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Biogenesis of Poxviruses: Inactivation of Host DNA Polymerase by a Component of the Invading Inoculum Particle

(vaccinia virus/viral DNase)

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ABSTRACT **Inhibition of nuclear DNA polymerase activity in cells infected with vaccinia virus parallels the development of a nuclease activity similar to one associated with the virus particles. Both phenomena occur in the absence of protein synthesis, implying that incoming particles are responsible for the effects observed. Experimental evidence is presented indicating that the nuclear DNA polymerase activity is inhibited coincidentally with, and perhaps as a consequence of, the hydrolysis of nascent, single-stranded DNA molecules. Should this interpretation prove to be correct, our observations may have revealed the first instance in virus cytopathology linking the inactivation of a specific host-cell function with an enzyme activity originating from the invading particle.**

Infection with vaccinia virus promptly inhibits host-cell metabolic activities, particularly DNA and protein synthesis (1–5). Synthesis and transport of nuclear RNA are affected later (6, 7). The inhibitory capability of the inoculum virions is retained by them after ultraviolet (UV) irradiation, but not after heating, implying that cores of the penetrating particles must enter the cytoplasmic matrix to elicit their effect (2, 3). Suppression of cellular DNA reduplication could arise as the indirect consequence of inhibition of protein synthesis of the host after infection with vaccinia virus. Since, however, under the conditions of the infection neither ongoing protein nor RNA synthesis is required for inhibiting host DNA, the evidence also suggests strongly that some constituent of the virion may be responsible (2, 3). Among the virion constituents, the obvious candidates are two DNase activities, previously shown by us to be integral components of the core in vaccinia and other poxviruses (8). Both enzymes hydrolyze single-stranded DNA, one acting as an acidic exonuclease and the other a neutral endonuclease. At about the time cores appear in the cytoplasm, the neutral endonuclease activity separates from the core, is found in the soluble cytoplasmic fraction (9), and in this state presumably becomes available for transfer to the nucleus where it could affect nascent single-stranded DNA molecules. We now present evidence supporting the idea that a DNase originating from the virion is the inhibitory factor affecting host nuclear DNA replication.

MATERIALS AND METHODS

Virus and cell cultures

The IHD strain of vaccinia virus and L2 cells were used in accordance with described experimental procedures (10). To initiate synchronous infection, 20 plaque-forming units (PFU) per cell were added to monolayer cultures containing 10^7 cells in 100-mm petri dishes.

The virus was inactivated by ultraviolet irradiation or heating as described (10) and resulted in a 10^4 reduction of titer. Virus, purified according to described methods, was degraded by steps into envelope-free units and cores (8).

Assays of DNA polymerase and DNase activities

Cells either in confluent monolayers or during their logarithmic growth were removed by scraping, centrifuged into pellets, washed with phosphate-buffered saline (11) and resuspended in a solution containing 10 mM Tris·HCl (pH 7.8)–1 mM $MgCl_2$–10 mM KCl (TMgK). After the cells remained for 10 min at 0°, Triton X-100 was added to a final concentration of 0.5%, and the cells were ruptured by 10 strokes of a Dounce homogenizer. The homogenate was centrifuged for 3 min at 800 × *g*, and the pellets of nuclei obtained were resuspended in buffers appropriate for each particular enzymatic assay.

DNA Polymerase. The assay measures conversion of radioactively labeled deoxyribonucleoside triphosphate into an acid-insoluble product. The standard incubation mixture (in 0.3 ml) contained dATP, dGTP, dCTP (0.2 μM each), 0.1 μM [^{3}H]TTP (specific activity 100 Ci/mol), 50 μg of native calf-thymus DNA, 10 μM $MgCl_2$, 5 μM 2-mercaptoethanol, and 50 mM Tris·HCl buffer at pH 8.0. The reaction, linear for 90 min, was allowed in the assays to proceed for 60 min at 37° and was stopped by addition of an ice-cold solution of 20% trichloroacetic acid with 50 mM sodium pyrophosphate. The precipitated material was collected on Millipore filters and washed repeatedly with 10% trichloroacetic acid before scintillation counting.

DNase Assay. A measure of the hydrolysis of ^{3}H-labeled DNA from L or HeLa cells was the conversion of single-stranded DNA to acid-soluble fragments, under the conditions described (8).

RESULTS

DNA polymerase activity in isolated nuclei

The DNA polymerase of controls or of cells after infection was determined in isolated nuclei. The activity in the uninfected material was constant throughout the time of sampling because the cells were in stationary phase of growth. In the presence of streptovitacin A, an inhibitor of protein synthesis (12), the activity declined slowly after 6 hr of treatment (Fig. 1). By comparison, in nuclei from infected cells there was a marked decrease in activity as early as 2 hr after infection

Abbreviation: PFU, plaque-forming unit.

