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# Shope Fibroma Virus

## I. Biological and Molecular Properties of a Cytocidal and a Noncytocidal Strain

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The biological and molecular properties of two strains of Shope fibroma virus (SFV) were compared. SFV-I was highly cytocidal to most of the cell lines tested and produced pocks in the chorioallantoic membrane of chick embryos. By contrast, SFV-W did not produce cytopathic effects in any of the cell lines or in the chorioallantoic membrane, but it induced characteristic foci 3 to 4 days after infection. Both strains produced tumors when inoculated into the skin of susceptible rabbits. Maximal infectivity in BSC-1 cells was reached by both strains between 24 to 48 h after inoculation. Viral DNA synthesis also took place at the same time, although cells infected with SFV-I incorporated three times more [$^3$H]thymidine than cells infected with SFV-W. Sedimentation analysis and hydroxylapatite chromatography of the two viral DNAs indicated that their molecular weights were similar and that both were naturally cross-linked. Digestion with three restriction endonucleases, however, revealed that they had different restriction sites. When SFV-I and vaccinia DNA were compared, the restriction patterns were more alike. Analysis of the virion structural proteins by gel electrophoresis indicated that SFV-I, SFV-W, and vaccinia virus had many polypeptides in common, although there were distinctive differences among the three viruses. Finally, the results of plaque neutralization tests with different antisera showed that SFV-I and SFV-W shared common antigens and that vaccinia antiserum inhibited SFV-I but not SFV-W. We conclude that the SFV-I genome contains information for both cytolysis and tumorigenesis. This unusual virus may be a recombinant between an orthopoxvirus and a leporipoxvirus.

The role of viruses in promoting cellular growth is now well established. Poxviruses belong to a group of agents that cause benign tumors in certain instances and cytocidal infections in others. Studies on the molecular biology of poxviruses have been successfully carried out with vaccinia virus, a member of the virulent orthopoxviruses. The biological and molecular properties of the oncogenic poxviruses, however, remain poorly understood.

Shope fibroma virus (SFV), a member of the leporipoxviruses, induces a self-limiting neoplasia in adult rabbits and malignant tumors in newborn rabbits (18). It has been shown that, in vitro, SFV produces persistently infected cell lines, promotes the growth of cells, and facilitates the growth of other viruses (6–8, 11, 19). In contrast, it has also been reported that SFV is able to inhibit host cell metabolism like other cytocidal poxviruses (4, 1). In the context of our studies concerning the effect of poxviruses on cell metabolism, we investigated the behavior of two SFV strains that were reported to cause different effects on host cells and compared their biological and molecular properties with those of vaccinia virus.

### MATERIALS AND METHODS

**Cells.** HeLa cells and mouse L fibroblasts were cultured as monolayers as described previously (2, 3, 14). The BSC-1 line of monkey kidney cells (from C. Basilico, New York University) was cultured as monolayers in minimal essential medium supplemented with 3% vitamin mixture, 2% nonessential amino acids, 1% sodium pyruvate, and 10% fetal calf serum, purchased from Microbiological Associates. Rabbit epidermal cells (Sf1Ep), from the American Type Culture Collection, were maintained as monolayers in minimal medium supplemented with nonessential amino acids and 10% fetal calf serum. Rabbit kidney cells (DRK) obtained from H. C. Hinze, University of Wisconsin, and N. Crouch, University of Rockland, Rockland, Ill., were cultured as monolayers in minimal medium with 10% fetal calf serum.

**Viruses.** Two samples of SFV originally derived from the Patuxent strain were used. The strain obtained from M. Hodes, Indiana University, was designated Shope fibroma virus, Indiana (SFV-I); the other, from H. C. Hinze, was designated Shope fibroma virus, Wisconsin (SFV-W). Both strains were propa-

