INFECTION AND IMMUNITY, Apr. 1973, p. 532-538
Vol. 7, No. 4
Printed in U.S.A.
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Mechanism of Shope Fibroma Virus-Induced Suppression of Host Deoxyribonucleic Acid Synthesis

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Received for publication 13 October 1972

The effects of treatment with live or inactivated Shope fibroma virus on host cell deoxyribonucleic acid (DNA) synthesis were determined. The incorporation of ^{3}H-thymidine into nuclear DNA was suppressed by both active and inactivated virus, although live virus was more effective. During the early phase of infection, stimulation of host nuclear DNA synthesis of up to 240% of control value was observed in cells infected with active virus. Inhibition of DNA synthesis began at about the 8th h and was maximal by 12 h postinfection. Virus inactivated by ultraviolet-irradiation or heat treatment did not induce viral DNA synthesis but was, nevertheless, able to suppress host DNA synthesis.

Poxviruses generally cause cell destruction and a decrease in host nuclear deoxyribonucleic acid (DNA) synthesis while replication of the viral DNA takes place in the cytoplasm. Some poxviruses induce tumors and can transform cells (12), attributes usually ascribed to oncogenic DNA viruses. These latter are known to stimulate cellular DNA synthesis. Although previous work from this laboratory suggests that infection with Shope fibroma virus (SFV) causes decreased synthesis of host nuclear DNA (2, 3), the studies of Tompkins et al. (17) suggest that SFV induces DNA synthesis in contact-inhibited X-irradiated rabbit kidney cells. We report here a reexamination of some of the facets of this problem, with particular emphasis on the possible mechanism of virus inhibition of nuclear DNA synthesis. Distinction is made between the possibility that the inhibitor of host DNA synthesis is induced by the virus and the possibility that the inhibitor is brought in by the virus. By rendering the virus inactive either by ultraviolet (UV) irradiation or heat treatment, it was found that under conditions where no viral DNA synthesis was induced, host DNA synthesis was still suppressed.

MATERIALS AND METHODS

Virus. The virus was a gift from Z. Zakay-Rones of the Hebrew University Medical School. It was reportedly Shope fibroma virus obtained from Ella Israeli, then at the Weizmann Institute of Science (20). The virus was passed once in primary rabbit kidney cells and an undetermined number of times in monkey kidney cells (BSC-1) by Z. Zakay-Rones. It was passed twice in BSC-1 cells by us. The virus produces visible plaques and cytoplasmic inclusions in BSC-1, HeLa, and rabbit kidney cells. In our hands, the virus produced an inflammatory lesion in rabbits and plaques in cultured cells within 48 h. Plaque formation was inhibited by both anti-SFV (Patuxent) serum and vaccinia-immune globulin. Its growth and appearance on electron microscopy are typical of a poxvirus. Stock virus was produced in BSC-1 cells. After 48 h of incubation at 36 C, infected cells were scraped and homogenized with a Virtis homogenizer, and the cell debris was removed by centrifugation at 1,000 × g for 10 min. The supernatant fluid was then ampouled and stored frozen at −90 C.

Virus purification. Purification of virus was based on the method of Joklik (5), with slight modification. Crude virus (low-speed supernatant fluid of cell lysate) was first pelleted through 5 ml of 36% (wt/vol) sucrose in 0.01 M NaCl buffered with sodium phosphate, pH 8.5 (PBS) (1), by centrifugation in rotor SW25.1 at 58,000 × g for 2 h. The pellet was resuspended in 0.001 M PBS (pH 8.5) and centrifuged in the same rotor through a 24-ml linear sucrose density gradient (15–80% wt/vol in 0.01 M PBS, pH 8.5) at 28,000 × g for 25 min. The virus band, clearly visible at about 2 cm from the bottom of the tube, was collected with a syringe from the top. Sucrose was removed either by dialysis against 0.001 M PBS (pH 8.5) or by first diluting the virus with 0.001 M PBS (pH 8.5) and then pelleting at 90,000 × g for 1 h.

