH 7.6 with 1% SDS and 1% β-mercaptoethanol, boiled for 5 min at 100°C before loading onto polyacrylamide slab gels containing SDS. Slab gel electrophoresis (Laemmli, 1970) was carried out for three hours at 100V. The gels were dried and exposed to Kodak-X-Omat-R film at -70°C. [^{14}C]-labeled proteins of known molecular weight from Bethesda Research Laboratories were used as standards to calculate the MW of the bands detected by radioautography. Purified SFV labeled with [^{35}S]-methionine was prepared by growing the virus in methionine-free medium containing 1 µCi/ml of [^{35}S]-methionine and 2% fetal calf serum.

Results

1. Establishment of cell lines transformed by SFV

Several experimental strategies were used to establish cell populations transformed by SFV: low multiplicity of infection (0.5 or lower FFU per cell) uv-irradiated virus and/or addition of a tumor promoter, 12-0-tetradecanoylphorbol-13-acetate, (TPA) (Pogo, 1983). Transformed cell lines were readily obtained with uv-irradiated virus. After the second or third passage, the cells lost contact inhibition and piled up forming distinctive foci as illustrated in Fig.1. Addition of 0.5 µg per ml of TPA increased the number of foci produced by the uv-irradiated virus by twofold in the first passages after infection. However, the phorbol ester had toxic effects on later passages. TPA can, therefore, only be added during the first 5-6 passages to increase the number of foci. Cell lines could be easily established by isolating cells from an individual focus. Most of the cells isolated from foci were viable as judged by trypan blue exclusion. A total of 6 cell lines have been developed to date. In this communication we mainly deal with cells infected with uv-irradiated virus and designated lines 96, 100 and 104 and with two cell lines derived from isolated foci from lines 96 and 100. Lines derived from isolated foci were designated F.

2. Production of virus by the cell transformants

Production of virus was followed after infection of the cells for several passages of the cells in culture. A very low rate of virus production was detected by the FFU assay during the first 10 passages

after which no virus production was recorded. Superinfection of the nonproductive cell lines revealed no increase in virus titer. To elucidate the mechanism whereby the nonproductive cells were resistant to superinfection, the presence of viral antigens and viral DNA sequences was investigated.

The presence of viral antigens in the cell transformants was sought by immunoprecipitation of labeled cell extracts followed by electrophoresis. Several polypeptides with MW similar to viral polypeptides were identified as seen in Fig. 2. Of the two passages studied of line 96 in this experiment, the earlier passage p37 showed more immunoprecipitated polypeptides than p49, indicating the the expression of viral polypeptides decreased with serial passages. Other passages studied yielded similar results. In addition, identification of viral antigens was investigated by immunofluorescence. Cells productively infected with native virus and cells from lines 96 and 100 until passage 10 gave a weak fluorescent signal with anti-SFV serum, while the same cell lines tested at later passages gave negative results (data not shown). These results again suggested that there was a loss of viral antigens with serial passage. The weakness of the immunofluorescence could be due to either low antiserum titer, or the amount of antigens expressed per cell was not enough to elicit a response. The antiserum used was obtained from tumor-bearing animals and it was expected to react with structural and non-structural proteins.

The presence of virus DNA sequences was also investigated in the nuclear and cytoplasmic fractions of cell transformants that no

longer produced infectious particles. Although no cytoplasmic viral DNA sequences were detected, Southern blots of restricted nuclear DNA hybridized with SFV DNA revealed that viral sequences were present in some of the cell transformants (Fig. 3 and 4). Thus, nuclear DNA from the same line (96) at different passages showed two distinct bands that hybridized to SFV DNA (Fig. 3, lanes a, b and c). One band migrating as 50 kb may represent the total viral genome, whereas the other around 23 kb, may correspond to Hind III terminal fragments B or C (Pogo, et al. 1982, Wills et al. 1983). However, by passage 52 the viral specific sequences were no longer present (Fig. 3, lane d). Another example of viral sequences in the nuclei of transformed cells is illustrated in Fig. 4. Nuclear DNA from another cell line (100) was analyzed at passage 10 showing a different and more complex pattern of hybridization to SFV DNA (Fig. 4, lane b) than that shown in Fig. 3. From this passage 10, a <u>focus</u> was isolated and a cell line established that was analyzed at passages 7 and 10 for viral specific sequences, when infectious particles were no longer detected. As seen in Fig. 4, few changes occurred in the complex pattern of hybridization between passages 7 and 10 (lanes c and d). However, when the same cell line was tested after 49 passages, the viral DNA sequences were lost (data not shown).

<u>3. Properties of the cells "transformed" by SFV</u>

The biological properties of the cell transformants were studied by determining cell growth, serum dependence, anchorage independence and tumorigenicity. To compare the rate of growth between control cells and transformants, thymidine incorporation was measured. In

Fig. 5, a representative experiment is shown. In the presence of 10% serum, it is clear that transformed cells (Fp42) incorporated more [H^3]-TdR than control cells at all times tested. This difference is more striking at lower serum concentrations.

To investigate further this serum independence, transformed cells were passaged in different serum concentrations along with the control cells. As shown in Table 1, SIRC cells could not grow in 2% serum and could only replicate two passages in 5% serum, while two transformed cell lines, Fp43 and 96 p49, were able to replicate 5 passages in 2% serum and required only 5% serum to continue growing. By contrast, line 104 p13 which was originally infected with uv-irradiated SFV but did not develop foci, failed to grow in 5% serum.

To investigate anchorage independence, cloning in soft agar was performed with several cell lines. While the efficiency of cloning of SIRC cells was found to be less than 1% in three independent experiments, that of transformed cell line 96 tested at passages 21, 30 and 43 was 10 to 15%.

To assess the tumorigenic potential of the transformants in the natural host of the virus, cells derived from two different passages of one of the lines (96) that showed all the characteristics of the transformed phenotype and from one line (104) that did not express those characteristics, were inoculated subcutaneously into rabbits. The results summarized in Table 2 indicated that neither control cells nor the cell line 104 induced tumors, whereas the two passages of line 96 and SFV were able to produce tumors at all the sites of inoculation (4 in each rabbit). The tumors induced by the transformants had an

average size of 2 cm and regressed after 2 weeks as did those induced by the virus inoculated alone.

4. Analysis of a cell line derived from an isolated focus

The experiments described above were carried out with different transformants derived either from a mass culture or from isolated foci. To investigate further the properties of these transformants, a line established from an isolated focus of line 96 was studied through 70 passages in tissue culture (approximately 280 cell generations). The results of these experiments are summarized in Table 3. As described before, the transformants did not produce infectious virus particles, were resistant to superinfection (titer obtained was probably residual virus), displayed high cloning efficiency in agar, could grow at low (2%) serum concentration and showed presence of DNA sequences in the nucleus for the first forty-two passages. However, when tested at passages 52 and 67, these transformants had lost the properties of the transformed phenotype as revealed by sensitivity to superinfection, lower cloning efficiency, serum dependency similar to the parental cell line and lack of viral DNA sequences in the nucleus.

5. Transfection of NIH 3T3 cells with SFV DNA

The results described above indicated that SFV was able to change the properties of the SIRC cells to a transformed phenotype in a transient manner. To investigate whether this poxvirus DNA has indeed oncogenic potential, transfection of NIH 3T3 cells with SFV DNA was performed. For these experiments, NIH 3T3 cells obtained from different laboratories were used.

The results of the experiments performed with NIH 3T3 cells

obtained from Cooper's laboratory are summarized in Table 4. In three separate experiments, SFV DNA was able to induce a five-fold increase in the number of foci/plate over calf thymus DNA or SIRC cell DNA, while DNA from the c-Ha-ras oncogene induced a six-fold increase. The foci induced by SFV DNA are of type 2 according to the classification of Perucho et al. (1981).

Results from experiments performed with NIH 3T3 cells obtained from other sources indicated that SFV DNA was unable to induce foci although transfection with the c-Ha-ras oncogene resulted in a higher degree of transformants (13.5 foci/μg DNA, results not presented). In both cells the efficiency of transfection of the neomycin gene in the presence of G418 (geneticin, Sigma) was 0.1%.

Attempts to increase the degree of transfection by using DMSO resulted in slight increase in the number of foci induced by SFV DNA but no increase in the foci induced by c-Ha-ras DNA (data not shown).

Discussion

Ultraviolet irradiation has been successfully used to demonstrate the oncogenic potential of herpes virus types 1 and 2, (Duff and Rapp 1971, 1973) and Yaba monkey virus (Rouhandeh and Vafai, 1982). The results reported in this communication indicated that uv-irradiated SFV can elicit the second step of carcinogenesis in vitro, or malignant transformation. Thus, inoculation of already immortalized rabbit fibroblasts with uv-irradiated virus resulted in cell lines that have acquired the properties characteristic of the malignant phenotype: lack of infectious virus particle production, low serum requirements, high efficiency of cloning, resistance to

superinfection, presence of viral DNA sequences in the nucleus, expression of some viral proteins and induction of tumors in rabbits. However, this cell transformation was not stable, since in all cell lines studied, a loss of the malignant phenotype was recorded after the 50th passage. The loss of the phenotype can be interpreted as: a) the number of transformed cells in the culture was small and the nontransformed cells overgrew them; b) the transformed cells reverted to the nontransformed state and c) SFV has weak oncogenic potential resulting in an unstable type of transformation.

The experiments carried out with cell lines derived from a single focus indicated that the same transient pattern of transformation evolved, thus eliminating the possibility that nontransformed cells overgrew transformed cells. On the other hand reversion of the malignant phenotype to the nontransformed one, although unlikely, can not be ruled out. Recently the reversibility of the malignant phenotype of rat fibroblasts transformed by mutant human ras oncogenes upon the loss of oncogene sequences has been described (Winter and Perucho, 1986). Most probably to maintain the transformed phenotype the constant expression of certain genes are required. Loss of that expression may result in the suppression of the malignant phenotype. In the case of SFV transformed cells, it is currently not possible to associate any particular region of the viral genome with the maintenance of the transformed phenotype. While several restriction fragments were detected in the nucleus of certain cell lines, only one or two fragments were present in others. Evidence for integration is also lacking, since restriction fragments detected in genomic DNA were

of similar size to those of restricted viral DNA. There is, however, one exception; the large MW fragment shown in Fig. 3. The significance of this fragment is not clear at the moment, although it has been frequently observed. Finally, it should also be borne in mind that the mechanisms operating in oncogenesis by nuclear DNA or RNA tumor viruses may not be functioning in the stimulation of cell growth by cytoplasmic DNA tumor viruses.

The results reported here are in agreement with those describing the transformation of Jinet cells by Yaba monkey virus (Rouhandeh and Vafai, 1982) and the abortive transformation of human fibroblasts by Molluscum contagiosum (Barbanti-Brodano et al., 1974). In the case of Yaba virus, however, no change in the malignant phenotype was reported.

Further evidence that SFV DNA contains potential oncogenic sequences was obtained by the transfection experiments performed with NIH 3T3 cells. These cells have been widely used for this type of study since they have a low degree of spontaneous transformation and provide favorable conditions for the expression of weak oncogenes. It is important to emphasize that not all the NIH 3T3 cell lines tested were capable of being transformed by SFV DNA. This may imply that a specific factor(s) is necessary for the expression of SFV DNA that may be absent in some of the NIH 3T3 cell lines. The degree of foci induction by SFV DNA was lower than that obtained with the c-Ha-ras oncogene. This could be due, in part, to the fact that poxvirus DNA lacks the promoter sequences recognized by the cell polymerases and, therefore, may not be efficiently or correctly transcribed. In spite

of that, it has been shown that L cells transfected with cloned restriction fragments of vaccinia virus DNA were able to express specific RNA sequences (Pellicer and Esteban, 1982, Boni et al., 1984).

Preliminary results indicated that NIH 3T3 cells transfected with SFV DNA contain viral DNA sequences and express viral RNA. Experiments are also in progress to establish the tumorigenicity of these transfected cells by inoculation into nude mice and to identify the site in the viral genome that codifies for the transforming activity.

One candidate for this activity could be the recently discovered growth factor of vaccinia virus (Blomquist et al., 1984, Brown et al., 1985). Similar nucleotide sequences have been found in SFV DNA (Chang et al., 1986). Attempts to transform NIH 3T3 cells with vaccinia DNA however, were negative (unpublished results). These observations can be explained by assuming that the mechanism of action of the vaccinia growth factor is different from that of the SFV growth factor, since orthopoxviruses do not cause tumors in animals but only a transitory hyperplasia at the site of inoculation (Dales and Pogo 1982). In conclusion, uv-irradiated SFV can establish transformed cell lines which express temporarily the malignant phenotype. The oncogenic potential of SFV DNA was shown by transfection of NIH 3T3 cells and the induction of <u>foci</u>.

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Table 1. Effect of Serum Concentration on Prolonged Passage of SIRC Cells Persistently Infected with SFV

Cell Line	Number of Passages		
	2%	5%	10%
SIRC	0	2	10
Fp43	5	10	10
96 p49	5	10	10
104 p13	0	1	10

Cells were propagated every three days in 60 mm dishes with medium containing different serum concentrations. Confluency was reached at 3×10^6 cells per plate after 3-4 days in culture. No increase in the number of seeded cells after 3-4 days was considered inability to grow at that serum concentration.

