Shope Fibroma Virus

II. Role of the Virion-Associated Nucleases

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The effect of Shope fibroma virus (SFV) infection on host DNA synthesis was investigated. The cytotoxic strain, SFV-I, inhibited the incorporation of [^3H]thymidine into nuclear DNA very shortly (2 h) after infection, whereas the noncytotoxic strain, SFV-W, did so later (10 h postinfection) and to a lesser extent. Furthermore, a two- to threefold stimulation of host DNA synthesis was recorded in SFV-W-infected cells 3 to 4 h after infection. Since virion-associated nucleases have been implicated in the shutoff of host synthesis, these and other enzymatic activities were measured in purified virion preparations. The SFV strains and vaccinia virus contained equivalent amounts of DNA-dependent RNA polymerase, ATPase, and protein kinase activities. However, in SFV-W the pH 4.5 exonuclease activity was lower than in SFV-I and vaccinia virus, and the level of pH 7.8 endonuclease was almost undetectable. To test whether the lack of endonucleolytic activity had some effect on the removal of the cross-links in the parental DNA that occurs after viral penetration, the fate of the virion SFV DNA was followed. The majority (80%) of the SFV-I and SFV-W DNA molecules extracted after viral adsorption sedimented in alkaline sucrose gradients as cross-linked. After 3 h of infection, 75% of the SFV-I DNA molecules lacked cross-links, whereas 78% of the SFV-W DNA still remained cross-linked. The same results were obtained when the presence of cross-links was tested in restriction fragments. Taken together, these results indicate that virion-associated nucleases are involved in the early shutoff of host DNA synthesis and in the elimination of cross-links from the parental viral DNA.

Inhibition of host DNA synthesis by vaccinia virus occurs very promptly after infection and is independent of virus replication (9, 10, 13, 14). However, with Shope fibroma virus (SFV), a leporipoxvirus that causes benign tumors in rabbits, both stimulation and depression of host DNA synthesis have been reported (3, 7, 11, 12, 29). In an effort to resolve this contradiction, the effect of host DNA synthesis by infection with two strains of SFV, one that promotes cell growth and one that lyses cells (26), was studied. Since the virion-associated nucleases have been implicated in the hydrolysis of newly synthesized host DNA (6, 22, 23) and in the removal of cross-links from parental viral DNA (18), the presence of nuclease activities in the virions of both strains was also investigated. The results indicated that there is a correlation between the shutoff of host DNA synthesis and the presence of virion-associated nucleases. Moreover, when nuclease activity is lacking, the cross-links in the parental DNA are preserved.

MATERIALS AND METHODS

Virus and cell culture. Two strains of SFV were used. One, obtained from M. Hodes (Indiana Univer-

sity), was designated SFV-I, and the other, from H. Hinze (University of Wisconsin), was designated SFV-W. Both viruses were grown in BSC-1 cells that were cultured as monolayers in minimal essential medium (Auto-Pow MEM; Flow Laboratories) supplemented with vitamins, sodium pyruvate, nonessential amino acids, and 10% fetal calf serum and maintained at 37°C in a gas mixture of air and CO₂ (26).

Infectivity of SFV-I was measured by the production of plaques in BSC-1 monolayers under agar as described for vaccinia virus (4, 26). SFV-W was measured by counting the number of foci produced in BSC-1 growing in Integrid petri dishes after staining of the cells with Giemsa-Wright stain (26).

To initiate synchronous infection, 5 PFU of SFV-I or 5 focus-forming units of SFV-W per cell was adsorbed to monolayer cultures containing 4×10^6 cells in 5-mm petri dishes for 1 h at 4°C. Unadsorbed virus was removed by repeated washing with medium before incubation at 37°C. After 1 h of infection, 2 μCi of [^3H]thymidine (TdR) per ml was added to the cultures. Incorporation of [^3H]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, suspended in a solution containing 0.01 M Tris-hydrochloride buffer, pH 7.8, 10 mM KCl, and 1 mM EDTA (TEK), and maintained for 10 min at 4°C. Then Nonidet P-40 was added to a 0.5% final concentration, and