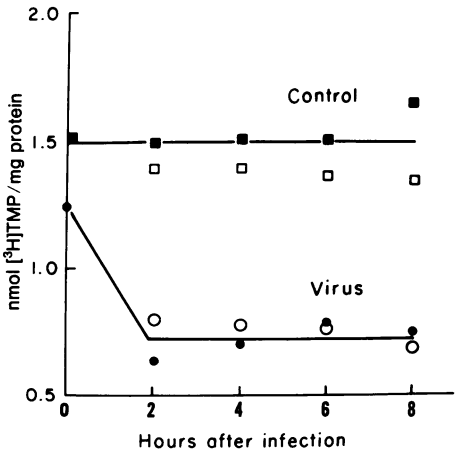

FIG. 1. Assay for DNA polymerase activity in nuclei isolated from uninfected and infected cells. Samples were prepared at the times indicated after initiation of penetration. ■- - -■, uninfected controls; □——□, uninfected, treated with 10 μg/ml of streptovitacin A; ●——●, infected; ○——○, infected, treated with 10 μg/ml of streptovitacin A.

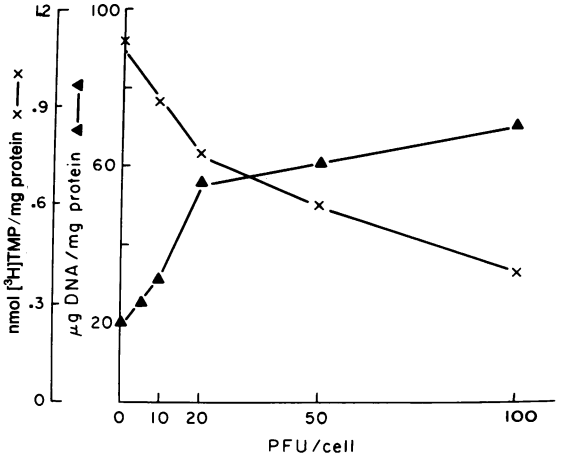

FIG. 2. Interrelationship between nuclear DNA polymerase and DNase activities at various multiplicities of infection. Nuclei were isolated 2 hr after inoculation and analyzed for both activities. ×——×, DNA synthesis; ▲——▲, DNA hydrolysis.

that was not influenced by the presence of this inhibitor of protein synthesis. The UV-irradiated virus depressed nuclear DNA polymerase to the same extent as active vaccinia virus (not shown in Fig. 1).

Effect of multiplicity of infection on nuclear-associated DNA polymerase and DNase activities

Monolayers of L cells were infected at various multiplicities. 2 hr after inoculation the cells were harvested and nuclear fractions were prepared from them. These fractions were assayed for DNA polymerase and DNase activities. There was a direct relationship between the degree of inhibition of DNA polymerase activity and the number of PFU per cell used for inoculation, in the range of 5–60 PFU per cell (Fig. 2). There was a parallel increase in the DNase activity (pH 7.8, single-stranded DNA) in proportion to the number of PFU per cell used. With single-stranded DNA as the substrate, the only hydrolytic activity evident in isolated nuclei was one at slightly alkaline pH that required Mg^{++} ions for activation, while addition of Ca^{++} failed to stimulate the reaction. This DNase activity was stimulated almost 3-fold after inoculation with 20 PFU per cell (Fig. 3). Addition of 10 μg/ml of streptovitacin A to the culture medium at the time of infection failed to affect either the appearance of DNase in nuclei (Fig. 3) or the inhibition of nuclear DNA polymerase (Fig. 1).

Effect of addition of viral cores on DNA polymerase activity

To test the effect of addition of cores on the DNA polymerase in isolated nuclei, cells were harvested in the logarithmic phase of growth, when maximum polymerase activity should prevail. A direct relationship was observed between the amount of core material added and inhibition of DNA synthesis, as determined by the number of counts of [³H]TMP incorporated into trichloroacetic acid-insoluble material (Fig. 4). However, even at the highest concentration of cores used, the DNA polymerase activity was not completely suppressed. The DNase activity of this core preparation was 900 μg of DNA solubilized per mg of core protein in 1 hr. In other experiments, addition of cores after the reaction was allowed to proceed for 60 min caused a decline in the additional synthesis during the extended 30 min of incubation in the presence of cores. It is highly significant that labeled DNA formed in the reaction *in vitro* before the addition of cores was not extensively hydrolyzed in the presence of added cores (Fig. 5).