Assay of virus infectivity. (i) Plaque assay:

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BSC-1 cells were grown on cover slips in Leighton tubes. Virus dilutions (0.5 ml/tube) were adsorbed onto cells for 2 h and unadsorbed virus was removed by washing. Cells were refed with 2 ml of medium and further incubated at 36 C for 48 h. Cells were fixed with methanol and stained with 0.1% crystal violet. The clearly visible plaques were then counted. (ii) Cytoplasmic inclusion body technique was performed essentially as previously described (4). Infected cells were stained with May-Grünwald-Giemsa stain at 20 h postinfection (PI).

Infection and pulse labeling. HeLa cell or LLC-RK_1 cell monolayers in Falcon flasks (25 cm^2) were infected with virus at a multiplicity of infection (MOI) of approximately 1 or 5 plaque-forming units (PFU) per cell. After virus adsorption at 36 C for 2 h, unadsorbed virus was removed by washing with medium. Cells were fed with 5 ml of medium and further incubated. Uninfected controls were treated with an equal volume of medium alone. At different times after infection, two flasks from each group were pulse labeled for 1 h with 5 μCi of ^{3}H-thymidine per ml (Amersham, Searle; specific activity = 14.1 Ci/mmol). Next, the cells were thoroughly washed four times with cold isotonic PBS (pH 7.4) and frozen until ready for processing.

Cell fractionation. Fractionation of HeLa cells into cytoplasm and nucleus was by the methods of Vesco and Penman (18). Cells were scraped off the glass with a rubber policeman into 5 ml of isotonic PBS (pH 7.4) and centrifuged at 1,000 $\times$ g for 10 min. The cell pellet was resuspended in 5 ml of hypotonic reticulocyte standard buffer (0.01 M tris(hydroxymethyl)aminomethane [Tris][pH 7.4], 0.01 M KCl, 0.0015 M $MgCl_2$) and allowed to swell for 10 min. Nonionic detergent, NP_{40} (Shell Oil Co.), was added to a final concentration of 0.5%, and the cell suspension was then agitated for 30 s with a Vortex mixer. The cells were ruptured with the release of the cytoplasm and without damage to the nucleus. Cell fractionation was monitored with a phase microscope. The nuclei were spun down at 1,000 $\times$ g for 3 min. The supernatant fluid containing cytoplasmic material was transferred and the nuclear material was resuspended in 5 ml of a buffer containing 0.5 M NaCl:0.01 M Tris (pH 7.4) and briefly sonicated with a Bronson sonifier at a current of 2 A. One milliliter each of cytoplasmic or nuclear suspension was precipitated with cold 5% trichloroacetic acid, filtered onto a Millipore filter (23 mm), washed with 5% trichloroacetic acid in 95% ethanol, and dried. The amount of radioactivity was determined by counting in a Packard liquid scintillation counter. The scintillant used was 0.4% BBOT in toluene.

Cells. HeLa cells, BSC-1 cells, or LLC-RK_1 cells were grown in medium 199:5% fetal calf serum (medium) and were subcultured weekly. Cells were grown in 16-oz prescription bottles (ca. 473 ml), in 25-cm^2 Falcon plastic flasks, or on cover slips (16 by 100 mm) in Leighton tubes.

Autoradiography. For autoradiography, the technique of Kato et al. (11) was modified. HeLa cells (300,000/tube) were grown to a monolayer on glass cover slips (16 by 100 mm) in Leighton tubes. Virus infection was carried out essentially as described earlier. At different times after infection, two cover slips from each group were labeled for 1 h with 0.1 μCi of ^{3}H-thymidine in 0.5 ml of medium and washed three times with 2 ml of ice-cold PBS (pH 7.4). Cells were fixed with methanol for 10 min and washed once in 2 ml of 2% perchloric acid at 4 C for 45 min to remove unincorporated label. The cover slips were then dipped in Kodak NTB-4 nuclear track emulsion prewarmed to 48 C, dried, and exposed in the dark for 3 days. Cover slips were then developed in Kodak D19 developer foı 90 s, washed in water containing a few drops of acetic acid, and fixed. The cover slips were stained with May-Grünwald-Giemsa stain. The number of labeled nuclei with an average of 20 or more grains per nucleus per 20 microscopic fields was counted under oil immersion.