the cells were disrupted with a Dounce homogenizer. The cell homogenate was spun down at $800 \times g$ for 2 min to sediment the nuclear fraction. The supernatant or cytoplasmic fraction was discarded, and the crude nuclear fraction was washed once with TEK to remove cytoplasmic contaminants and then spun down at the same speed. The pellet containing the nuclear fraction was resuspended in cold 10% trichloroacetic acid; the precipitate was spun down and washed once with 10% trichloroacetic acid and once with alcohol. The pellet was treated with 0.5 N perchloric acid for 20 min at 70°C. The amount of DNA in this extract was measured by the Burton's procedure (2), and the radioactivity was measured by scintillation counting.

Virion purification and enzymatic reactions. Virus purification was carried out by a procedure similar to that described for vaccinia virus purification (5), involving sonication of the infected cells, differential centrifugation, trypsin digestion, discontinuous sucrose gradients, and continuous gradients of potassium tartrate.

Enzymatic activities in purified virion preparations were measured under the conditions described for DNA-dependent RNA polymerase activity by Kates and McAuslan (15), for ATPase activity by Gold and Dales (8) and Paoletti and Moss (17), for protein kinase by Kleiman and Moss (16), and for the DNases by Pogo and Dales (21).

Infection of cells with labeled virus and extraction of viral DNA. [^3H]TdR- or [^{14}C]TdR-labeled virus was prepared by addition of 0.5 μCi of [^3H]TdR or [^{14}C]TdR per ml to the culture medium of infected cells from 2 to 8 h after infection, when viral DNA synthesis was maximal. At the end of this period the isotope was removed, and the cells were incubated in normal medium for an additional 16 h for SFV-I and 72 to 96 h for SFV-W. Then the cells were harvested and the virus was purified as described above.

The conditions for infection of cells with ^3H -labeled virus, preparation of cell fractions, and extraction of parental DNA were similar to those previously described for vaccinia virus-infected cells (17, 18). Parental viral DNA was extracted from cytoplasmic fractions, sedimented through alkaline sucrose gradients (17), or digested with *Hind*III, and the restriction fragments were separated by agarose gel electrophoresis as described previously (18).

RESULTS

Host DNA synthesis in virus-infected cells. The rate of host DNA synthesis was measured in virus-infected and control cells by incorporation of [^3H]TdR into the nuclear fraction after pulse-labeling for 15 min (Fig. 1). In cells infected with SFV-I, inhibition was observed by 3 h and was complete by 12 h. The cells were lysed by 48 h. By contrast, in cells infected with SFV-W, no inhibition of host DNA synthesis occurred during the first 10 h of infection. By 12 h, a 30% inhibition was observed which reached 50% after 16 h and remained at this level for 24 h. After 48 h, however, the cells appeared to recover; the host DNA synthesis returned to the

FIG. 1. Inhibition of host DNA synthesis after infection. Monolayers containing 8×10^6 cells were infected with 5 PFU of SFV-I or 5 focus-forming units of SFV-W. At the times indicated, 1 μCi of [^3H]TdR per ml was added to the culture medium, and the cells were incubated for 15 min at 37°C. Nuclear fractions were prepared as described in Materials and Methods, and DNA synthesis was measured by the amount of [^3H]TdR incorporated per microgram of DNA. The results are expressed as percentage of inhibition over the control nuclei. Symbols: Δ , Cells infected with SFV-I; $\bullet$, cells infected with SFV-W.

level of the control and by 72 to 96 h had further increased two- to threefold (data not shown).

Virion-associated enzymes. Since nucleases had been implicated in the shutoff of host DNA synthesis, a comparative study of these activities as well as those of other enzymes known to be present in poxviruses virions was carried out (Table 1). Both SFV-I and SFV-W strains had equivalent activities of ATPase, DNA-dependent RNA polymerase, and protein kinase. However, in SFV-W the pH 4.5 exonuclease activity was lower than in SFV-I, and the level of the pH 7.8 endonuclease was almost undetectable. By contrast, SFV-I, the cytotidal strain, contained nuclease activities in amounts close to those found in vaccinia virus virions. Attempts to activate the pH 7.8 endonuclease from SFV-W virions by means of trypsin or heat (25) yielded negative results.