Fp43 is a line derived from an isolated focus of line 96.

Table 2. Induction of Tumors in Rabbits by SIRC Cells Persistently Infected with SFV

Inoculum	No. of Cells	Size of Tumor	Regression
SIRC cells (1)	2.0×10^7	none	-
96 p35b (2)	3.0×10^7	2 cm	15 days
96 p39 (2)	1.5×10^7	2 cm	15 days
104 p10 (1)	1.5×10^7	none	-
SFV (3)	1.0×10^6 FFU	4 cm	16 days

Male adult New Zealand white rabbits were shaved in the back and inoculated subcutaneously in 4 different places with the amount of cells indicated. Purified SFV was inoculated intradermally. The progression and regression of the tumors were monitored daily for 2 weeks. In parenthesis is the number of animals used in each group.

Table 3. Properties of a Cell Line Established from an Isolated Focus

Passage Number	Virus Replication FFU/ml	Superinfection FFU/ml	Cloning Efficiency	Growth in 2% Serum	SFV DNA in nuclei
0	0	1×10^7	1%	-	-
2	5×10^5	5×10^6	N.D.	N.D.	N.D.
4	4×10^5	5×10^5	N.D.	N.D.	N.D.
6	4×10^5	N.D.	N.D.	-	N.D.
8	2×10^3	N.D.	N.D.	N.D.	N.D.
10	0	4×10^5	10%	+	+
16	0	5×10^5	15%	+	+
22	0	4×10^5	10%	+	+
27	0	N.D.	15%	N.D.	N.D.
34	0	N.D.	10%	+	+
42	0	3×10^5	15%	+	+
52	0	2×10^7	1%	-	-
67	0	2×10^7	1%	-	-

A cell line established from an isolated focus of line 96 was propagated in vitro for 70 passages. At the passages indicated, 1×10^7 cells were analyzed for virus infectivity, cloning efficiency in soft agar, growth in 2% serum for three passages, presence of viral DNA sequences in the nucleus and were superinfected with 1×10^7 FFU. N.D. = not done.

Table 4. Transfection of NIH 3T3 Cells with Shope Fibroma Virus DNA

Source of DNA	Experiment Number	µg DNA	Foci/µg DNA	Average Foci/plate
SFV	1	10	2.40	2.27
	2	5	1.60	
	3	5	2.20	
H-ras	1	2	8.00	3.0
	2	2	5.50	
Calf thymus	1	20	0.10	
	2	15	0.06	0.45
	3	15	0.13	
SIRC	1	20	0	0
No DNA	1	0	-	
	2	0	-	0.46
	3	0	-	

NIH 3T3 cells (Cooper's line) were transfected with DNA as indicated in material and methods and monitored for the presence of foci. After 3 weeks, the cells were fixed with methanol, stained with Giemsa and the number of foci counted.

Figure 1

Focus formation induced by uv-irradiated SFV. SIRC cells were infected with uv-irradiated virus as described in material and methods. Micrographs were taken after the second passage in vitro. A: area of the culture showing a typical focus; B: area of the culture showing cells with normal morphology. Magnification 100X.

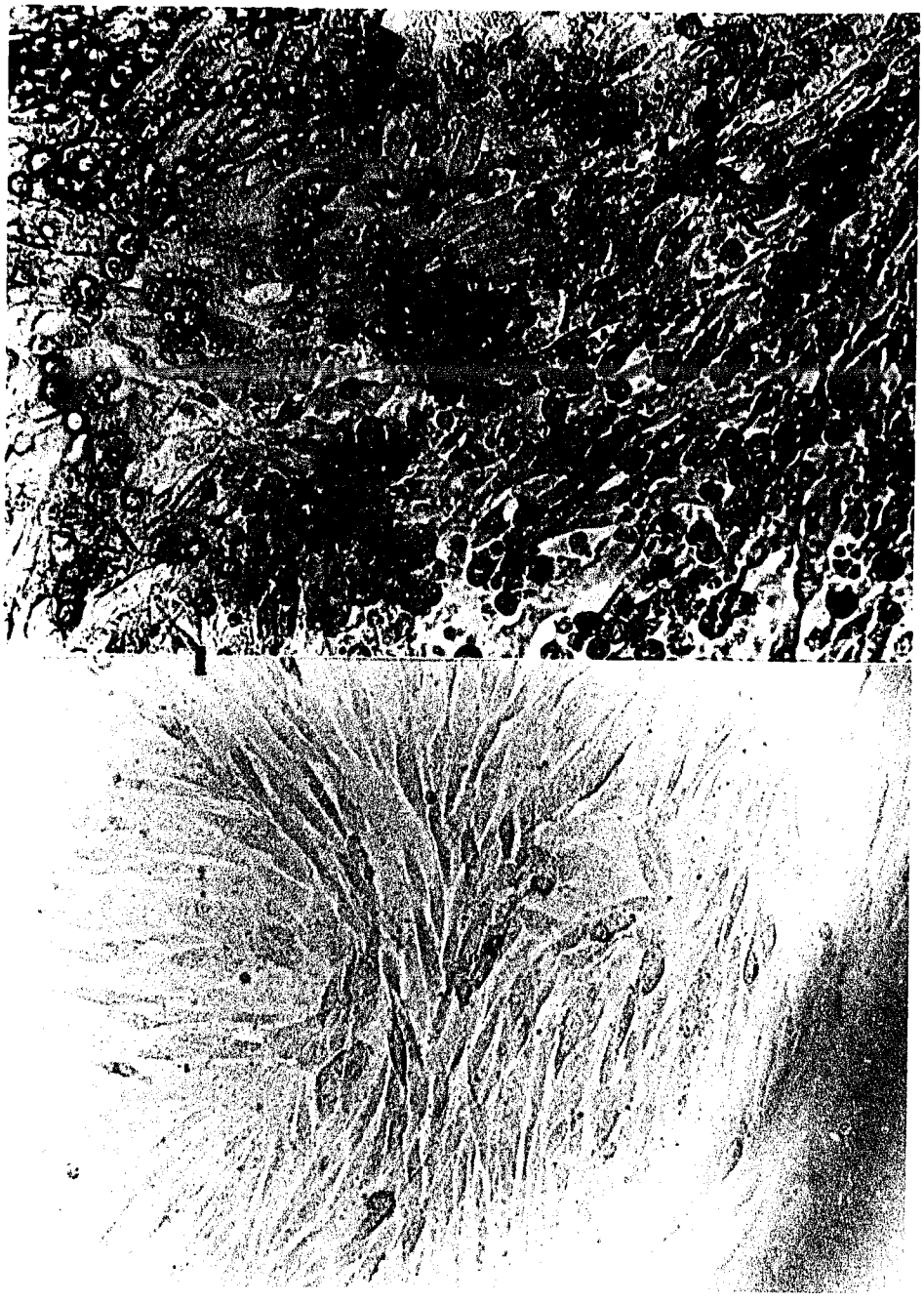

A
B

Figure 2

Immunoprecipitation of [^{35}S]-methionine labeled cytoplasmic proteins with SFV antiserum. Conditions as described in material and methods. Lane a: cytoplasmic extract from line 96 p49, lane b: same as in a, but precipitated with SFV antiserum, lane c: same as in a, but precipitated with preimmune serum, lane d: cytoplasmic extract from line 96 p37, lane e: same as in d, but precipitated with preimmune serum, lane f: same as in d, but precipitated with SFV antiserum, lane g: [^{35}S]-methionine-labeled SFV, lane h: [^{14}C]-labeled molecular weight markers 43K: albumin; 25K: α-chymotrypsinogen; 18K: lactoglobulin.


a b c d e f g h

Figure 3

Southern hybridization of restricted nuclear DNA of transformed cells to [^{35}S]-labeled SFV DNA. DNA extracted from purified nuclear preparations was digested with Hind III, electrophoresed in 0.6% agarose gels, transferred to nitrocellulose filters, hybridized to nick-translated SFV DNA and radioautographed. Lane a: line 96 pass 3, lane b: pass 10, lane c: pass 34, lane d: pass 52, lane e: uninfected SIRC cells nuclear DNA, lane f: SFV DNA restricted with Hind III. Numbers (kb) indicated the position in the gel of restriction fragments of λ DNA digested with Hind III.

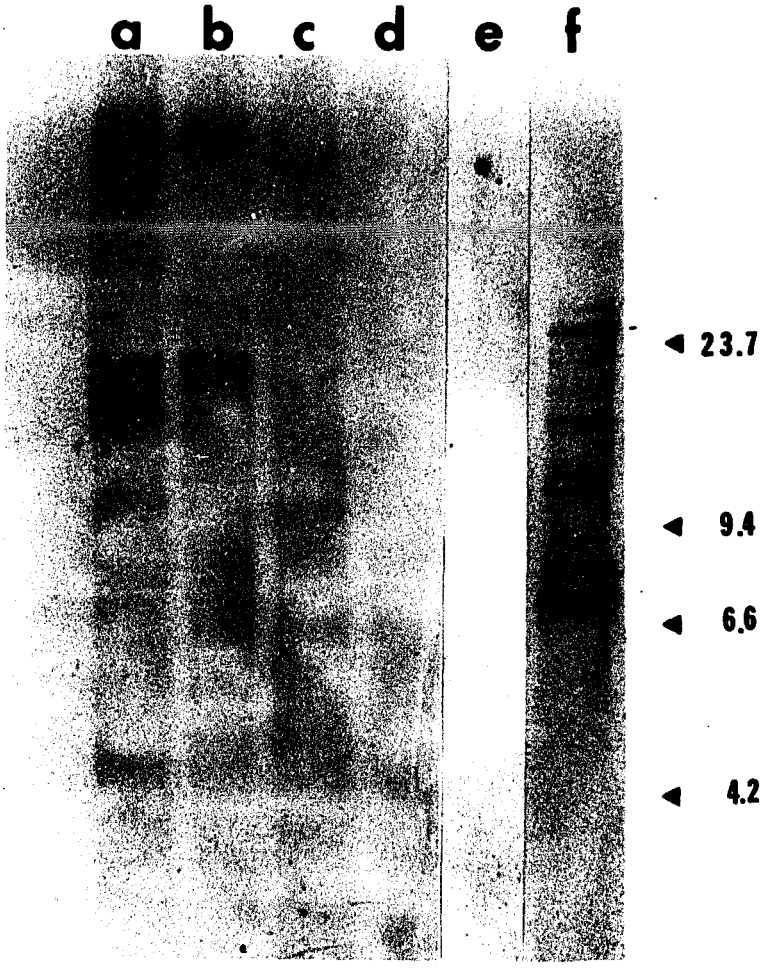

a
b
c
d
e
f
23.7
9.4
6.6
4.2

Figure 4

Southern hybridization of Hind III restricted nuclear DNA from transformed cells to [^{35}S]-labeled SFV DNA. Conditions as described in Fig. 3. lane a: SFV DNA, lane b: line 100 pass 10, lane c: Fp7, a cell line derived from an isolated focus of line 100 pass 10, lane d: Fp10 as in c, lane e: control cell DNA.

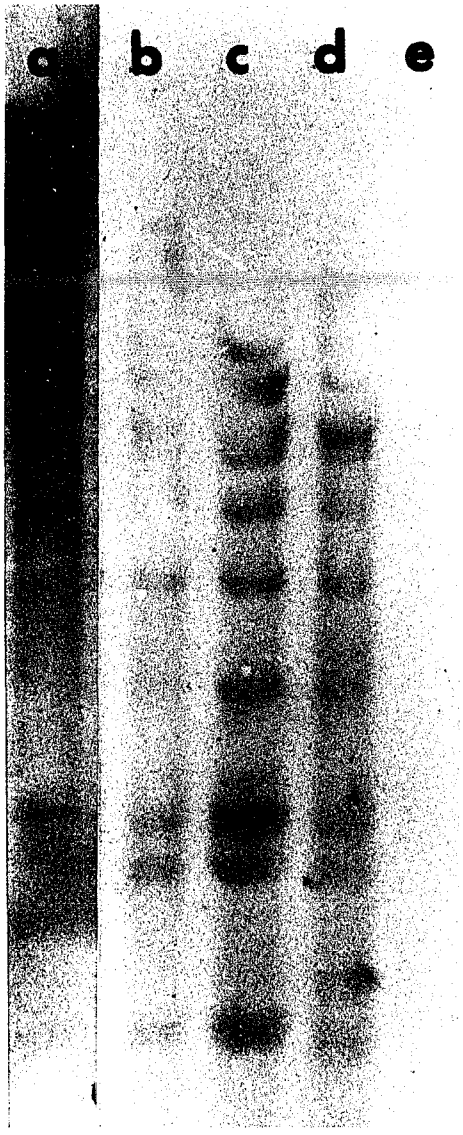
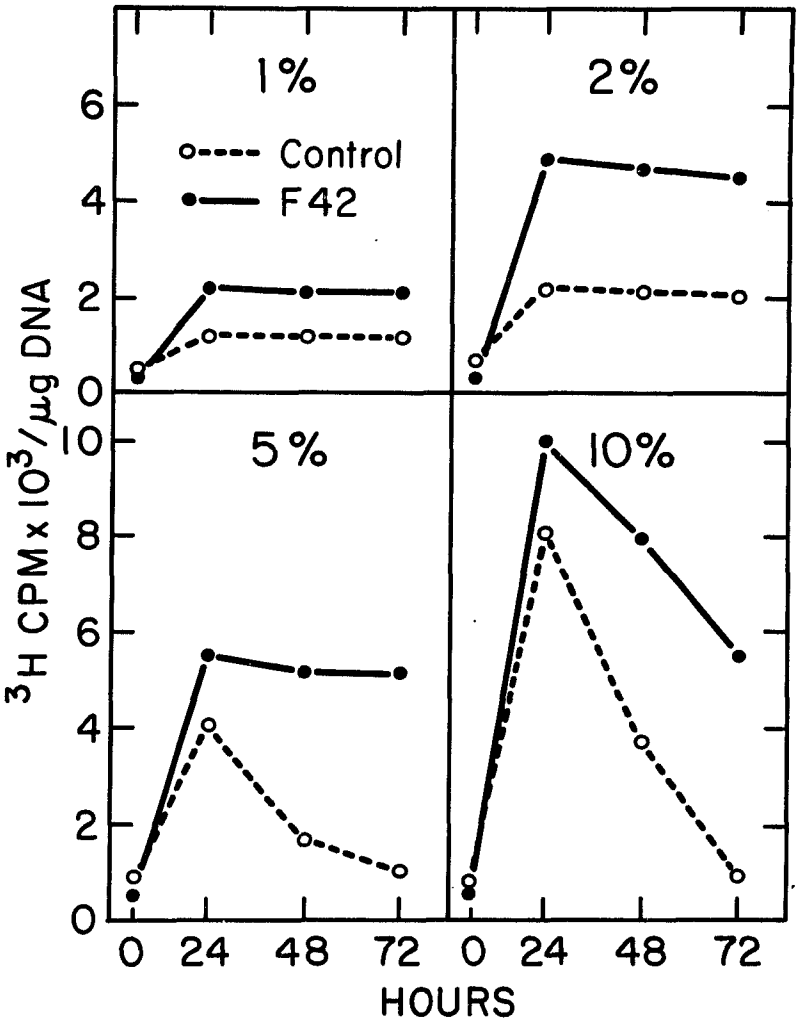