RESULTS

Viral DNA synthesis in HeLa cells. As shown in Fig. 1A, cytoplasmic DNA synthesis in HeLa cells infected at a MOI of 1 PFU/cell was already active at 4 h PI, peaked around 6 h and sharply declined after 6 h PI. No comparable synthesis was observed in mock-infected cells.

Host nuclear synthesis in HeLa cells. In the nuclei of the cells of Fig. 1A, there was an enhanced incorporation of ^{3}H-thymidine (about 130 to 170% of the mock-infected control in this and other experiments) at 4 and 6 h PI (times concomitant with active viral DNA synthesis) (Fig. 1B).

Beginning at 8 h PI, a sharp suppression of host DNA synthesis was observed. By 24 h PI, the rate of nuclear DNA synthesis in the infected cells had declined to a level equivalent to approximately 25% of the mock-infected control.

Cytoplasmic and nuclear DNA synthesis in rabbit kidney cells. To check whether the early increase in nuclear DNA synthesis was unique to infected HeLa cells, a different cell line, LLC-RK1 (rabbit kidney cells), was used. Experiments were carried out as in HeLa cells, and the MOI was 1 PFU/cell. DNA synthesis after infection is summarized in Fig. 2. Again the pattern of cytoplasmic viral DNA synthesis was found to be similar to that in HeLa cells (Fig. 2A). Early increase in nuclear DNA synthesis at about 4 and 7 h PI was also noted. Furthermore, the extent of increase was significantly higher (about 240% of the mock-infected control) (Fig. 2B). This was followed by a more gradual suppression of nuclear DNA synthesis than that observed in HeLa cells in Fig. 1B.

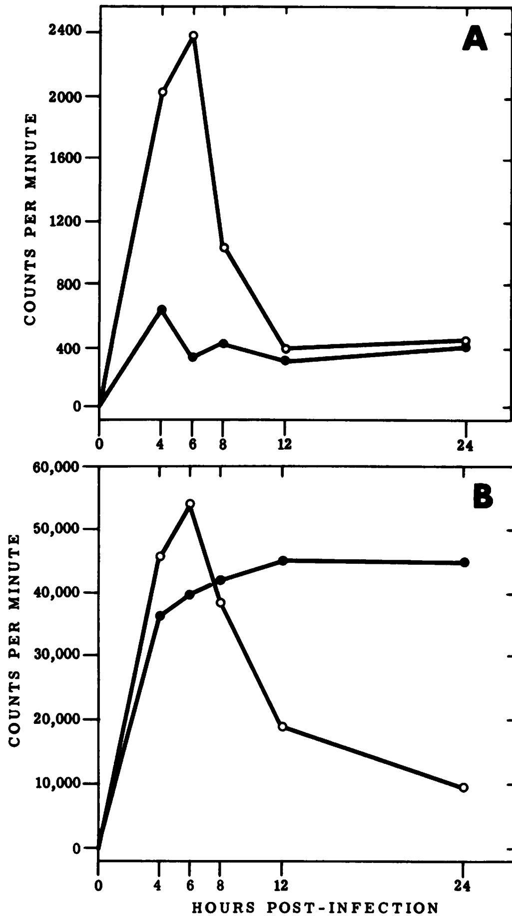

FIG. 1. *A, Viral (cytoplasmic) and B, host (nuclear) DNA synthesis in SFV-infected HeLa cells. Cells were infected at an MOI of 1 PFU/cell with live virus (○) or were mock-infected (●) and pulse labeled for 1 h with 5 μCi of ³H-thymidine per ml. Time on graph refers to beginning of pulse. The cells were fractionated into cytoplasm and nucleus by a detergent method. One milliliter (of the total 5 ml) of each sample was precipitated with trichloroacetic acid and counted. Cytoplasmic and nuclear counts per minute are directly comparable.*