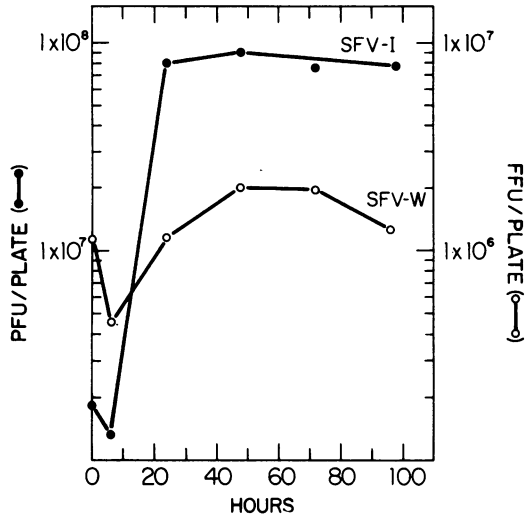


FIG. 1. Multiplication of SFV-I and SFV-W in BSC-1 cells. Monolayers containing $4 \times 10^6$ cells were infected with either 5 PFU of SFV-I or 5 FFU of SFV-W. At the time indicated, samples of the infected cells were processed to determine the amount of virus present either by plaque assays or by counting the foci induced as described in Materials and Methods.

gated in BSC-1 monolayers. SFV-I was assayed by the number of plaques produced in BSC-1 monolayers under agar, as described for vaccinia virus (2). SFV-W was assayed by the number of foci induced in the same cell monolayers. For this purpose, the cells were plated in Integrid plates, infected when they reached confluence, and stained with Giemsa-Wright 5 days after infection. The foci per square in the plates were counted and multiplied by the total number of squares and dilution factors to express the infectivity as focus-forming units (FFU) per milliliter.

The two strains were also inoculated into the chorioallantoic membrane of chicken embryos according to the technique described by Overman and Tamm (10). The IHD-W strain of vaccinia virus was used in all experiments under the conditions already published (2).

**Inoculation into rabbits.** Rabbits were inoculated intradermally in the shaved skin of the back with serial dilutions ($10^2$ to $10^6$) of each virus strain to be tested. The titers of the stocks were $8 \times 10^7$ PFU/ml for SFV-I and $6 \times 10^7$ FFU/ml for SFV-W. Tumors developed 1 week after the inoculation with SFV-I, reached a maximal size (20 mm) after 15 days, and regressed by the 3rd week. The tumors induced by SFV-W reached the same maximal size after 2 weeks but did not regress until the 4th or 5th week. No major macroscopic differences were observed between the tumors induced by the two viruses, except that those produced by SFV-I were more necrotic, leaving a darker scar. After regression of the fibroma lesions, the rabbits were challenged with an additional inoculation of virus, but no tumors developed, and they were bled 2 and 3 weeks after the second inoculation. Rabbits inoculated with vaccinia virus developed only ulcerations of the skin.

To test antibody levels, virus preparations were incubated with serial dilutions of each antiserum at 37°C for 1 h and then assayed for infectivity. Vaccinia antiserum was a gift of S. Dales, University of Western Ontario.

**Synthesis of viral DNA and other procedures.** To establish when viral DNA was synthesized, the incorporation of $^3$H-labeled thymidine ([$^3$H]TdR) into the cytoplasmic fraction of virus-infected cells was measured at different times after infection. To initiate synchronous infection, cell monolayers containing $8 \times 10^6$ BSC-1 cells were infected with either 5 PFU of SFV-1 or 5 FFU of SFV-W per cell. After adsorption for 1 h at 4°C, the unadsorbed inoculum was removed by repeated washings with medium, and the cells were incubated at 37°C. After 1 h of infection, 1 μCi of [$^3$H]TdR per ml was added to the culture. Incorporation of [$^3$H]TdR was terminated by removal of the medium and repeated washings with unlabeled medium. Thereafter, the cells were scraped off the plates, washed with phosphate-buffered saline, and suspended in a solution composed of 0.01 M Tris-hydrochlo-