a
b
c
d
e

Figure 5

Effect of serum concentration on the growth of SIRC cells and a transformed cell line, Fp42. Cells were seeded at 2 x 10^5 per ml in 60 mm petri dishes, in culture medium containing different serum concentrations. At the times indicated, 1 µCi per ml of [^{3}H]-TdR was added to the culture medium and the cells incubated for 1 h at 37°C. Cells were processed as described in materials and methods and DNA synthesis was measured by the amount of [^{3}H]-TdR incorporated per µg of DNA. Numbers in percent indicated serum concentrations.


1%
2%
5%
10%
Control
F42
^{3}H CPM x 10^3/µg DNA
0
2
4
6
8
10
0 24 48 72 0 24 48 72
HOURS

Chapter 3

Tumorigenic Conversion of NIH 3T3 Cells by Transfection With Shope Fibroma Virus DNA

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ABSTRACT

Shope fibroma virus (SFV) is a leporipoxvirus that induces benign tumors in adult rabbits but fibrosarcomas in newborns and immunocompromised adults. In vitro, SFV stimulates cell growth and formation of foci. We have previously reported that ultraviolet-inactivated SFV causes a transient transformation of immortalized rabbit cornea fibroblasts and that calcium-phosphate precipitated SFV DNA induces focus formation in NIH 3T3 cells. In this communication the biological and molecular properties of cell lines derived from individual focus induced by transfection of NIH 3T3 cells were investigated. Results indicated that five of the six lines studied induced tumorigenesis in nude mice and that four lines were anchorage independent. At early passages all cell lines contained SFV DNA sequences and two of three lines tested expressed SFV DNA. Transfection of NIH 3T3 cells with cloned Bam HI fragments of SFV DNA containing terminal sequences of the molecule, either alone or in combination, did not result in the formation of foci with the exception of fragment C. This fragment which comprises 17.6 kilobases of the right termini contains the gene for the SFV growth factor. However, focus-derived cell lines from this transfection failed to induce tumors in nude mice. It is concluded that SFV DNA can induce transformation and tumorigenic conversion of NIH 3T3 cells and that the growth factor alone is not responsible for this effect.

(Shope fibroma virus, tumorigenic conversion, growth factor, NIH 3T3 cells, DNA transfection)

INTRODUCTION

Infection by a poxvirus *in vivo* initially results in cell proliferation that may be due to the expression of an epidermal growth factor (EGF)-like protein (6,7,28). In most instances, the pox or "microtumors" produced during infection regress quickly due to either viral induced cell necrosis, or to an immunological response against the infected cells. But in the case of *Molluscum contagiosum*, Shope fibroma virus, rabbit myxoma and Yaba monkey tumor virus, cell proliferation is more pronounced, resulting in tumors that can persist for weeks or months before regressing or lead to the death of the host. Although transformation of cells *in vitro* by poxviruses has been reported with vaccinia virus (13), *Molluscum contagiosum* (4), Yaba monkey tumor virus (23) and Shope fibroma virus (17), the mechanism(s) whereby poxviruses induce oncogenesis is(are) unknown.

Shope fibroma virus (SFV) is a leporipoxvirus that induces benign tumors in adult rabbits (24) but fibrosarcomas in newborns (2) and in immunocompromised adults (1,3). Infection of cells in culture with SFV results in the stimulation of cell growth and formation of *foci* (11,20). We recently reported that ultraviolet-inactivated SFV causes a transient transformation of immortalized rabbit corneal fibroblast cells (SIRC) and that calcium-phosphate precipitated SFV DNA can induce *focus* formation in NIH 3T3 cells, whereas vaccinia virus DNA failed to do so (17).

To gain a better understanding of how SFV DNA transforms NIH 3T3 cells, we have established cell lines derived from individual *foci* and investigated their biological properties and the presence and

expression of SFV DNA. The results indicated that five of the six lines studied induced tumorigenesis in nude mice and that four of the six lines were anchorage independent. At early passages all lines contained SFV DNA sequences and two of three lines tested expressed SFV DNA.

To determine which region(s) of the genome is(are) responsible for in vitro transformation, cloned Bam HI fragments of SFV which contained the terminal sequences of the molecule were transfected into NIH 3T3 cells. The data indicated that only one of the fragments, C, which comprises 17.6 kilobases (kb) of the right termini of the genome and contains the gene for the SFV growth factor (7) could induce incomplete transformation of NIH 3T3, cells as evidenced by focus formation, but no tumorigenesis in nude mice.

MATERIALS AND METHODS

Cells

NIH 3T3 cells obtained from Dr. G. Cooper (Harvard University) were maintained and transfected with calcium-phosphate precipitated DNA as previously described (Obom and Pogo, 1987). To determine if 12-0-tetradeconyl phorbol-13-acetate (TPA) will increase the number of foci after transfection, the cells were split 1:5 and maintained in Dulbecco's modified essential media (DME) supplemented with 5% fetal bovine serum and 30 μM TPA.

Focus-derived cell lines (designated F) were established by picking foci from NIH 3T3 cells transfected with SFV DNA maintained in the absence, (F2, F3, F4) or presence, (F5, F6, F7) of 30 μM TPA. Three lines designated controls were established from spontaneously

arising foci in NIH 3T3 cells that had been transfected with calf thymus DNA. Control line 1 derived from a transfection in the absence of TPA, whereas control lines 2 and 3 originated from transfections made in the presence of TPA. The focus-derived cell lines were maintained in DME supplemented with 5% FCS and passaged twice a week. TPA was removed from the medium after the foci were isolated to establish cell lines.

Virus and plasmids

Shope fibroma virus was grown, assayed and DNA extracted from purified virions as previously described (5).

The 21 Bam HI fragments of the Shope fibroma virus genome have been cloned by Wills et al. (30). Dr. Grant McFadden (University of Edmonton, Alberta) has kindly provided us with the clones. In this study, cloned DNA from SFV Bam HI fragments I, E, H, N, F2, A (pKB/HE, pKHE, pKB/HJ), G and C was transfected into NIH 3T3 cells. Plasmid pSIC-9 DNA that contains host and virus sequences (27) was also transfected.

Nucleic acid hybridizations

Nuclear DNA from the focus-derived cells was extracted as previously described (17), digested with restriction enzymes according to the manufacturers recommendations, electrophoresed on a 0.5% agarose gel in Tris-Borate-EDTA buffer (90 mM Tris, 90 mM borate, 2.5 mM EDTA) for 18 hr at 50 V, denatured and transferred to nitrocellulose paper (25) or dot blotted onto nitrocellulose filters (12). DNA hybridization and washings were carried out according to the procedure of Wahl et al. (29). Blots were exposed to Kodak XAR

(XOMAT) film at -70° C.

Total RNA was extracted from focus-derived cells purified by the guanidium isothyocyanate/cesium chloride method (16), dotted onto nitrocellulose filters and hybridized to ^{35}S-labeled SFV DNA.

DNA was nick-translated according to the procedure of Rigby et al., (22). Enzymes for nick-translation were purchased from BRL, restriction enzymes were purchased from Boehringer Mannheim and ^{35}S-dATP was purchased from New England Nuclear.

Soft agar cloning

Cloning in soft agar was performed according to the procedure of Rhim (21). 1 x 10^5 cells in duplicate were seeded into Earle-Clay (Novato, CA) ultraclone media containing 10% fetal bovine serum and 0.375% agar and overlayed onto a 60 mm plate containing ultraclone media supplemented with 10% fetal bovine serum and a final concentration of agar of 0.5%. The cells were incubated at 37° C in 5% CO_2 for two weeks and then screened for colonies containing more than 8 cells.

Tumorigenesis in nude mice

Four to six week old female nude mice were inoculated subcutaneously with 1 to 2 x 10^6 cells per site. Cells were removed from the plates with trypsin:EDTA, washed twice with sterile cold PBS and resuspended in PBS for injection. Each mouse was inoculated in each side of the back and monitored daily for development of tumors for 8 weeks. Tumors were excised, processed for histologic examination and nucleic acid extraction. Mice were purchased from Charles River Laboratories, Inc., Wilmington, MA.

RESULTS

Biological characterization of focus-derived cell lines

SFV DNA induced focus formation in NIH 3T3 cells (17). The morphology of the foci-induced by SFV DNA was compared to that induced by an activated c-Ha-ras gene (Fig. 1). While SFV DNA produced an increase in cell density, the cells did not become spindle shaped or refractile as did the ras-induced foci, instead the SFV foci were smaller than the ras foci, and the cells were rounder. This type of morphology was similar to that displayed by rabbit fibroblasts infected with SFV. In this experiment, the number of foci/μg DNA were 2.4 for SFV, 8.0 for H-ras and 0.10 for calf thymus DNA.

Since the phorbol ester TPA increases the number of foci in cells infected with adenovirus (9) and Shope fibroma virus (19), NIH 3T3 cells transfected with SFV DNA were maintained in DME supplemented with 5% FCS and 30 μM TPA. The results (not shown) indicated that TPA increases twofold the number of foci-induced by SFV DNA as well as the spontaneously arising foci.

Cell lines were developed from individual foci as described in materials and methods and assayed for the phenotypic changes associated with complete transformation, mainly anchorage independence and induction of tumors in nude mice. The results of the cloning experiments are summarized in Table 1. Although all the lines displayed a low degree of cloning efficiency, (with exception of F4) lines derived from foci-induced by SFV DNA were able to form a larger number of colonies in agar than control lines. Whereas lines F3 and F6 produced only twice the number of colonies over controls which was

not statistically significant by the student's t test, lines F2, F4, F5 and F7 seemed to be anchorage independent.

The most sensitive assay for transformation is the ability of cells to induce tumors in nude mice. For that purpose, 1 to 2 x 10^6 cells were inoculated into female nude mice and the animals monitored for the development of tumors for eight weeks. In Table 2 the results from this experiment are summarized. Five of the six focus-derived lines induced tumors although they differed in time of latency and in numbers. Of the six lines tested, only line F4 was not tumorigenic, although it was anchorage independent. Fig. 2 shows a nude mouse with tumors developed after inoculation with cells from line F5. All tumors reached a size of 1 cm in diameter or larger and then histologic examination revealed that they were fibrosarcomas. Some of the F lines were tested for tumorigenesis at different passages and the results indicated that they were always tumorigenic.

To investigate if SFV genomic sequences were present in the tumors, DNA was extracted, dotted onto nitrocellulose filters and hybridized to ^{35}S-dATP labeled SFV DNA. All four tumors tested, contained DNA sequences which hybridized to the SFV DNA probe. However, by Southern blot hybridization they did not show sequences homologous to SFV DNA (data not shown).

Molecular characterization of focus-derived cell lines

To determine if SFV genomic sequences were present in the focus-derived cell lines, nuclear DNA was extracted from cells at different passages, dotted onto nitrocellulose filters and hybridized to ^{35}S-dATP labeled SFV DNA. As shown in Fig. 2, all focus-derived

lines tested contained sequences that hybridized to SFV DNA. To ascertain the size of the SFV genome sequences present, cell lines after 8 passages or more were analyzed by Southern blot hybridization. We were unable to show presence of SFV DNA sequences in the cell lines by this method, although it allowed the detection of one cellular gene, c-myc, that is known to be present at one copy per cell (data not shown).