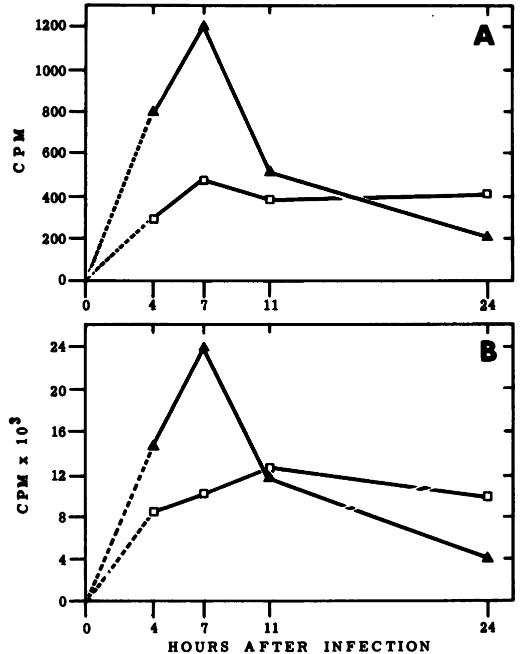

FIG. 2. *A, Viral (cytoplasmic) and B, host (nuclear) DNA synthesis in SFV-infected RK_1 cells. Cells were infected at an MOI of 1 PFU/cell with virus (▲) or were mock-infected (□), and pulse labeled for 1 h with 5 μCi of ³H-thymidine per ml. Cell fractionation was performed as in Fig. 1.*

Ability of inactivated virus to suppress host DNA synthesis. In separate tests, it was established that UV irradiation for 2 min or heating at 60 C for 15 min reduced the titer from 27.5×10^6 PFU/ml to 0 at 1:10 dilution. In this experiment, crude virus was diluted to contain 1 PFU/cell. One portion of the virus was exposed to a UV germicidal lamp (Labcono Hood) for 2 min, another portion was heated in a 60 C water bath for 15 min, and the remainder was used as live virus. HeLa cells were infected with these preparations and pulse labeled as previously described, and the cytoplasmic and nuclear DNA synthesis was measured. Again, live virus-induced cytoplasmic DNA synthesis which peaked at 6 h was seen (Fig. 3A). In this experiment, there was also an early increase in the amount of cytoplasmic DNA synthesis in the other three groups (mock-infected, UV virus-infected groups, and the group infected with heated virus). This could be due to factors such as medium change, cell cycle, etc. However, neither UV nor heated virus induced a significantly higher amount of cytoplasmic viral DNA synthesis than the mock-infected control. This confirmed separate experiments which indicated that either treatment completely inactivated viral infectivity. At an MOI of 1 PFU/cell, UV-inactivated virus rapidly suppressed nuclear DNA synthesis in HeLa cells (Fig. 3B). The rate of nuclear incorporation of ³H-thymidine in cells infected with live virus was again higher at 6 h PI (about 170% of the uninfected control), but from 8 h onward there was a gradual suppression. Heat-inactivated virus produced only a partial suppression of nuclear DNA synthesis at this