Fate of the parental DNA after infection. Experiments with labeled vaccinia DNA suggested that the virion-associated endonuclease activity was involved in the elimination of cross-links of viral DNA after viral penetration into cells (17). As previously shown, the DNA of both strains of SFV contained cross-links as judged by sedimentation through alkaline sucrose gradients and hydroxylapatite chromatography (26). Therefore, the fate of SFV parental DNA after infection was studied. Table 2 summarizes the

TABLE 1. Enzymatic activities in purified SFV^a

Expt no.	Condition	ATPase activity (pmol of ATP hydrolyzed/ μg of protein)			RNA polymerase activity (pmol of UMP incor- porated/μg of protein)			Protein kinase activity (pmol of acid-insoluble ATP/μg of protein)			DNase activity (μg of DNA hydrolyzed /mg of protein)		
		Vac- cinia	SFV-I	SFV-W	Vac- cinia	SFV-I	SFV-W	Vac- cinia	SFV-I	SFV-W	Vac- cinia	SFV-I	SFV-W
1	-ssDNA	100.0	78.0	78.0									
	+ssDNA	130.0	162.0	85.0									
2	-ssDNA	36.0	65.0	74.0									
	+ssDNA	74.0	126.0	95.0									
1					2.6	3.9	4.5						
2					2.0	2.4	3.9						
1	15 min							5.8	5.9	5.2			
2	30 min							15.2	15.9	14.7			
1	pH 4.5										500.0	560.0	36.0
	pH 7.8										354.0	450.0	2.8
2	pH 4.5										320.0	360.0	24.0
	pH 7.8										270.0	260.0	0.5

^a Aliquots containing 10 μg of purified virion protein were incubated with the appropriate buffers and substrates, as described in Materials and Methods, to measure enzymatic activities.

TABLE 2. Distribution of labeled parental DNA in alkaline sucrose gradients^a

Time (h) after infection	Size	% cpm		
		SFV-I	SFV-W	Vaccinia virus
0	106S-90S	80	80	100
	68S	20	20	0
2	106S-90S	51	84	10
	68S	49	16	90
3	106S-90S	25	78	
	68S	75	22	

^a Cells were infected with [³H]TdR- or [¹⁴C]TdR-labeled SFV-I or SFV-W virions. At the time indicated, cytoplasmic fractions were prepared, and the viral DNA was extracted as described in Materials and Methods. The DNA was sedimented through 5 to 20% alkaline sucrose gradients in an SW41 rotor at 40,000 rpm for 180 min. The distribution (percentage) of labeled DNA into the different peaks of the gradient was then calculated. The data for vaccinia virus DNA were taken from reference 18.

distribution of faster-sedimenting (90S to 106S; cross-linked) and slower sedimenting (68S; non-cross-linked) molecules in alkaline sucrose gradients to DNAs extracted at different times after infection with SFV-I and SFV-W. For comparison, the results of a previous study carried out with vaccinia virus DNA (18) are also included. The majority (80%) of the SFV-I and SFV-W DNA molecules extracted immediately after adsorption sedimented as cross-linked molecules. After 2 h of infection, half of the DNA molecules of SFV-I were cross-linked, whereas SFV-W remained the same. After 3 h of infection, 75% of the SFV-I DNA molecules were converted to slower-sedimenting species (non-cross-linked); by contrast, 78% of the SFV-W molecules remained cross-linked.

Since the changes in sedimentation rate could be accounted for by nicks or gaps and not necessarily by the loss of cross-links, the DNAs were digested with restriction endonuclease, and the presence of cross-links was determined after denaturation by the "snap-back" fragments (19) (Fig. 2 and 3). Figure 2 shows the analysis of the three larger fragments (A, B, and C) of the *Hind*III digest of the SFV-I DNA, as separated by agarose gel electrophoresis. Fragments B and C are the terminal fragments containing the cross-links as shown by their ability to snap back after denaturation. In Fig. 2a, the DNA extracted from the virion showed the two fragments after denaturation. After adsorption to the cells, the parental DNA also contained the cross-links (Fig. 2b), but by 3 h after infection only a small proportion of fragment B contained the cross-links (Fig. 2c).