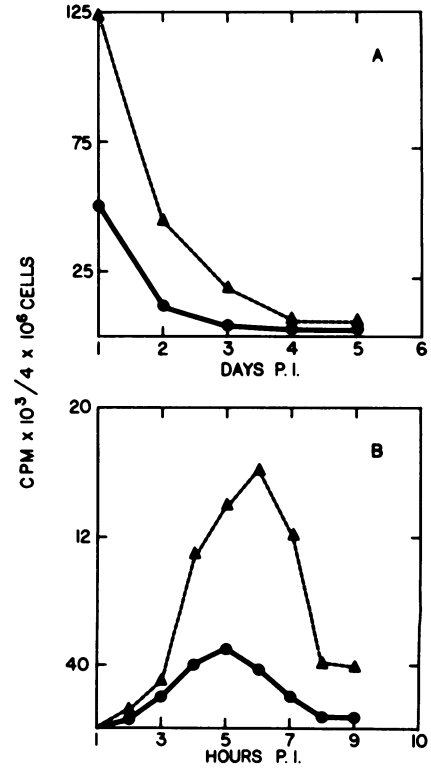


FIG. 2. Synthesis of viral DNA. (A) Cells infected with either 5 PFU of SFV-I or 5 FFU of SFV-W were incubated with 1 μCi of [$^3$H]TdR per ml for 3 h. At the times indicated, the cells were fractionated, the cytoplasmic fraction was isolated, and the acid-insoluble radioactivity was determined. (B) Cells were infected as in (A) 1 h after infection, 1 μCi of [$^3$H]TdR per ml was added to the culture medium. Samples were taken at 1-h intervals and processed as in (A). Symbols: ●, SFV-W; ▲, SFV-I.

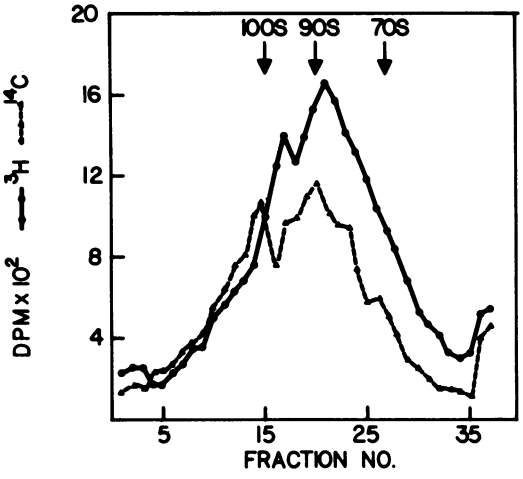


FIG. 3. Sedimentation analysis of SFV DNA. Aliquots of $^3$H-labeled SFV-W DNA and $^{14}$C-labeled SFV-I DNA were mixed, treated with 0.1 N NaOH for 10 min at 37°C, neutralized, and sedimented through 5 to 20% alkaline sucrose gradients in an SW41 rotor at 40,000 rpm for 180 min. Symbols: ●, SFV-W DNA; ▲, SFV-I DNA. Arrows indicate position of vaccinia DNA, sedimented under the same conditions.

ride buffer, pH 7.8, 10 mM KCl, and 1 mM EDTA, maintained for 10 min at 4°C, and then disrupted with a Dounce homogenizer. The cell homogenate was spun down at 800 × *g* for 2 min to sediment the nuclear fraction. The supernatant (cytoplasmic fraction) was brought up to 10% trichloroacetic acid in the cold, and the precipitate was collected on membrane filters. After several washings with trichloroacetic acid, the filters were dried and the radioactivity was measured by scintillation counting.

Preparation of [$^3$H]TdR-labeled virus was carried out as previously described (12). Extraction of DNA from purified virus preparations was also performed according to published procedures (15, 16). Sedimentation analysis of the DNA was accomplished by centrifugation in an SW41 rotor at 40,000 rpm for 3.5 h in alkaline 5 to 20% (wt/vol) sucrose gradients containing 0.9 M NaCl, 0.1 N NaOH, 0.15% Sarkosyl, and 0.001 M EDTA. The gradients were fractionated into approximately 40 fractions; the DNA in each fraction was precipitated with cold trichloroacetic acid, the precipitates were collected on membrane filters, and the radioactivity was determined by scintillation counting.

Hydroxylapatite chromatography of the virion DNA was performed as previously described (15). Adsorption of the DNA to hydroxylapatite was carried out in 0.01 M sodium phosphate at pH 6.9. Elution was performed by increasing the buffer molarity stepwise from 0.05 to 0.35 M. Single-stranded DNA eluted at 0.15 M, and double-stranded DNA eluted at 0.25 M. When necessary, viral DNA was denatured by incubation with 0.1 N NaOH for 10 min at 37°C followed by neutralization with HCl, or by heating at 100°C for 5 min and rapid cooling. Uncoating of the virion DNA was determined by sensitivity of the parental DNA to pancreatic DNase under the conditions described previously (12).

Restriction endonuclease digestions and separation of the fragments by agarose gel electrophoresis were carried out according to procedures already published (13). The restriction enzymes *Bam*I, *Hin*dIII, and *Kpn*I were purchased from Bethesda Research Laboratories and used according to the conditions recommended by the supplier.