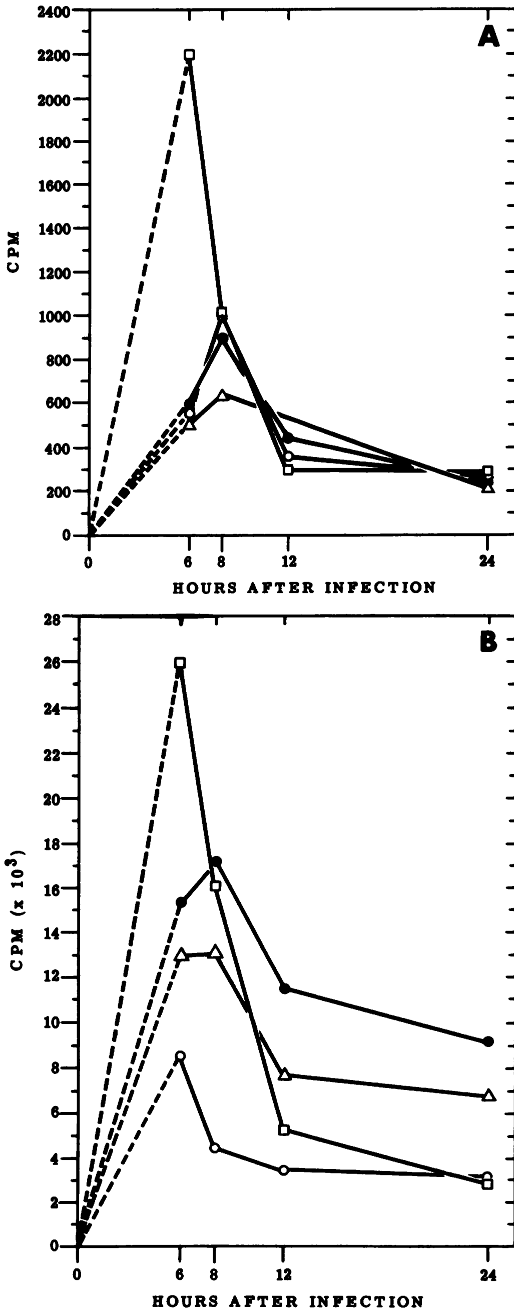

FIG. 3. *Ability of UV- or heat-inactivated SFV to suppress host nuclear DNA synthesis (B) in the absence of viral (cytoplasmic) DNA synthesis (A) in infected HeLa cells. Cells were infected with crude live (□), UV-inactivated (○), or heat-inactivated (△) virus at an MOI of 1 PFU per cell, or they were mock-infected (●). Cell fractionation was performed as in Fig. 1.*

MOI. The ability of inactivated crude virus to suppress host DNA synthesis was confirmed by another experiment in which HeLa cells were treated with UV- or heat-inactivated virus and were labeled with ^{3}H-thymidine between 4 and 24 h PI to measure total DNA synthesis. Results summarized in Table 1 indicate that under conditions where no viral DNA synthesis took place, the amount of total ^{3}H-thymidine incorporated into nuclei was significantly suppressed in cells treated with UV-inactiviated virus and was suppressed to a lesser extent with heat-inactivated virus. In the case of live virus-infected cells, the total amount of ^{3}H-thymidine incorporated during this period was about the same as in the uninfected control. This can be explained by the fact that the inhibitory effect was probably offset by the early stimulation of nuclear DNA synthesis seen previously in Fig. 1B and 2B. As stated previously, the reasons for the early increase in nuclear incorporation of ^{3}H-thymidine induced by live virus are not clear.

Autoradiographic studies with purified virus. To further confirm the previous results and to ascertain whether the inhibitor of the host DNA synthesis was associated with the virus particle, the experiments were repeated but autoradiography was used to measure DNA synthesis, and virus purified in a sucrose density gradient was substituted for crude virus. The autoradiographic technique was used so that the MOI could be increased to 5 without requiring a large amount of purified virus. Results sum-

TABLE 1. *Effect of live and inactivated SFV on host DNA synthesis in HeLa cells*

Treatment	^{3}H-thymidine incorporated (4–24 h after infection)[a]	
	Cytoplasmic (counts/min)	Nuclear (counts/min)
Infected with live virus[b]	14,006	41,494
Infected with UV virus[b]	850	17,356
Infected with heated virus[b]	1,187	23,884
Mock-infected	812	41,425

[a] After treatment with virus for 4 h, cells were washed and refed with medium containing 1 μCi of ^{3}H-thymidine per ml and further incubated for 20 h at 36 C. Cells were fractionated into cytoplasm and nucleus as described previously.

[b] Crude virus, MOI = 1 PFU per cell.