Similar experiments were carried out with DNA from SFV-W. In Fig. 3a, the three large

fragments of the *Hind*III digest of virion DNA are represented. The presence of cross-links in fragments B and C could be demonstrated after denaturation. The same results were obtained when the DNA was extracted after adsorption to

FIG. 2. Fractionation of parental SFV-I DNA restriction fragments by agarose gel electrophoresis. Duplicate portions of ³H-labeled parental DNA were incubated with 5 U of *Hind*III in a mixture containing 20 mM Tris-hydrochloride, pH 7.4, 7 mM MgCl₂, 60 mM NaCl, and 25 µg of bovine serum in 50 µl. After 2 h at 37°C, one set of samples was heated at 60°C for 5 min, and 10 µl of a solution containing 0.15% bromophenol blue, 60% sucrose, and 0.1 M EDTA was added. The other set of samples was treated with 0.1 N NaOH for 15 min at 37°C and neutralized with 0.1 N HCl, and the dye solution was added. Both sets of samples were applied to 12-cm cylindrical gels and electrophoresed for 16 h at 20 V and for 2 h at 50 V. The gels were sliced into 100 fractions and incubated with 0.5 ml of *N,N'*-dimethylformamide for 2 h at 40°C. Radioactivities were determined in a scintillation counter, using 5 ml of Biofluor (New England Nuclear Corp.). Only the three largest fragments of the digest are represented. Parental DNA extracted at (a) time zero; (b) after 1 h of infection; (c) after 3 h. Symbols: ●, *Hind*III digest; ○, *Hind*III digest denatured with alkali.

FIG. 3. Fractionation of parental SFV-W DNA restriction fragments by agarose gel electrophoresis. Experimental conditions were similar to those for Fig. 2. Parental DNA extracted (a) at time zero; (b) after 1 h of infection; (c) after 3 h. Symbols are as in Fig. 2.

cells (Fig. 3b). Only fragment C seemed to have lost some of the cross-links 3 h after infection (Fig. 3c). These results are in good agreement with those obtained by sedimentation through alkaline sucrose gradients (Table 2).

DISCUSSION

The results reported here confirm the correlation between the shutoff of host DNA synthesis and the presence of nuclease activities in virions. Infection with the cytotidal strain of SFV results in the prompt arrest of host DNA synthesis, in a manner similar to that which occurs after infection with vaccinia virus. By contrast, infection with the noncytotidal strain affects

host DNA synthesis at a later time, to a lesser degree, and only temporarily. In addition, stimulation of host DNA synthesis is detected 3 days after infection with SFV-W, as has been reported by others (11, 12, 29). The differences in the host response to the two strains do not seem to be related to the uncoating or replication of viral DNA, since these processes take place at the same time with the two viruses (26); rather, they seem related to the content of nuclease activity in virions. Virion-associated nucleases have been found in different genera of poxviruses (1, 21, 24, 28), and one of them, the pH 4.5 activity, has been purified from vaccinia virus virions (27). Both enzymes are also present in the cytotidal strain of SFV; however, in the noncytotidal strain, the pH 4.5 exonuclease is low and the pH 7.8 enzyme is almost undetectable. The release of virion endonuclease in the soluble fraction of vaccinia virus infected shortly after penetration has been described and correlated with the early inhibition of host DNA synthesis (20). This early inhibition is not observed in cells infected with the noncytotidal strain that lacks endonuclease activity in virions. The inhibition that occurs later, 10 h after infection, may be caused by the arrest of host protein synthesis that follows virus replication.

Another postulated function for the virion-associated nucleases is the elimination of the cross-links of parental viral DNA before synthesis (18, 19). The lack of endonucleolytic activity in SFV-W resulting in preservation of most of the cross-links in the viral genome can explain the relative poor efficiency of replication of this strain (26). Insufficient nuclease activity may result in less available functional templates for replication or transcription.

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