Polyacrylamide gel electrophoresis of the virion-dissociated proteins was carried out in 12-cm cylindrical gels containing 10% acrylamide for 3 h at 100 V. The resulting polypeptides were stained with Coomassie blue. Molecular weight standards were purchased from Bio-Rad Laboratories.

## RESULTS

**Effect of SFV on different cells.** Cells of several lines were infected with vaccinia virus, SFV-I, and SFV-W, and the virus growth was monitored by the cytopathic effects. Vaccinia virus produced cytopathic effects in all cells tested. Rapid cytolysis occurred when HeLa, BSC-1, DRK, and Sf1ep cells were infected with SFV-I; L-cell fibroblasts showed no cytopathic effects, and the chorioallantoic membrane of chick embryos showed characteristic pocks. When cells were infected with SFV-W, no cytopathic effects were observed in HeLa cells, L cells, or chorioallantoic membrane. By contrast, BSC-1, DRK, and Sf1ep cells showed foci after 3 or 4 days of infection. These observations indicate that SFV-I is highly cytocidal and that SFV-W induces cell growth.

**Growth of SFV and viral DNA synthesis.** A comparison of the multiplication rates of SFV-I and SFV-W in BSC-1 cells showed that they were very similar (Fig. 1). Both viruses reached maximal infectivity during the first 24 to 48 h of infection, although the assays by which the infections were quantitated were different, as noted in Materials and Methods.

The rate of viral DNA synthesis was also compared. In some experiments, the cells were pulse-labeled for 3 h with [$^3$H]TdR at different

TABLE 1. Hydroxylapatite chromatography of SFV DNA[a]

| DNA type | % cpm eluted as double-stranded DNA | |
|---|---|---|
| | Expt 1 | Expt 2 |
| SFV-I native | 98 | 97 |
| SFV-I denatured | 78 | 73 |
| SFV-W native | 97 | 96 |
| SFV-W denatured | 75 | 70 |

[a] Aliquots of radioactively labeled native or heat-denatured SFV were subjected to hydroxylapatite chromatography as described in Materials and Methods. Double-stranded DNA was eluted with 0.25 M phosphate buffer.