Expression of the SFV genome was investigated by dot-blot hybridization of total RNA from three cell lines (F2, F3 and F4) to ^{35}S-dATP labeled SFV DNA (Fig. 3). Of the three cell lines tested, only two, F2 and F3, contain RNA sequences which hybridized to the probe.

Transfection of NIH 3T3 cell with cloned SFV genomic fragments

To ascertain which region(s) of the SFV genome were necessary for transformation, cloned Bam HI fragments of the SFV DNA were used for transfection of NIH 3T3 cells. It was first established that restriction of the SFV genome with Bam HI did not destroy its transforming activity, (data not shown). Therefore, NIH 3T3 cells were transfected with calcium-phosphate precipitated cloned DNA fragments. The fragments tested were mostly those located at the ends of the genome, since it is known from studies with orthopoxviruses that genes concerned with virus-cell interactions are located terminally. From the fragments tested, only one fragment, the Bam HI C fragment, was able to induce focus formation in NIH 3T3 cells (Table 3).

Three cell lines were established from foci-induced in NIH 3T3

cells by transfection with the SFV Bam HI fragment C (Table 4). These cell lines were also tested for their ability to induce tumors in nude mice, to develop colonies in soft agar and for the presence of SFV DNA. The results indicated that some of the cell lines contained DNA sequences which hybridized to an SFV Bam HI fragment C probe, but none of them induced tumors in nude mice or anchorage independent growth. In addition, plasmid pSIC-9 originally isolated from uninfected rabbit cells (27) and contains sequences common to SFV and the host genome, was able to induce focus formation although to a lesser extent. When cells from these foci were inoculated in nude mice, they were not tumorigenic.

DISCUSSION

The results reported here indicated that SFV DNA can induce transformation of NIH 3T3 cells and tumorigenic conversion. Cells derived from the foci obtained after transfection with SFV DNA displayed some of the characteristic properties of the malignant phenotype, such as anchorage independence and tumorigenesis in nude mice. The presence of SFV DNA sequences was detected in early cell passages by dot blot hybridization but attempts to identify the sequences by Southern blot hybridization using the entire SFV genome as probe were, however, negative. These results can be explained by a lack of sensitivity of the technique, a complete loss of the viral sequences or a partial loss that left behind sequences which could not be detected by the method employed. It has been previously estimated that between 4 to 8 fmoles of viral DNA can be detected in 50 μg of nuclear DNA (18). Furthermore, using similar Southern hybridization

conditions, we were able to detect one cellular gene (c-myc) that is present at a level of one copy per cell. However, when the probe used is of large MW like the SFV genome (160 kb), it is possible that the distribution of radioactivity in the entire genome may not be homogenous, thus hindering the detection of a small integrated sequence. This defficiency might be overcome by the use of individual cloned fragments as probes. These experiments are now in progress. Similar considerations can explain, in part, the failure to detect RNA sequences specific for SFV in some cell lines. Alternatively, the SFV mRNA may be present in very low copy number.

Failure to detect integrated viral DNA sequences has been reported in cells transformed by herpes simplex virus (10) adenovirus (14) and by bacterial plasmids containing oncogenes (15). These results imply that the presence of viral DNA is not needed to maintain the transformed phenotype and suggest a hit and run phenomenon. In spite of the fact that viral sequences were not detected, cells transformed by SFV remained highly tumorigenic as shown by their repeated ability to induce tumors in nude mice.

Transfection of NIH 3T3 cells with cloned fragments of SFV DNA corresponding to the terminal parts of the molecule alone or in combination brought about induction of <u>foci</u> only with pSIC-9 and Bam HI fragment C. This latter result is of significance because fragment C contains the sequences that encode for SFV growth factor (7). Cell lines derived from these <u>foci</u>, however, failed to induce tumors in nude mice or colonies in soft agar. These results are not unexpected in view of the fact that the transformation growth factor α gene could

transform NIH 3T3 cells but did not convert them to a tumorigenic phenotype (8). Taken together the results reported here suggest that tumorigenic conversion of NIH 3T3 cells by SFV DNA may not be due to the growth factor alone and that it may require the presence and expression of more than one viral gene. Experiments are now in progress to identify the location of these genes.

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Table 1. Soft agar cloning of focus-derived cell lines

Cell line	Exp No.	No. of Colonies/ 10^5 Cells	(Avg. No. of Colonies/10^5 cells) ± SE
F2	1	25, 30	
	2	42, 31	25.8 ± 3.8
	3	22, 15	
F3	1	7, 9	
	2	6, 4	4.3 ± 1.5*
	3	0, 0	
F4	1	770, 795	
	2	216, 274	413.0 ± 105.79
	3	310, 592	
F5	1	13, -	
	2	11, 15	14.6 ± 2.23
	3	23, 11	
F6	1	3, 10	
	2	10, 13	6.8 ± 1.95*
	3	4, 1	
F7	1	73, 75	
	2	32, 65	61.2 ± 9.98
	3	ND	
NIH 3T3	1	1, 2	
	2	3, 0	2.0 ± 0.51
	3	3, 3	

1 x 10^5 cells were seeded in 0.375% agar as described in material and methods. After 2 weeks, colonies containing 8 cells or more were counted.

ND: Not done

SE: Standard error of the mean

*Not significant by student's t test

Table 2. Induction of tumors in female nude mice by focus-derived cell lines

Cell line	Number of tumors/number of sites of inoculation	Latency
F2	15/18	2 weeks
F3	3/20	4-6 weeks
F4	0/18	neg. for 8 weeks
F5	7/10	3 weeks
F6	3/10	5 weeks
F7	10/10	4 weeks
Control 1	0/27	neg. for 8 weeks
Control 2	0/10	neg. for 8 weeks
Control 3	0/10	neg. for 8 weeks

$1 - 2 \times 10^6$ cells/site were inoculated subcutaneously into 4 - 6 week old female nude mice into the two hind limb sites. Development of tumors was monitored for 8 weeks.

Table 3. Transfection of NIH 3T3 cells with SFV DNA fragments

DNA	Foci/μg test DNA	Foci/μg genome equiv.	Total foci/ total plates
A_1 (pKB/HE)	0	0	0/10
A_2 pKHE	0.50	0.70	1/10
A_3 pKB/HJ	0	0	0/10
C	27.0	33.0	54/10
E	0	0	0/13
F_2	0.50	0.72	1/10
G (Bam HI)	0.50	0.83	1/10
H	1.00	2.00	2/10
N	1.00	2.50	2/10
pSIC-9	7.20	10.31	13/09
E,F_2	4.00	5.48	8/05
E,F_2,G,pSIC-9	2.40	3.60	27/14
E,F_2,G,I,pSIC-9	2.25	4.00	9/04
pSIC-9,F_2	2.50	8.10	5/05
Salmon sperm	0.03	0.03	2/21
No DNA	-	-	3/40

Transfection of NIH 3T3 cells with cloned fragments of SFV DNA. 3×10^5 cells were seeded 24 hours prior to transfection with 1 μg of cloned DNA; 24 hours later they were split 1:5 and maintained in Dulbecco's modified minimal essential media supplemented with 5% fetal bovine serum. The number of foci was determined after 2 weeks.

Table 4. Properties of focus-derived lines from fragment C transformants

Cell line	Focus Formation	Fragment C DNA	Tumorigenesis	Soft Agar Cloning
C3	+	-	-	-
C5	+	+	-	-
C7	+	-	-	-
control	-	-	-	-

Focus formation was evident in cells 4-6 days post-transfection. The presence of SFV DNA was determined by Southern blot hybridizations of fragment C to nuclear DNA; female nude mice were inoculated subcutaneously with 1×10^6 cells/site and monitored for 8 weeks for tumor formation; 1×10^5 cells were seeded in soft agar and were scored after 2 weeks for colony formation.

Figure 1: Morphology of NIH 3T3 cells transfected with SFV DNA and H-ras DNA. NIH 3T3 cells were transfected with calcium-phosphate precipitated DNA as described in material and methods. Two weeks later, they were fixed with methanol and stained with Giemsa-Wright. Cells transfected with A: calf thymus DNA, B: SFV DNA and C: H-ras DNA.

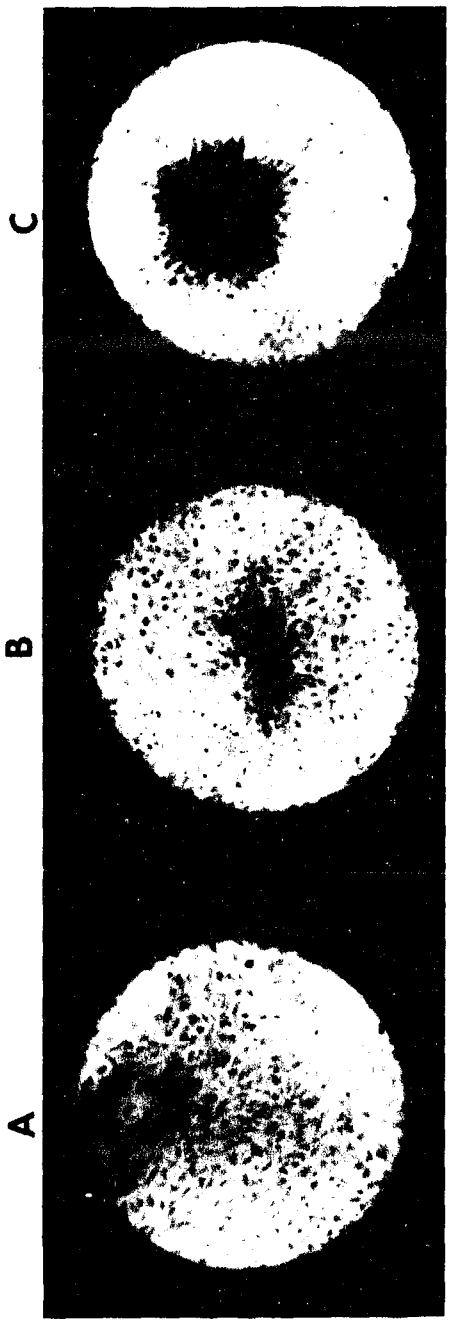
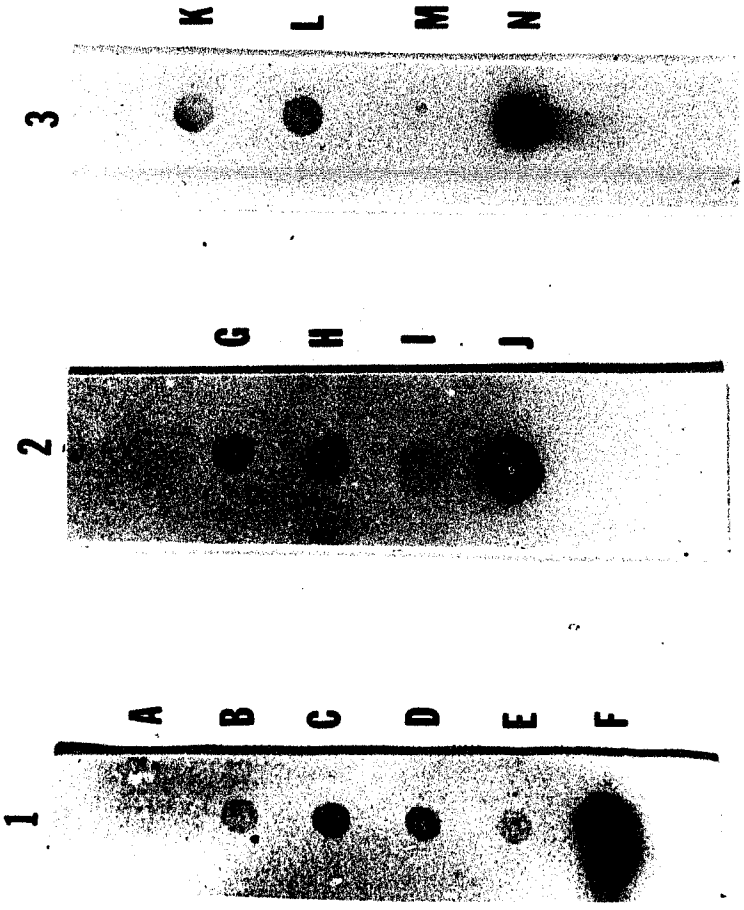

C
B
A

Figure 2: Nude mouse showing tumors induced by cell line F5.

Figure 3: Dot blot analysis of DNA extracted from focus-derived cell lines. Aliquots containing 10 µg of nuclear DNA were dotted onto nitrocellulose and hybridized with ^{35}S-dATP-labeled SFV DNA. Column 1, DNA from a: NIH 3T3 cells, b: line F2p3, c: line F2p4, d: line F3p3, e: line F3p4, f: 100 ng SFV DNA. Column 2, DNA from g: line F4p3, h: cells transfected with SFV DNA but not expressing foci, i: NIH 3T3 cells, j: 100 ng SFV DNA. Column 3, DNA from k: line F5p3, l: line F6p3, m: NIH 3T3 cells n: 100 ng SFV DNA.