TABLE 2. *Nuclear DNA synthesis in SFV-infected HeLa cells (measured by autoradiography)*

Cell treatment	No. of labeled nuclei[a]			
	8[b]	12	24	48
Purified virus (5 PFU/cell)				
Live virus	10 (29.4)[c]	1 (0.16)	1 (0.98)	0 (0)
UV virus	ND[d]	16 (26.2)	20 (19.6)	21 (30)
Heat-intactivated virus	ND	11 (18.0)	20 (19.6)	23 (32.8)
Mock-infected	34 (100)	61 (100)	102 (100)	70 (100)

[a] Two cover slips from each group at the designated times were pulse labeled with 0.1μCi of ^{3}H-thymidine per 0.5 ml, washed, dipped in emulsion, and developed in the dark for 3 days. After staining with May-Grünwald-Giemsa, the number of cell nuclei containing 20 or more grains each were counted in 20 microscopic fields under oil immersion.

[b] Numbers across represent hours after infection.

[c] Figures in parentheses represent percentage of mock-infected control.

[d] ND, not done.

marized to Table 2 indicate that at an MOI of 5, suppression of host DNA synthesis by live virus proceeded more rapidly than previously observed at an MOI of 1 (Fig. 1). At the higher MOI, UV- and heat-inactivated virus both suppressed host DNA synthesis to about the same extent by 12 h PI.

Fate of host DNA. To find out whether host DNA might be degraded as a result of virus infection to provide precursors for viral DNA synthesis, HeLa cells were prelabeled with 1 μCi of ^{3}H-thymidine for 20 h, washed twice, and then infected with virus. Cells were maintained in cold medium for 48 h at 36 C and homogenized in 5 ml of 0.001 M PBS (pH 8.5). Approximately 3 ml of homogenate was layered onto a 24-ml linear sucrose density gradient (15–80% wt/vol in 0.01 M PBS, pH 8.5). After centrifugation at 28,000 × *g* (rotor 25.1) for 25 min, 2-ml fractions were collected from the bottom of the tube. Infectivity of fractions was assayed by the inclusion body assay method. From each fraction, 0.1 ml of material was dried onto a Whatman filter disk (3 mm), washed with cold 5% trichloroacetic acid and 5% trichloroacetic acid in 95% ethanol, and the amount of radioactivity was determined as previously described. As shown in Fig. 4, no significant amount of ^{3}H-thymidine was found to be associated with the virus band, indicating that the ^{3}H-thymidine incorporated into host DNA was not utilized for viral DNA synthesis.

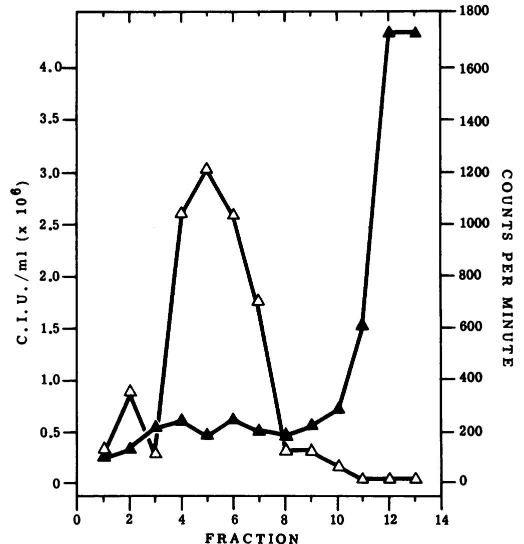

FIG. 4. *Lack of incorporation of ^{3}H-thymidine into viral progeny from prelabeled host cell DNA. HeLa cells were prelabeled with ^{3}H-thymidine and subsequently infected with SFV at an MOI of 1 PFU/cell. After 48 h at 36 C, cells were homogenized and the homogenate was layered onto a linear sucrose density gradient (15–80% wt/vol in 0.001 M PBS, pH 8.5). Centrifugation was carried out at 28,000 × g for 25 min. See Material and Methods for details. Symbols: ▲, counts per minute; △, infecting units.*