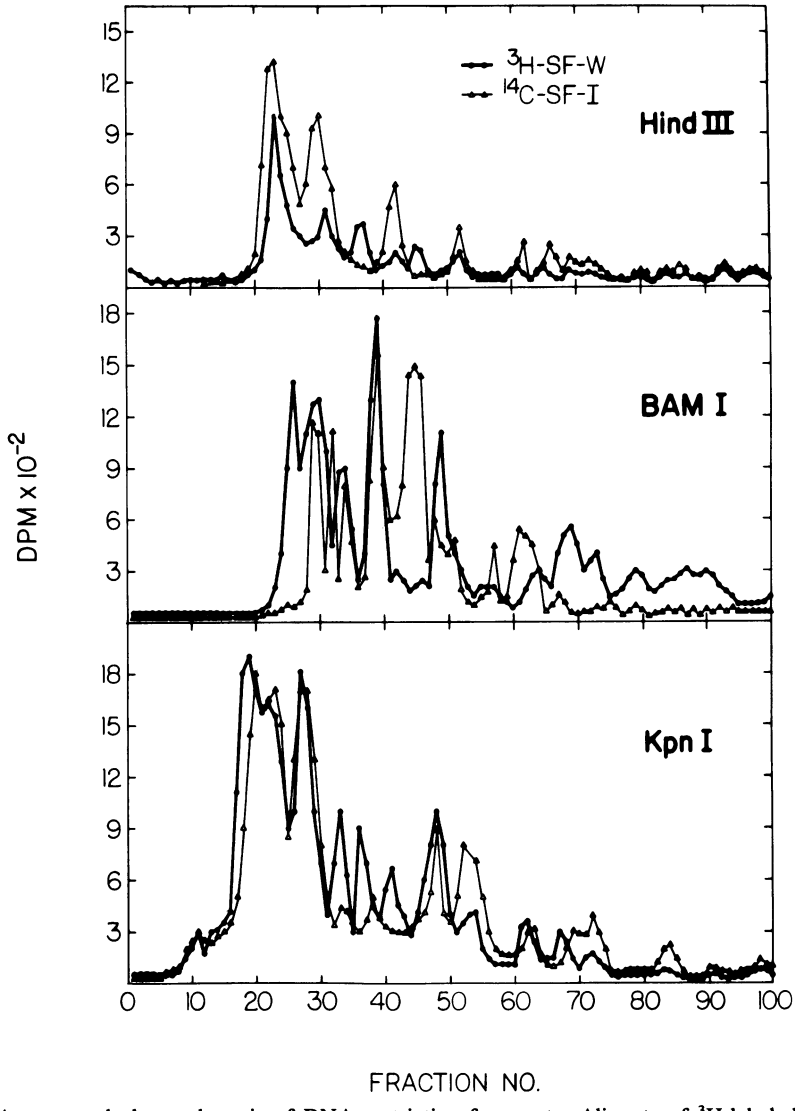


FIG. 4. Agarose gel electrophoresis of DNA restriction fragments. Aliquots of $^{3}$H-labeled SFV-W DNA and $^{14}$C-labeled SFV-I DNA were mixed and incubated with three restriction endonucleases, *Hin*dIII, *Bam*I, and *Kpn*I, independently. After 3 h of incubation at 37°C, the digests were electrophoresed in 0.8% agarose gels in cylindrical tubes for 17 h at 20 V and for 2 h at 50 V. The gels were fractionated into 100 slices, and the radioactivity was determined in each fraction. Arrows indicate the main differences among the genomes.

days after infection, and the radioactivity was measured in the cytoplasmic fraction (Fig. 2A). It is clear that the viral DNA synthesis occurred mainly during the first 24 h of infection for both strains. Cells infected with SFV-I, however, incorporated twice the amount of [$^{3}$H]TdR as cells infected with SFV-W.

Figure 2B shows the incorporation of [$^{3}$H]TdR into cytoplasmic fractions during the first 8 h of infection. The [$^{3}$H]TdR was added 1 h after infection, and samples were taken at 1-h intervals. The kinetics of incorporation were similar for the two viruses, but the amount of [$^{3}$H]TdR incorporated was three times higher in cells infected with SFV-I.

**Uncoating of viral DNA.** To find out whether the low incorporation of [$^{3}$H]TdR by SFV-W-infected cells was due to inefficient virus uncoating, the efficiency of uncoating by both virus strains was also compared. The results indicated that SFV-W parental DNA was sensitive to nuclease digestion to the same extent as SFV-I DNA or vaccinia virus DNA (data not shown).

**Characterization of the viral DNA.** To estab-

lish the molecular weight of the SFV genome, the DNA extracted from TdR-labeled virions was sedimented through alkaline sucrose gradients. Equal portions of SFV-W DNA labeled with [$^3$H]TdR and SFV-I DNA labeled with [$^{14}$C]TdR were mixed and run in the same gradient (Fig. 3). Most of the viral DNA sedimented at 106 to 90S as compared with vaccinia virus DNA, indicating that under denaturing conditions they were in the form of circles or circles with a nick (5). On the basis of these results, the molecular weight of SFV DNA was calculated to be between $120 \times 10^6$ and $130 \times 10^6$, with SFV-I DNA sedimenting slightly faster than SFV-W DNA. The presence of cross-links at the terminal ends of the DNA molecules of both viruses is supported by this sedimentation behavior. Furthermore, the elution pattern of denatured DNA in hydroxylapatite chromatography also indicated the presence of cross-linked molecules (Table 1). After denaturation, cross-linked molecules "snap back" and behave as double-stranded molecules. As shown in Table 1, 70 to 78% of the viral DNA, after denaturation, eluted as double-stranded DNA.

**Restriction endonuclease analysis.** Analysis of the viral genome by three restriction endonucleases was carried out by digesting the $^{14}$C-labeled SFV-W DNA and the $^3$H-labeled SFV-I DNA together and separating the restriction fragments