K
L
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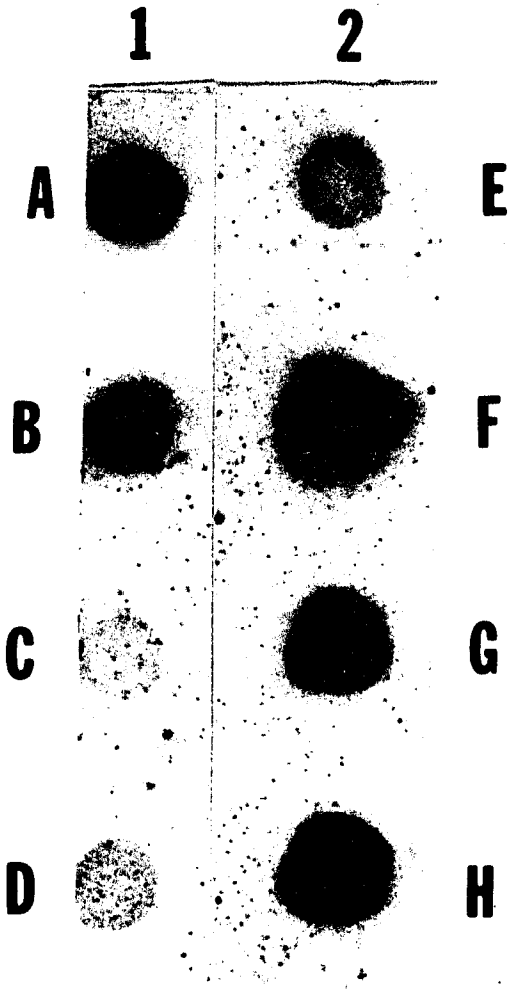
1

Figure 4: Dot blot of RNA extracted from focus-derived cell lines. Aliquots containing 20 µg of total RNA were dotted onto nitrocellulose and hybridized to ^{35}S-dATP labeled SFV DNA. Column 1, RNA from focus-derived lines a: F2p8, b: F3p8, c: F4p7, d: control p8. Column 2, RNA from SIRC cells infected with SFV for e: 8 hr, f: 24 hrs, g: 32 hrs, h: 48 hrs.


1
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Chapter 4

Association of Mitochondria DNA with Viral DNA in Purified Preparations of Poxviruses

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Summary

The presence of cellular material in purified preparations of Shope fibroma virus (SFV) and two orthopoxviruses, (vaccinia and Indiana) was investigated. Mitochondria were observed in purified preparations of SFV by electron microscopy and mitochondrial (mt) DNA was identified in restricted viral DNA by Southern blot hybridization with mouse cloned mt DNA. Mitochondria DNA was also detected in vaccinia and Indiana DNAs extracted from purified virions or cores. The viral and mt DNAs could be separated on the basis of their size by agarose gel electrophoresis but not by their buoyant density by centrifugation in cesium chloride gradients. These findings led us to re-examine previously reported results showing some homology between SFV, a leporipoxvirus and Indiana an orthopoxvirus (Berkowitz and Pogo, Virology 142:437-440, 1985) using cloned fragments of SFV DNA instead of the entire viral DNA. The results indicated that cross hybridization between SFV and Indiana DNAs was due in part to mt DNA but they also revealed an unrecognized region of homology between the two poxvirus genera.

Poxviruses/mitochondrial DNA/sequence homology

Introduction

Poxviruses are large DNA viruses which replicate in the cytoplasm of infected cells and remain largely host-cell associated after maturation. Purification of these viruses requires breakage of the cells and isolation of virions from crude cell extracts by differential centrifugations (Joklik, 1962, Dales and Mosbach, 1968). These purification procedures were designed for orthopoxviruses such as vaccinia, which are highly cytolytic and may not be appropriate for purification of poxviruses which are not cytocidal. Studies using electron microscopy indicated that there were no major cell contaminants in vaccinia preparations obtained after two rounds of density gradient centrifugation (Stern and Dales, 1974). However, the presence of host-derived material in purified virus preparations, has been recently reported. Thus, Franke and Hruby (1987) showed incorporation of a host thymidine kinase (tk) into tk- vaccinia virions, and Morrison and Moyer (1986) demonstrated the presence of a host polymerase subunit in rabbitpox virus cores. In addition, a plasmid containing Shope fibroma virus (SFV) and host DNA sequences has been isolated from rabbit cornea fibroblasts in culture, raising the possibility that these endogenous elements may contaminate viral preparations (Upton and McFadden, 1986a).

These results have led us to question whether cellular DNA may be present in purified preparations of the three poxviruses currently under study in our laboratory (vaccinia virus, Indiana virus and SFV) that are grown in different cell types and display different degrees of cytopathogenicity. In this communication we report that

mitochondrial (mt) DNA was present in all virus preparations regardless of the cell type in which the viruses were grown as determined by Southern blot hybridization. We found that mitochondria and poxvirus genomes could be separated on the basis of their size by agarose gel electrophoresis, but not by improving the purification procedure or by centrifugation in cesium chloride density gradients.

In light of these findings, we have reexamined hybridization studies on the homology of Indiana virus, an orthopoxvirus and SFV, a leporipoxvirus (Berkowitz and Pogo, 1985) by using cloned fragments of the SFV genome as probes, instead of the entire viral genome. The results indicated that the cross hybridization between Indiana and SFV DNAs was in part due to mt DNA, but they also revealed a previously unrecognized region of homology between orthopoxviruses (vaccinia and Indiana) and a leporipoxvirus (SFV).

Material and Methods

Viruses and Cells

The IHD-W strain of vaccinia virus was used in all experiments and was grown and assayed in L cells as described by Dales (1963). Shope fibroma virus obtained from H. C. Hinze, University of Wisconsin and the Indiana isolate obtained from M. Hodes, Indiana University were cultured in SIRC or BGMK cells and assayed as described (Pogo et al., 1982, Berkowitz and Pogo, 1985).

Mouse L fibroblasts were cultured as monolayers as described previously (Dales, 1963). Baby green monkey kidney fibroblasts (BGMK) obtained from S. Dales, (University of Western Ontario) were cultured as monolayers in Minimum Essential Media supplemented with 5% fetal

bovine serum. Cells were released from the surface with Trypsin:EDTA and passaged twice a week. Rabbit cornea fibroblasts (SIRC) were cultured as described (Obom and Pogo, 1987).

Virus Purification

Initially all viruses were purified according to the method of Stern and Dales (1974) which consists briefly in releasing virus from cells by sonication followed by a low speed centrifugation to remove cell debris, treatment of the supernatant with trypsin and high speed centrifugation to sediment the crude virus preparation. This preparation is then subjected to sedimentation through a 20% to 40% discontinuous sucrose gradient and the resulting virus pellet centrifuged through a continuous potassium tartrate gradient. In an effort to eliminate cellular contaminants from SFV grown in SIRC cells, two modifications in the differential centrifugation steps were introduced based on conditions used for the isolation of mitochondria from cells in culture (Vesco and Penman, 1969).

Method I: The viral material was loaded onto a continuous 20% - 50% potassium tartrate gradient (0.01 M Tris-HCl pH 8.5) and centrifuged in a Beckman SW 50.1 rotor at 18,000 rpm for 60 min at 4°C. The resulting bands were isolated from the gradient, resuspended in 0.01 M Tris-HCl buffer pH 8.5 and centrifuged at 23,000 rpm for 30 min at 4°C. The pellets from the two bands were resuspended in a small volume of 0.01 M Tris-HCl pH 8.5 and loaded onto a continuous 20% to 30% potassium tartrate gradient with a 40% cushion (0.01 M Tris-HCl pH 8.5) and centrifuged at 18,000 rpm for 60 min at 4°C. Two distinctive bands were isolated from these gradients, they were resuspended in

0.01 M Tris-HCl pH 7.2 buffer and centrifuged at 23,000 rpm for 30 min at 4°C. The resulting virus pellets were resuspended in 0.01 M Tris-HCl buffer pH 7.2 for further analysis.

Method II: The virus preparation was centrifuged in a 10% - 40% continuous sucrose gradient (0.01 M Tris-HCl pH 7.2) for 60 min at 18,000 rpm in a Beckman SW 50.1 rotor at 4°C. Three diffuse bands were identified. The middle and lower bands were removed from the gradient resuspended in 0.01 M Tris-HCl pH 7.2 and centrifuged at 25,000 rpm for 30 min at 4°C. Each pellet was resuspended in 0.01 M Tris-HCl pH 8.5 and loaded onto a continuous 20% - 50% potassium tartrate gradient (0.01 M Tris-HCl pH 8.5) for 60 min at 18,000 rpm, 4°C. Two distinctive bands were isolated from each gradient, resuspended in 0.01 M Tris-HCl pH 7.2 and the purified virus was centrifuged as described above. Virus cores were prepared as previously described (Dales and Pogo, 1969). Viral DNA was extracted from purified virions and virus cores as described (Berkowitz and Pogo, 1985).

Purification of viral DNA by cesium chloride centrifugation

The conditions employed were essentially those described by Radloff, Bauer and Vinograd (1967). A 50% wt/vol solution of cesium chloride and 0.01 M Tris-HCl pH 7.6 0.001 M EDTA was made and ethidium bromide was added to a concentration of 300 µg/ml. DNAs were added to the gradients and centrifuged at 50,000 rpm for 16 h at 20°C in a Beckman VTi 65 rotor. Two bands were visualized by ultra-violet illumination, the bands were removed and the refractive index was determined using an Aby refractometer.

Agarose gel electrophoresis and Southern hybridization

DNAs were digested with restriction enzymes following the conditions recommended by the manufacturer. Restriction fragments were separated by electrophoresis in 0.5% agarose gels for 18 h at 50 volts in a buffer composed of 90 mM Tris-borate, 90 mM boric acid and 2.5 mM EDTA. After denaturation, the DNA fragments were transferred to nitrocellulose paper by the Southern procedure (Southern, 1975). Prehybridization and hybridization conditions for high stringency were carried out following the procedure described by Maniatis et al. (1982). For low stringency conditions, the blots were prehybridized in a solution containing 6X SSC (1X SSC:0.15 M NaCl, 0.015 M Na citrate), 0.5% SDS, 5X Denhardt's (0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin) and 100 µg/ml denatured calf thymus DNA for 2 hr at 37°C. Hybridization was carried out in a solution containing 6X SSC, 0.5% SDS, 5X Denhardt's, 0.01 M EDTA, 100 µg/ml denatured calf thymus DNA and 10^6 cpm/ml of labeled DNA probe for 16 h at 37°C. The blots were then washed in 2X SSC, 0.5% SDS, at room temperature for 5 min, in 2X SSC, 0.1% SDS at room temperature for 15 min and in 1X SSC, 0.5% SDS at 37°C for 2 h, finally they were air-dryed and exposed to Kodak XAR film at -70°C.

Nick-translation was performed following the conditions described by Rigby et al. (1977). Restriction enzymes and enzymes for nick-translation were purchased from Boehringer Mannheim and BRL respectively. [^{35}S]-thio-dATP was purchased from New England Nuclear.

Plasmids

Cloned Bam HI fragments of Shope fibroma virus (Wills et al., 1983) and the endogenous cellular plasmid, pSIC-9 (Upton and McFadden, 1986a) were kindly provided by Dr. G. McFadden, University of Alberta. Cloned mouse mt DNA, plasmid pAMI, (Martens and Clayton, 1979) was a gift from Dr. David Clayton, Stanford University. To eliminate vector sequences from the pKB/HE plasmid containing the left 10 kb of the SFV Bam HI A fragment, the plasmid was restricted with Hind III and Bam HI, electrophoresed in 0.5% agarose gel and the insert SFV DNA electroeluted from the gel using an IBI unidirectional electroelutor following the conditions recommended by the manufacturer.

Results

Comparison of DNA restriction pattern of viruses grown in the same cell line

When DNA from vaccinia virus grown in L and SIRC cells, Indiana and SFV grown in SIRC cells was restricted with Bam HI and electrophoresed in 0.5% agarose gels, it was observed that vaccinia DNA from virus grown in SIRC cells showed an extra 5 kb fragment as compared to DNA from vaccinia grown in L cells (Fig. 1A a and b). This 5 kb fragment was also present in the DNAs of Indiana and SFV also grown in SIRC cells (Fig. 1A c and d). After blotting and hybridization with [^{35}S]-labeled SFV DNA, it was found that the DNA of vaccinia grown in SIRC cells contained sequences that hybridized to SFV DNA (Fig. 1B b), also present in the DNA of Indiana (Fig. 1B c) but absent from the DNA of vaccinia grown in L cells (Fig. 1B a). Since it was previously reported (Wills et al., 1983, Berkowitz and

Pogo, 1985) using stringent hybridization conditions that vaccinia DNA from virus grown in L cells and SFV grown in SIRC cells have no sequences in common, these findings suggested that the viral DNA preparations might contain DNA sequences from SIRC cells.