DISCUSSION

The effect of a BSC-1 cell-adapted SFV on incorporation of ^{3}H-thymidine into host nuclear DNA is biphasic. During the first 4 to 6 h after infection, which corresponds to the period of viral DNA synthesis, there is an apparent increase in nuclear DNA synthesis. This is of considerable magnitude (about 130 to 170% of mock-infected control in the case of HeLa cells and 240% in the case of RK_1 cells). This increase is followed by a decrease in incorporation by 8 h. The results are similar to those obtained previously with the rabbit-adapted Indiana strain of SFV, although in consonance with its slower growth rate, the peak of stimulation of ^{3}H-thymidine uptake oc-

curs much later (3). Inhibition of host cell DNA synthesis after poxvirus infection has been reported by others (see review by Joklik, reference 6). None of the authors has noted an antecedent increase in nuclear uptake of ^{3}H-thymidine. The closest parallel to our observation is that of Tompkins et al. (17), who noted an early decrease (through day 1) and then a marked increase in cellular DNA synthesis after infection of rabbit kidney cells with the Patuxent strain of SFV. The ability to stimulate host DNA synthesis might be unique to the SFV, a tumorigenic poxvirus. However, thus far no in vitro transformation by SFV has been documented. SFV, therefore, does not behave in vitro as do oncogenic DNA viruses, such as SV_{40} or polyoma viruses.

As to the observed stimulation of host DNA synthesis, several other explanations can be entertained. McAuslan and Smith (13) noted that cytoplasmic DNA from FV-3 infected cells was associated with the nuclear membrane, and in our experiment, some of the cytoplasmic ^{3}H-label could not be removed by the Penman detergent method. However, this explanation is not a likely one in the present case, since previous cytological staining or autoradiographic studies showed that SFV viral DNA synthesis was not associated with the nuclear membrane (4, 16). Furthermore, Joklik and Becker (7) reported that the total amount of vaccinia viral DNA synthesized is equivalent at best to only about 50% of the host cell's DNA complement, whereas an increase of 240% in nuclear ^{3}H-thymidine incorporated was observed in virus-infected RK_1 cells (Fig. 2B). Recently, Walen (19) claimed that poxvirus DNA synthesis begins in the nucleus. This is also an unlikely explanation, since in one of our experiments no precursors from prelabeled nuclear DNA were incorporated into viral DNA (as monitored by autoradiographic and sucrose density gradient techniques). The exact nature of this "stimulation" of early nuclear DNA synthesis remains to be determined.

The phase of suppression of host cell DNA synthesis is accompanied by clear-cut differences in the rate of nuclear DNA synthesis in control and infected cells. These results are essentially in keeping with the findings of previous authors (8–10, 15, 16).

At an MOI of 1, live and UV-inactivated SFV are more effective at suppression of host DNA synthesis than heat-inactivated virus, although as found with cowpox and vaccinia by Jungwirth and Launer (8), inhibition does occur in the latter instance. This difference in the degree of maximum inhibition may be due to the partial sensitivity of inhibitor to thermal denaturation or to poor adsorption after heat treatment (8). Cells treated with live virus or UV-inactivated virus reached the point of maximum inhibition by 12 h after infection.

At an MOI of 5, UV- or heat-inactivated purified virus inhibited nuclear DNA synthesis to about the same extent by 12 h PI. However, live virus suppressed nuclear DNA synthesis to a greater extent. Since neither UV- nor heat-inactivated virus induced viral DNA synthesis, the results could be taken to mean that the inhibitor of nuclear DNA synthesis is a component brought in by the purified virion. The fact that live virus suppressed to a greater extent could be reconciled by the explanation of Magee and Levine (14) that the inhibitor released from the virion and the newly synthesized inhibitor (induced by live virus) synergise their inhibitory effect. The properties of this virion-associated inhibitor remain to be determined.

ACKNOWLEDGMENTS

This investigation was supported by research grants from the Marion County Cancer Society, Inc., Indianapolis, Indiana, the American Cancer Society (institutional grant 57-821-05), and St. Joseph County Cancer Society, Inc., Indiana.

The assistance of D. A. Zavela and E. Jones is acknowledged.

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