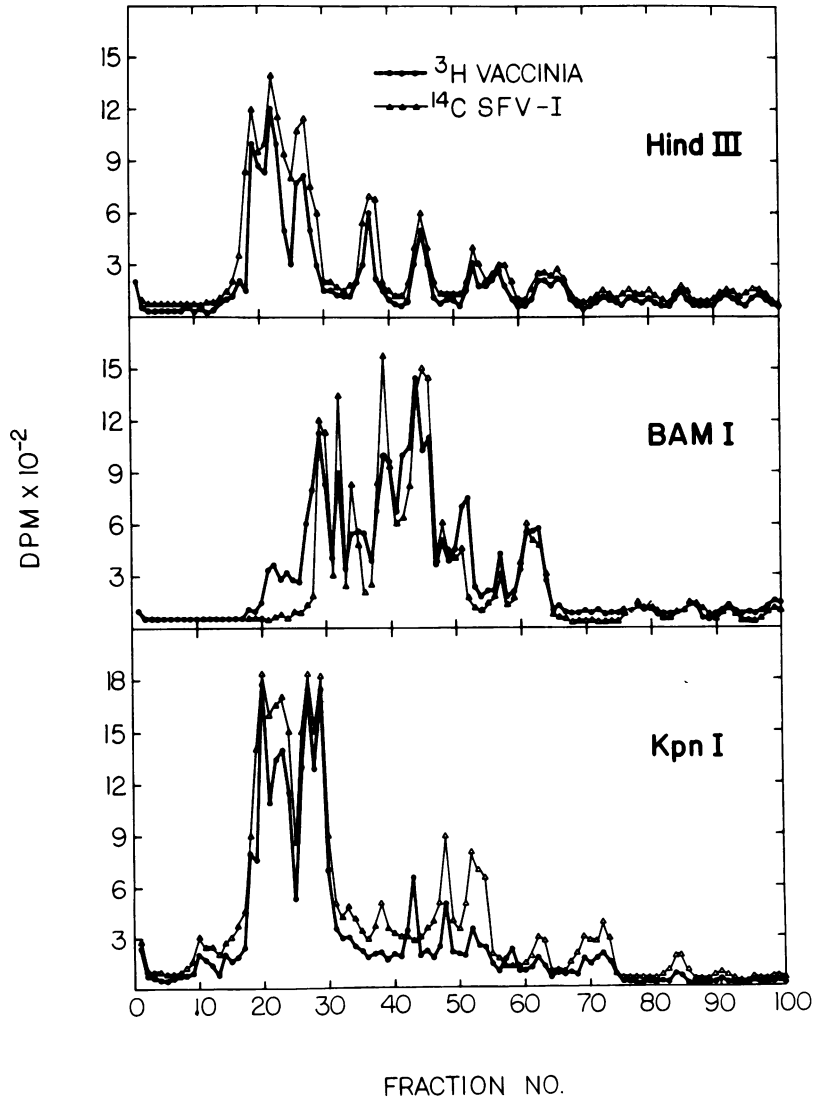


FIG. 5. Agarose gel electrophoresis of DNA restriction fragments. Aliquots of $^3$H-labeled vaccinia DNA and $^{14}$C-labeled SFV-I DNA were treated as described in Fig. 4.

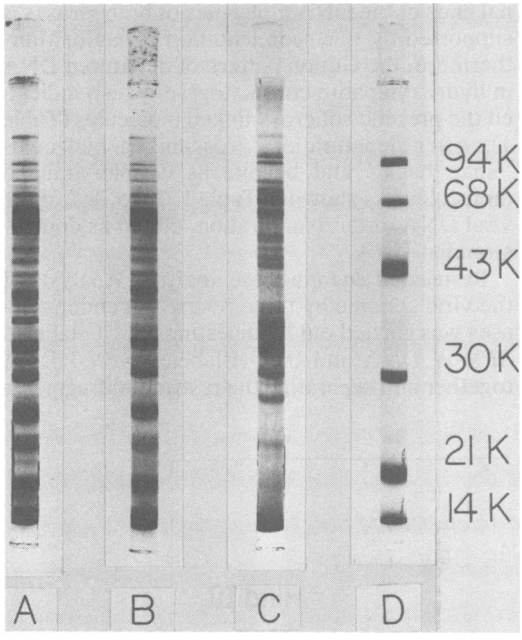


FIG. 6. Polyacrylamide gel electrophoresis of virion proteins. Aliquots containing 100 μg of viral proteins were dissociated with 0.1% sodium dodecyl sulfate and 0.05% β-mercaptoethanol at 100°C for 5 min, electrophoresed in 10% acrylamide gels for 3 h at 100 V, and stained with Coomassie blue. (A) Vaccinia virus; (B) SFV-I; (C) SFV-W; (D) molecular weight markers (from top to bottom: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

by agarose gel electrophoresis (Fig. 4). It is clear that SFV-I and SFV-W had different restriction sites, since there were changes in the number and size of the fragments when the viruses were subjected to digestion with different enzymes. For comparison, SFV-I and $^3$H-labeled vaccinia virus DNA were also analyzed (Fig. 5). There seemed to be homology between SFV-I and vaccinia virus DNAs when digested with *Hin*dIII; however, there were distinctive differences when *Bam*I or *Kpn*I was used.