Isolation of a cellular component from uninfected SIRC cells using standard virus purification methods

To elucidate if a cellular component was present in the purified virus preparations, uninfected SIRC cells were subjected to the same purification procedure used to isolate virus, (Stern and Dales, 1974). One distinctive band was observed in the position of the potassium tartrate gradient corresponding to the viral band. DNA isolated from this band was restricted with Bam HI or Eco RI and electrophoresed in 0.5% agarose gels as shown in Fig. 2A. Uncut DNA migrated at approximately 50 kb, 25 kb and 17 kb (Fig. 2A c) while restricted DNA showed 2 major bands with Bam HI, 12 kb and 5 kb, and three bands with Eco RI, 17 kb, 11 kb and 6 kb (Fig. 2A a and b). The DNA was blotted onto nitrocellulose and hybridized with labeled cloned mouse mt DNA at low stringency (Fig. 2B), because preliminary results indicated that mt DNA from SIRC cells (rabbit) and mouse cloned mt DNA hybridized only at low stringency as expected for species with low homology (data not shown). The molecular size of the isolated cellular DNA as well as the hybridization signal indicated that mt DNA was present in the DNA preparations of virus grown in SIRC cells when using the standard purification procedure. Furthermore, hybridization of this cellular DNA with pSIC-9 DNA a plasmid which contains both SFV and cellular sequences and bands at the same buoyant density than mt DNA (Upton and

McFadden, 1986a) showed no homology, thus eliminating the possibility that endogenous plasmids were also present (data not shown).

The presence of mt in "purified" virus preparations from SIRC cells was confirmed by EM examination. Intact and broken mitochondria to a different extent were observed in "purified" preparations of vaccinia, Indiana and SFV grown in SIRC cells. Figure 3 shows an electron micrograph of material present in the virus band of potassium tartrate gradients of uninfected SIRC cells (A) and SIRC cells infected with Shope fibroma virus (B). Even at this final stage of purification, mitochondria and other subcellular structures are present.

To determine if vaccinia virus DNA from virus preparations grown in L cells also contained mt DNA, vaccinia DNA was hybridized to cloned mouse mt DNA. The results shown in Fig. 4 indicated that vaccinia DNA contained mt DNA, in spite of the fact that EM examination of similar preparations revealed no major cell contaminants (Stern and Dales, 1974). Identical results were obtained with DNA extracted from core preparations (Fig. 4B).

Attempts to eliminate mitochondrial DNA from purified virus preparations

Three different approaches were used in an attempt to eliminate mt DNA from virus preparations: 1) changes in the purification procedures, 2) isopycnic centrifugation in cesium chloride to separate the viral DNA from the mt DNA and 3) electrophoretic separation of the DNAs. Figure 5 illustrates one of the gradient separation procedures we employed to remove mitochondria from the virus (method II). After the crude virus was centrifuged through a 10% to 40% continuous

sucrose gradient, three diffuse bands were visible. The middle and lower bands were removed from the gradient, and centrifuged through a 20% to 50% continuous potassium tartrate gradient as described in materials and methods. Lanes a and b contain viral DNA isolated from the middle band in the sucrose gradient, followed by potassium tartrate gradient centrifugation. Lanes c and d contain DNA from viruses isolated from the lower band of sucrose gradient followed by potassium tartrate gradient centrifugation. Fig. 5 represents Southern blot hybridization of these DNAs with mt DNA. It is clear that mt DNA is present in all fractions, even those subjected to two gradients. Both methods employed resulted in virus preparations containing mt DNA.

When SFV and Indiana DNAs and the DNA isolated from uninfected SIRC cells were centrifuged in cesium chloride gradients containing ethidium bromide, two bands were isolated for each type of DNA having the following densities (gm/ml): 1.537 and 1.547 for SFV, 1.539 and 1.550 for Indiana and 1.538 and 1.546 for uninfected SIRC cell DNA. Southern hybridization of these DNAs at low stringency to labeled cloned mouse mt DNA indicated that all fractions contained mt DNA (data not shown).

Finally we attempted to separate the two DNAs by agarose gel electrophoresis. As seen in Fig. 6, undigested SFV DNA resolves into three species after electrophoresis in 0.5% agarose gels for 18 hours at 50 volts, (panel A). When the DNA was transferred to a nitrocellulose filter and hybridized to labeled cloned mt DNA at low stringency, only two species hybridized to mt DNA (Fig. 6B a).

However, when the DNA was hybridized to labeled cloned SFV Bam HI fragment C DNA, only one species, unique to SFV was identified (Fig. 6B b).

Identification of a region of SFV DNA which hybridizes to vaccinia DNA

On the basis of the findings reported above, it can be concluded that most of the cross hybridization between SFV and Indiana previously observed (Berkowitz and Pogo, 1985), could be accounted for by the presence of contaminating mt DNA sequences. However, a 3.9 kb Hind III fragment of Indiana DNA which hybridized to SFV at high stringency conditions, did not hybridize to cloned mouse mt DNA (data not shown). To elucidate the nature of this fragment, Indiana and vaccinia DNAs were hybridized to cloned fragments of SFV DNA. Results from these experiments indicated that one SFV cloned fragment from the left portion of the Bam HI fragment A (plasmid pKB/HE) was able to hybridize to vaccinia and Indiana DNAs at low stringency conditions. As shown in Fig. 7, this plasmid hybridized to vaccinia Hind III fragments G, J and L. To avoid spurious hybridization, the SFV DNA insert was cut from the plasmid, electrophoresed in 0.5% agarose gel and electroeluted before nick-translation.

Discussion

From the results reported here, we conclude that mt DNA is present not only in purified SFV preparations obtained from SIRC and BGMK cells but also in vaccinia virions purified from L cells. Attempts to improve the purification procedure for SFV by using different density gradients were not successful. Results from experiments in which SFV and vaccinia DNAs were extracted from

purified viral cores, indicate that mitochondria DNA was also present in such preparations, suggesting that cellular DNA may be encapsidated in the virion during morphogenesis.

While SFV and Indiana DNAs banded at the same buoyant density as SIRC cell mt DNA in cesium chloride isopycnic gradients, the viral and cellular genomes could be separated on the basis of their size by agarose gel electrophoresis. Thus, when SFV DNA isolated from "purified" virions is electrophoresed in 0.5% agarose, three DNA species are visible, two of which are mitochondria and one which is unique to SFV.

The presence of contaminating single stranded DNA bound to the surface of virions has been extensively discussed by Holowczak (1982). In this report we have shown that cellular double standard DNA is also a major contaminant of "purified" preparations of poxviruses. The results reported here are particularly relevant to studies comparing DNA restriction patterns of poxviruses grown in different cell types, to the determination of homology between unrelated poxviruses (Berkowitz and Pogo, 1985, Rouhandeh et al., 1982, Drillien et al., 1987), or to the significance of cellular material present in virus preparations (Morrison and Moyer, 1986, Franke and Hruby, 1987).

The presence of mt DNA in all the virus preparations tested led to reevaluation of previously reported results on the Indiana isolate (Berkowitz and Pogo, 1985). It was shown that there was no cross-hybridization between orthopox (vaccinia grown in L cells), and leporipoxviruses (SFV grown in SIRC cells) at conditions of high stringency of hybridization (Berkowitz and Pogo, 1985, Wills et al.,

1983). However, the DNA from Indiana virus which we have classified as an orthopoxvirus was found to hybridize partially to SFV DNA, when total viral DNA was used. By contrast, when equivalent amounts of cloned restriction fragments of SFV were used to hybridize to Indiana DNA at high stringency conditions, no homology was detected (G. McFadden, personal communication). The current studies suggest that the hybridization was due, in part, to the presence of mt DNA. It is significant, however, that at low stringency conditions Indiana and vaccinia DNAs hybridized to a specific region of the SFV genome, the left portion of the Bam HI fragment A. This region which contains the viral thymidine kinase gene and the immediate environment around this gene, appears to be conserved between the orthopoxviruses and leporipoxviruses (Upton and McFadden, 1986b). The SFV subgenomic fragment revealed homology to three contiguous vaccinia Hind III fragments, G, J, L; the vaccinia Hind III J fragment encompassed the vaccinia thymidine kinase gene. In this context, it is worthwhile mentioning that Drillien et al. (1987) have recently reported the cross hybridization between a region of fowlpox virus DNA, an avipoxvirus, and the vaccinia Hind III J fragment. These highly conserved regions of the poxvirus genome may encode gene products that are involved in functions common to all poxviruses.

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Figure 1:

Southern hybridization of vaccinia virus, Indiana virus and SFV DNAs to labeled SFV DNA. 1-2 μg of poxvirus DNA was restricted with Bam HI, electrophoresed, blotted onto nitrocellulose paper and hybridized to ^{35}S-dATP labeled SFV DNA (SFV grown in SIRC cells) at high stringency as described in materials and methods. Panel A: Ethidium bromide stain of agarose gel. Panel B: Southern hybridization. Lane a: DNA from vaccinia virus grown in L cells, lane b: DNA from vaccinia virus grown in SIRC cells, lane c: DNA from Indiana virus grown in SIRC cells, lane d: DNA from SFV grown in SIRC cells. Arrow indicates the position of an extra band in DNA from virus grown in SIRC cells.

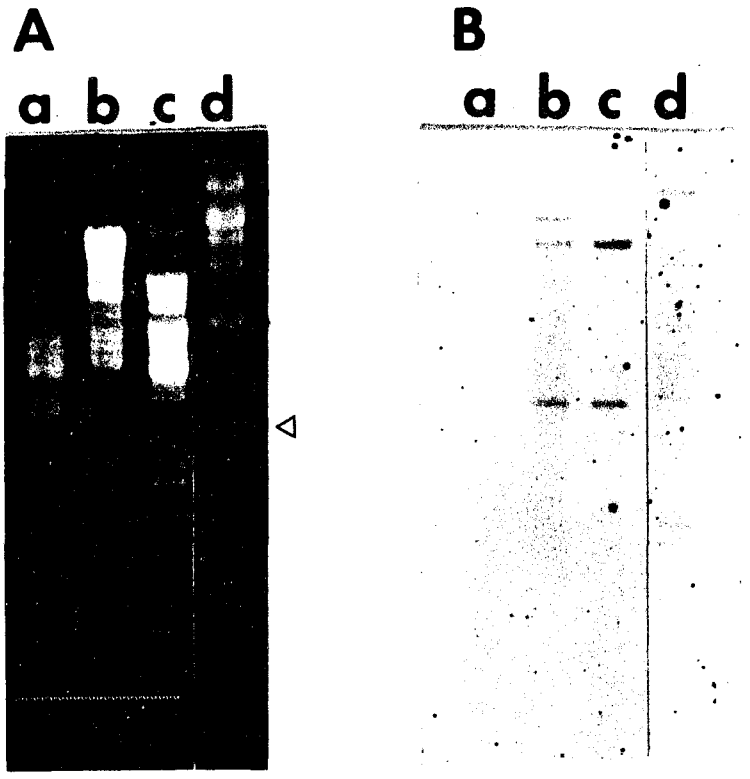

A
a b c d
B
a b c d

Figure 2:

Identification of cellular DNA in preparations of uninfected SIRC cells after being subjected to virus purification procedure. DNA was extracted from a band isolated in tartrate gradients as described in material and methods. 1-2 µg of DNA was restricted, electrophoresed, blotted and hybridized to ^{35}S-dATP labeled cloned mouse mitochondrial DNA at low stringency. Panel A: Ethidium bromide stain of agarose gel. Panel B: Southern hybridization. Lane a: DNA from uninfected SIRC cells restricted with Bam HI, lane b: same with Eco RI, lane c: same unrestricted. Numbers indicate molecular weight of λ DNA restricted with Hind III.

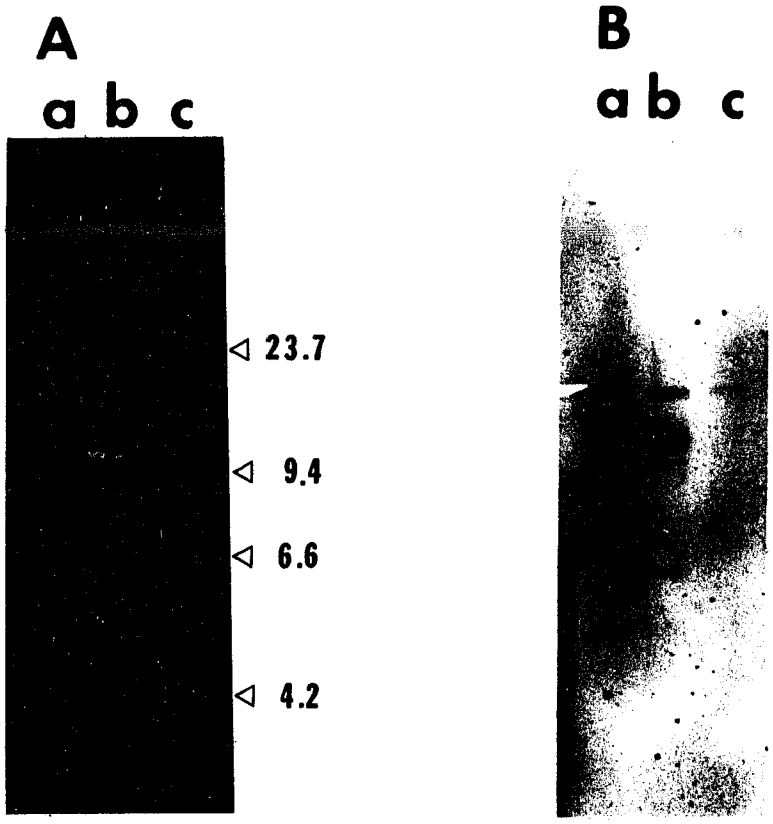
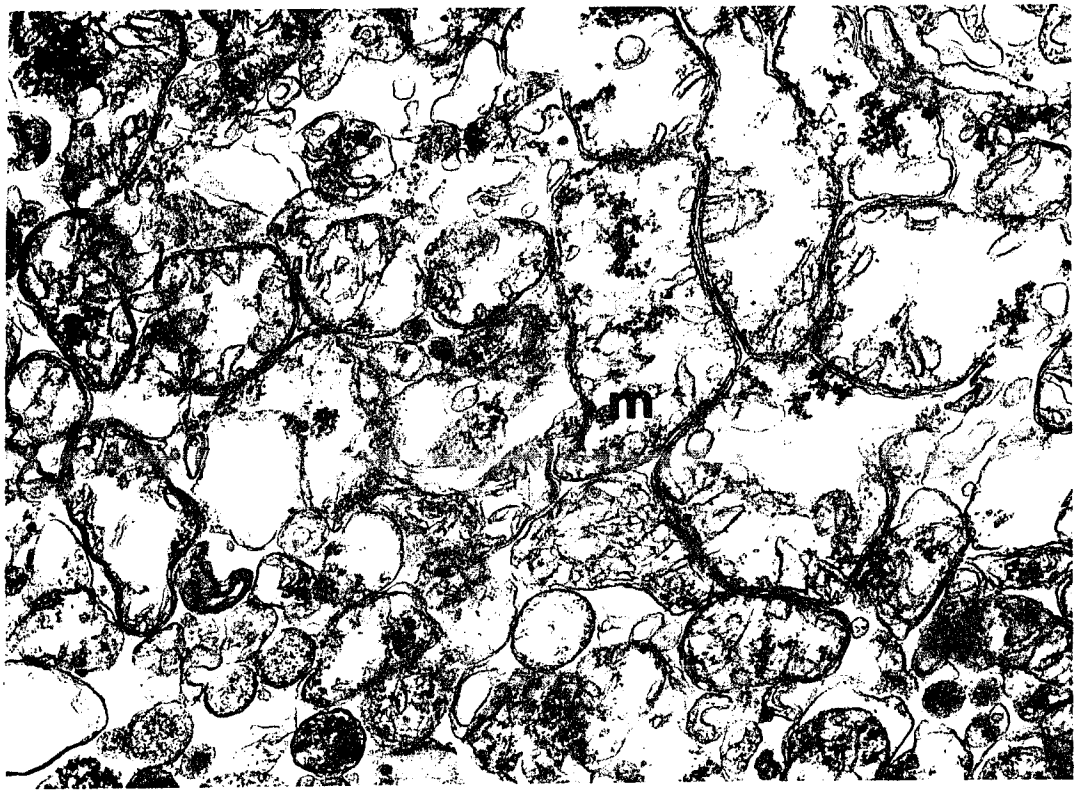

A
a b c
23.7
9.4
6.6
4.2
B
a b c

Figure 3:

Electron micrograph of material isolated from tartrate gradients of uninfected and Shope fibroma virus infected SIRC cells. Uninfected and SFV infected SIRC cells were fractionated according to standard procedures. Material isolated from the "virus" band of potassium tartrate gradients was fixed and sectioned for electron microscopy. Panel A: uninfected SIRC cells (27,600X). Panel B: SFV infected cells (43,700X). M: mitochondria, V: virus.


A
m

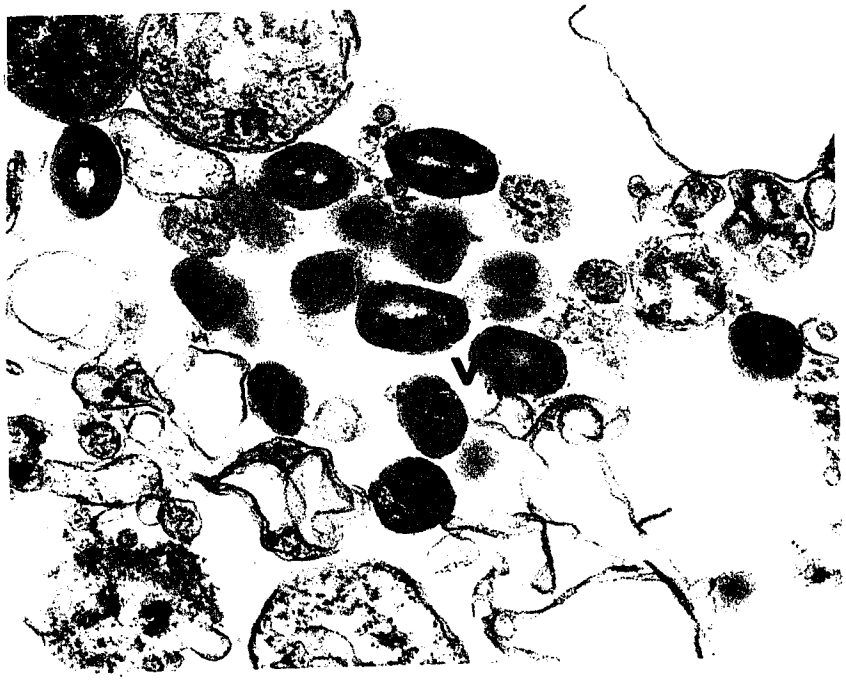

B
v

Figure 4:

Presence of mitochondria DNA in vaccinia virus DNA. 1-2 μg of vaccinia virus DNA was restricted, electrophoresed, blotted and hybridized to ^{35}S-dATP labeled cloned mouse mitochondria DNA at low stringency. Panel A: Ethidium bromide stain of agarose gel. Panel B: Southern hybridization. Lane a: unrestricted vaccinia DNA, lane b: vaccinia DNA restricted with Bam HI, lane c: cloned mouse mitochondria DNA restricted with Bam HI. Lane d: λ DNA restricted with Hind III. Numbers indicate MW of restriction fragments of λ DNA.

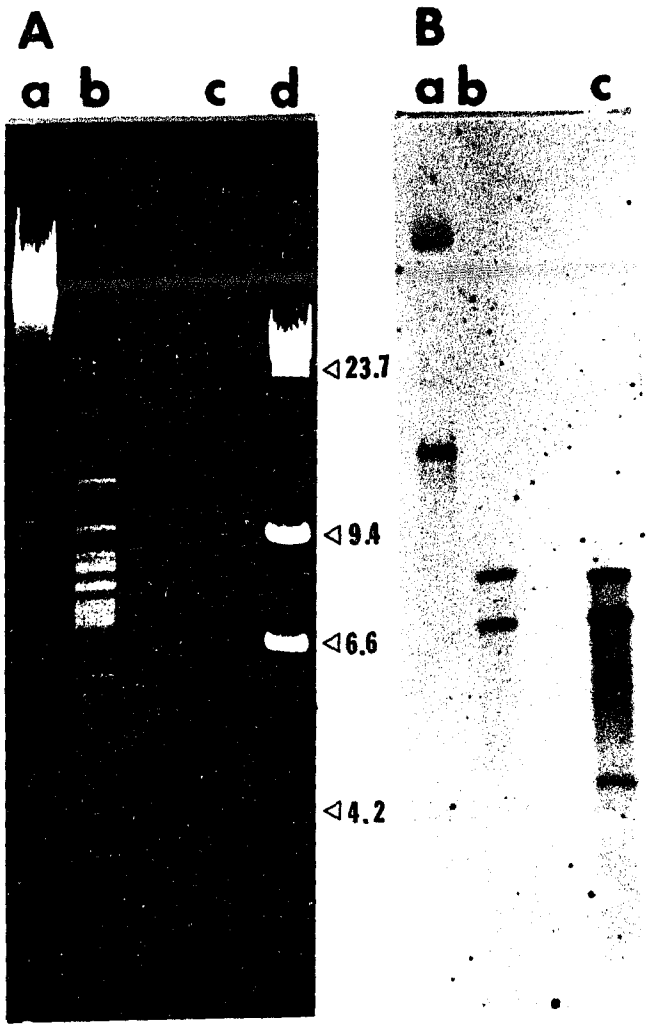

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Figure 4B:

Presence of mitochondria DNA in preparations of vaccinia and SFV DNA isolated from purified cores. 1-2 µg of poxvirus DNA was restricted with Bam HI, electrophoresed in a 0.5% agarose gel transferred to a nitrocellulose filter and hybridized to ^{35}S-dATP labeled cloned mouse mt DNA. Panel A: Ethidium bromide stain of agarose gel. Panel B: Southern hybridization, lane a: vaccinia DNA (grown in L cells), lane b: SFV DNA (grown in SIRC cells), lane c: uncut cloned mouse mt DNA.

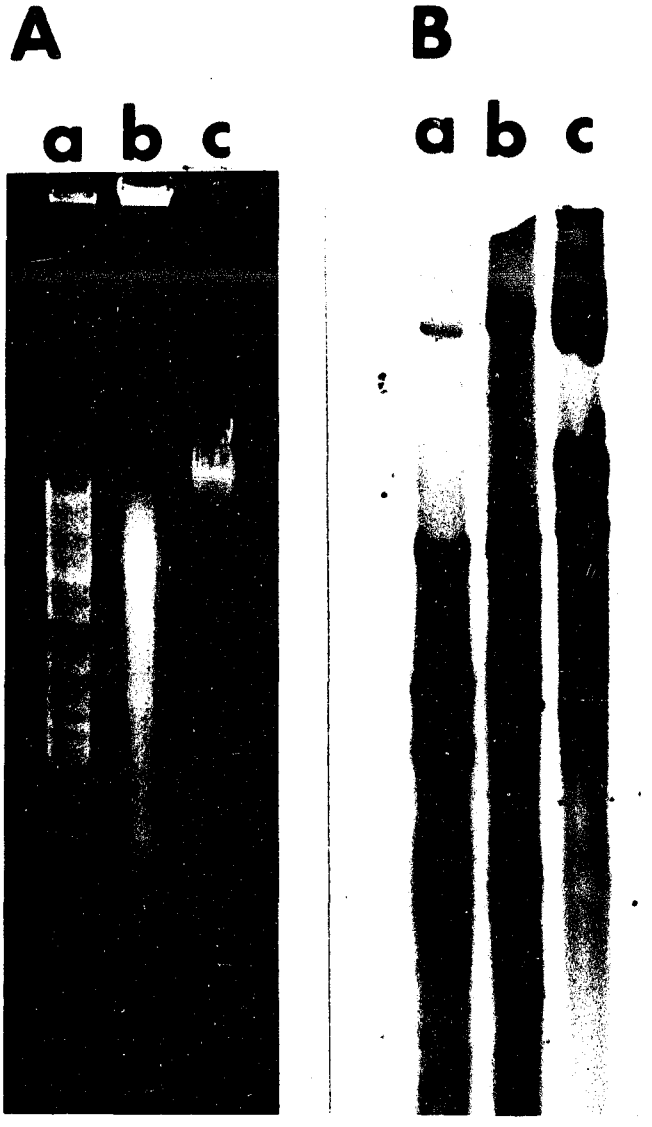

A
a b c
B
a b c

Figure 5:

Purification of Shope fibroma virus by Method II. DNA was extracted from virus isolated at different stages of purification and restricted with Bam HI, blotted onto nitrocellulose filter and hybridized to ^{35}S-dATP labeled mouse mitochondria DNA at low stringency. Panel A: Ethidium bromide stain of agarose gel. Panel B: Southern hybridization. Virus from middle sucrose band was centrifuged in tartrate gradients, lane a: upper band, lane b: lower band. Virus from lower sucrose band was centrifuged in tartrate gradients, lane c: upper band, lane d: lower band, lane e: virus from upper sucrose band, lane f: virus from lower sucrose band, g: λ DNA.

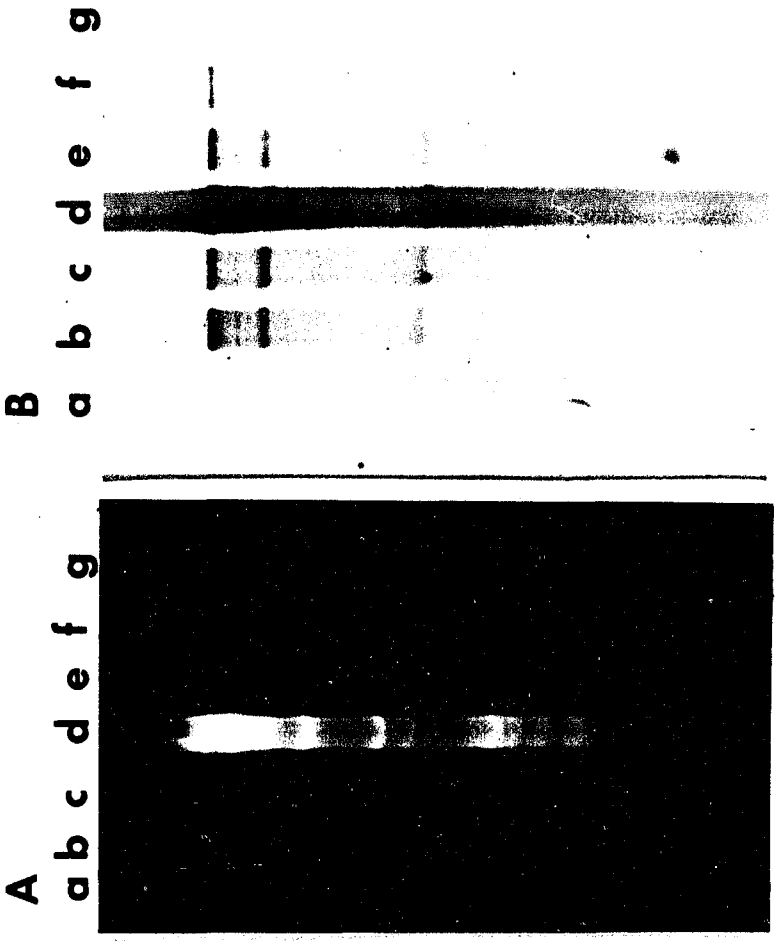

B
a b c d e f g
A
a b c d e f g

Figure 6:

Separation of SFV DNA and rabbit mitochondria DNA by agarose gel electrophoresis. Unrestricted SFV DNA was electrophoresed in 0.5% agarose gel, transferred to nitrocellulose paper and hybridized at low stringency to ^{35}S-dATP labeled mouse mitochondria DNA (lane a), or ^{35}S-dATP labeled cloned SFV fragment C DNA (lane b). Panel A: Ethidium bromide stain of agarose gel. Panel B: Southern hybridization.