**Virion proteins.** The structural proteins of the two SFV virus strains were compared with those of vaccinia virus by polyacrylamide gel electrophoresis. SFV-I, SFV-W, and vaccinia virus had many polypeptides in common (Fig. 6); however, each virus had distinctive polypeptides which were either of different molecular weight or not present.

**Effect of antiserum on virus infectivity.** Table 2 summarizes the results of the plaque neutralization tests with three antisera. Vaccinia antiserum was able to reduce the plaque efficiency of the homologous virus, vaccinia, by 2 logs and of SFV-I by 2 to 3 logs, and it did not affect the focus production of SFV-W. In contrast, SFV-I antiserum had no effect on the plaque efficiency of vaccinia virus, although it reduced growth of the homologous virus and the growth of the SFV-W by 2 to 3 logs. Antiserum against SFV-W was effective against both strains of SFV, but it did not inhibit vaccinia virus growth. These results have been confirmed by using diluted preparations of vaccinia virus and SFV-I.

## DISCUSSION

Our results indicate that there are striking differences in the biological and molecular properties of the two strains of SFV analyzed. Their distinctive behavior in tissue culture, one strain producing a cytocidal infection and the other stimulating cell growth, is paralleled by differences in the structure of the genome and the virion proteins. The activities of some of the virion-associated enzymes have also been found to be different (17). We found, however, some similarities between SFV-I and SFV-W: (i) both strains induced viral DNA synthesis at the same time after infection; (ii) the sizes of the viral DNAs were found to be similar, about $120 \times 10^6$ daltons (it should be noted that this finding is not in disagreement with a previous report that estimated the molecular weight of SFV DNA as $153 \times 10^6$ when compared with vaccinia DNA [9], since the molecular weight of vaccinia DNA was then considered to be $160 \times 10^6$); (iii) both SFV strains shared common antigens as revealed by the results of the cross-neutralization tests; and (iv) both induced tumors when inoculated into rabbits.

Of particular interest are the similarities between vaccinia virus and SFV-I. They both produced cytocidal effects on all cells tested, with the exception of the lack of response of L cells to SFV-I. The restriction patterns of their DNAs when treated with *Hin*dIII were also identical, although differences in restriction sites were found with *Bam*I and *Kpn*I. Finally, vaccinia virus antiserum had an inhibitory effect on SFV-I growth, but this effect was not reciprocal with antiserum to SFV-I. This was an unexpected finding because there is no cross-activity

TABLE 2. Effect of antisera on virus infectivity[a]

| Addition | Infectivity titer | | |
|---|---|---|---|
| | Vaccinia (PFU/ml) | SFV-I (PFU/ml) | SFV-W (FFU/ml) |
| None | $1 \times 10^8$ | $5 \times 10^8$ | $1.5 \times 10^5$ |
| Anti-vaccinia (1:20) | $6 \times 10^5$ | $2 \times 10^5$ | $1.5 \times 10^5$ |
| Anti-SFV-I (1:5) | $1 \times 10^8$ | $1 \times 10^5$ | $5 \times 10^4$ |
| Anti-SFV-W (1:5) | $1 \times 10^8$ | $1 \times 10^5$ | $2 \times 10^3$ |

[a] Virus preparations were incubated with serial dilutions of the antisera for 1 h at 37°C and then assayed for infectivity as described in Materials and Methods.

between leporipoxviruses, to which SFV belongs, and the orthopoxviruses, to which vaccinia virus belongs. Taken together, these results suggest that SFV-I may be a recombinant between members of the two genera. A detailed study of the homology of both genomes is necessary to identify the parts of the genome common to both strains and to determine to what extent SFV-I is a recombinant. These studies are now in progress in this laboratory.

Thus, SFV-I has unique biological properties in that it induces cytocidal infections in cell cultures and is able to produce tumors in rabbits. The genes responsible for inducing the cytocidal and neoplastic responses seem to be present and expressed in the same genome. It remains to be determined whether cytocidal genes are also present but repressed in SFV-W and whether the tumorigenic genes are also present in vaccinia virus.

## ACKNOWLEDGMENTS

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