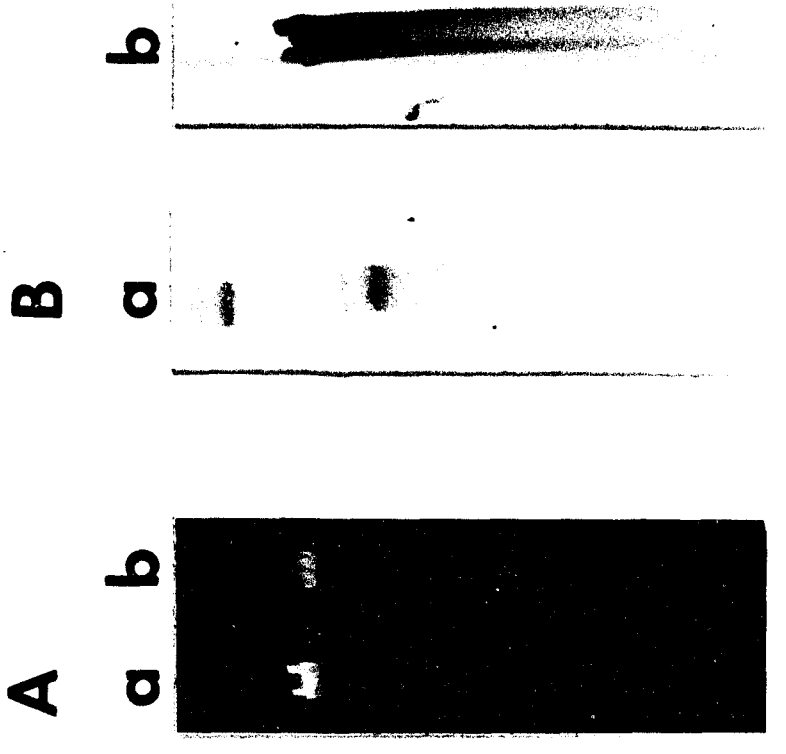
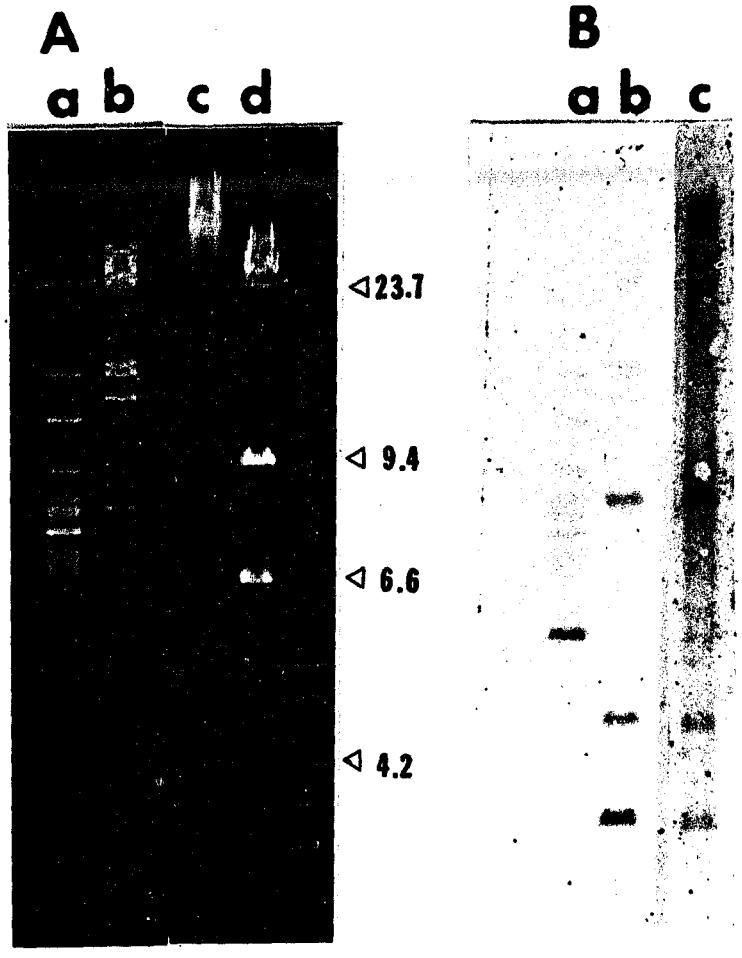

A
a
b
B
a
b

Figure 7:

Hybridization of SFV Bam HI fragment A to orthopoxvirus DNA. 1-2 µg of poxvirus DNA was electrophoresed in 0.5% agarose gel, transferred to nitrocellulose filters and hybridized to plasmid pKB/HE at low stringency. Panel A: Ethidium bromide stain of agarose gel. Panel B: Southern hybridization. Lane a: vaccinia DNA restricted with Bam HI, lane b: vaccinia DNA restricted with Hind III, lane c: Indiana DNA restricted with Hind III, lane d: λ DNA restricted with Hind III. Numbers indicate MW of λ DNA restricted with Hind III.


A
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Chapter 5

DISCUSSION

The studies on SFV presented here have resulted in several principal findings: i) uv-irradiated SFV caused a transient transformation of an immortalized rabbit cell line (SIRC), ii) SFV DNA induced tumorigenic conversion of NIH 3T3 cells while the Bam HI fragment C containing the SFV growth factor gene, induced only *focus* formation, iii) mitochondrial DNA is present in purified poxvirus preparations and may be packaged during virus morphogenesis, iv) a region of common homology is shared between the genomes of leporipox and orthopox viruses.

The oncogenic potential of SFV was demonstrated by uv-irradiation of the virions prior to infection of immortalized SIRC cells. This treatment decreases the cytolytic effect of the virus by inhibiting replication without apparently destroying the transforming ability (Duff and Rapp, 1971, 1973). Transformants were characterized by loss of contact inhibition, high efficiency of cloning in soft agar, ability to grow in low concentrations of serum, induction of tumors in rabbits, the presence of SFV DNA sequences in the nucleus and the expression of SFV proteins. The transformation of SIRC cells by SFV was transient; after approximately 50 passages the cells lost the transformed phenotype. Several possibilities could account for the loss of the malignant phenotype. First, non-transformed cells may have overgrown the transformed population. This possibility seems unlikely since cell lines derived from single *foci* also lost the

transformed phenotype after approximately 50 passages. Second, SFV may have a weak oncogenic potential resulting in an unstable type of transformation. Finally, transformation of SIRC cells by SFV may require the continued presence and expression of SFV DNA. This last possibility is more likely, because the loss of viral sequences and disappearance of SFV proteins correlated with loss of the transformed phenotype.

The finding that uv-inactivated SFV is able to induce cell transformation is consistent with previous reports that showed that Yaba monkey tumor virus, vaccinia virus and Molluscum contagiosum cause cell transformation. Rouhandeh and Vafai (1982) demonstrated the transforming capacity of Yaba virus by using uv-irradiated virus to infect JINET cells. However, these investigators followed the infection for only 50 passages, so it is not known whether Yaba virus induced transformation has the same transient characteristics as that induced by SFV. Koziorowska and co-workers (1971) have shown that vaccinia virus can transform primary mouse cells and that the transformed phenotype was maintained for over 90 passages. Finally, Barbanti-Brodano et al., (1974) reported that abortive infection of human embryo fibroblasts by Molluscum contagiosum resulted in transient transformation.

Further evidence for the oncogenic potential of SFV was obtained by transfection of calcium-phosphate precipitated SFV DNA into NIH 3T3 cells. SFV DNA induced complete transformation of the cells as characterized by focus formation, anchorage independent growth, tumorigenesis in nude mice and presence of SFV DNA and RNA sequences

in transformants. To our knowledge, this is the first demonstration of the transforming ability of poxvirus DNA in the NIH 3T3 cell assay. By contrast, vaccinia virus DNA was unable to induce transformation in this assay.

The mechanism by which SFV DNA causes cell transformation remains unclear at the moment. It was difficult to determine whether SFV sequences were lost or undectable in the NIH 3T3 cell transformants. Southern blot analyses of genomic DNA from transformants after several passages did not detect SFV DNA sequences, although the cells remained transformed. Failure to detect SFV DNA sequences could be due to limitations in the sensitivity of the Southern procedure. Employing the entire SFV genome as probe, 4 to 8 fmoles of SFV DNA were detectable in 50 µg of cell DNA and single copy genes could be identified. This method may not be sensitive enough, however, to identify short sequences of the SFV genome. In addition, incorporation of labeled nucleotides during nick-translation within the 160 kb SFV genome may not be equally distributed. Use of sub-genomic fragments as probes in future studies may improve the sensitivity of detection.

If SFV sequences are actually lost, a hit and run phenomenon may have occurred. In this situation, viral DNA is thought to mutate or activate a cellular proto-oncogene which becomes transforming. Hit and run phenomenona have been observed in cells transformed by herpesvirus (Galloway, 1982) and adenovirus (Kuhlmann et al., 1982).

Of the SFV fragments tested, only the Bam HI fragment C, which contains the SFV growth factor, induced <u>focus</u> formation. This

fragment was not fully transforming since it failed to induce anchorage independent growth or tumorigenesis in nude mice. Thus genes present in fragment C may play a role in transformation but not in tumorigenic conversion. Recent results obtained by transfection of the EGF and TGF-α genes may help to explain why the SFV DNA fragment containing the growth factor was not tumorigenic. Finzi et al., (1987) found that transfection of NIH 3T3 cells with the TGF-α gene resulted in increase saturation density but not in tumorigenesis, while Rosenthal et al., (1986) observed that Rat-1 cells transfected with TGF-α were able to increase slightly the number of tumors in nude mice over controls. The data suggested that EGF, TGF-α and possibly other EGF-like proteins may play only an enhancing role in transformation of certain cells. In the case of SFV the results may imply that other regions of the SFV genome, either independently or in association with fragment C, are necessary for tumorigenic conversion.

The genetic relatedness of orthopoxviruses and SFV was investigated by Southern blot analysis using cloned Bam HI SFV fragments as probes. During the course of this investigation, the association of SFV DNA with mitochondria DNA was uncovered. Results from these experiments revealed that the cellular DNA was actually packaged into virions. The two DNAs could be separated by size in agarose gel electrophoresis but not by their density in isopycnic density gradients.

It is unclear whether mitochondria DNA participates in the life cycle of the virus or if this finding indicates non-specificity in the packaging of the virion DNA. In any event, the data indicate that the

comparison of different poxviruses, based on restriction enzyme analysis and Southern hybridization using total genomic probes, could give spurious results. Therefore, a better procedure for comparing virus genomes would be Southern hybridization using cloned fragments as probes.

While much of the hybridization initially observed between SFV and Indiana virus (orthopoxvirus) appeared to be due to mitochondria DNA sequences present in both viruses, one of the reacting restriction fragments was clearly not of mitochondrial origin. To determine the nature of this fragment, individual cloned fragments of the SFV genome were hybridized to vaccinia and Indiana DNAs under low stringency conditions. The results indicated that the left 10 kb of the SFV Bam HI fragment A, hybridized to the Hind III fragments G, J and L of the two orthopoxviruses. It is significant that SFV fragment A and vaccinia fragment J contain the viral thymidine kinase (tk) gene. It has been previously determined by nucleic acid sequence analysis (Upton and McFadden, 1986) that the region immediately surrounding the SFV tk gene was highly homologous to the vaccinia Hind III J fragment. This data is also consistent with the finding of Drillien et al., (1987) that a fragment in fowlpox (an avipoxvirus) DNA also hybridized to the vaccinia Hind III J fragment. It is also relevant that transcriptional mapping has revealed that the Hind III L fragment codes only for late transcripts (Belle-Isle et al., 1982). Since late messages are known to codify for structural proteins, the conserved region of SFV and vaccinia DNA may encoded a common virion protein.

In conclusion, the oncogenic potential of SFV was demonstrated by

infecting already immortalized cells with uv-irradiated virus and by transfection of NIH 3T3 cells with calcium-phosphate precipitated DNA. The data also suggest a role for the Bam HI fragment C in transformation. Mitochondria DNA was found to be present in preparations of purified virus and a region of common homology was found between the genomes of viruses from two different genera.

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