Further characterization and influence of vaccinia components on the DNA polymerase reaction *in vitro*

Nuclei isolated from cells in logarithmic growth were used for DNA polymerase assays in the presence of virion components, RNase, DNase, and actinomycin D. A summary of the results shown in Table 1 reveals that: (*a*) omission of an exogenous DNA template from the reaction mixture reduced the activity by 65%; (*b*) omission of any two nucleotides inhibited the reaction by 96%, ruling out the likelihood of terminal addition; (*c*) the presence of RNase had no affect on the polymerization of TTP; (*d*) by contrast, the presence of DNase and actinomycin D inhibited the synthesis; (*e*) addition of intact virions had no influence; (*f*) but the presence of either envelope-free or, more specifically, the naked

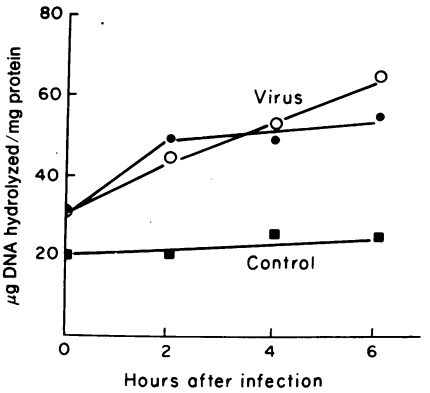

FIG. 3. Time course of appearance of DNase activity within nuclei. Nuclei were prepared at the times indicated and assayed. ■——■, uninfected; ●——●, infected; ○——○, infected in the presence of 10 μg/ml of streptovitacin A.

TABLE 1. *Effect of inhibitors and virus components on DNA polymerase activity of nuclei in vitro*

Additions	DNA polymerase activity as % of the basic reaction mixture
Complete	100 (5 nmol TMP per mg of protein)
(−) dATP and dCTP	4
− DNA	35
+ DNase (20 μg)	1
+ RNase (10 μg)	95
+ Actinomycin D (20 μg)	25
+ Virus (30 μg)	100
+ Stripped virus (30 μg)	75
+ Cores (40 μg)	5
+ Heated cores (40 μg)	90

Each tube of the reaction mixture contained in 0.3 ml 100 μg of nuclear protein, and where appropriate, virus components or inhibitors in amounts indicated. Amounts of the other reactants are given in *Methods*.

cores, was inhibitory; and (*g*) after heating of cores for 15 min at 85°, the DNA polymerase inhibitor was inactivated.

Site of action of the cores' inhibitory factor of the DNA of polymerase reaction

Two types of experiments were designed to determine whether the inhibitor of the nuclear DNA polymerase reaction from virus cores was acting upon the exogenous DNA template. In one series, ^{3}H-labeled DNA was used as template and at the end of the inoculation the DNA was extracted by means of hot phenol and recovered from the aqueous phase after ethanol precipitation. As shown in Table 2, 95% of the counts were recovered in the aqueous phase, suggesting that this template was unaffected in the presence of cores added in sufficient amounts to inhibit by 60% the nuclear polymerase reaction in a parallel experiment.

TABLE 2. *Conservation of the exogenous, double-stranded DNA template, after in vitro incubation of nuclei with vaccinia cores*

Conditions	[^{3}H]DNA (cpm)		nmol of [^{3}H]TMP per mg of protein
	Aqueous phase	Phenol phase	
Nuclei alone	18,900	100	2.20
Nuclei + cores (20 μg)	17,500	70	0.88

Nuclei were isolated from uninfected cells. DNA polymerase was assayed in the presence of 20 μg of ^{3}H-labeled DNA from L cells (specific activity 1200 cpm/μg) and unlabeled nucleotides. After 1 hr of incubation, nuclei were extracted with phenol and the amount of radioactivity in the phenol or aqueous phases was determined. The amount of [^{3}H]TMP incorporated into macromolecular material (DNA) was ascertained in parallel samples of nuclei.

In the second series of experiments, various amounts of DNA were added to the reaction mixture to determine whether the concentration of the template influences the inhibition of DNA polymerase by cores. The data summarized in Table 3 show that while the nuclear polymerase activity increased as a function of the amount of DNA template added, activity remaining in the presence of cores regardless of template concentration was almost constant. Presumably, an inhibitor of the DNA polymerase from cores did not affect the double-stranded DNA template.

Since the cores also contain a nucleotide phosphohydrolase activity (13), the possibility was considered that the substrate nucleotides were being affected directly and therefore the site upon which the core inhibitor acted. In one type of experiment, although two disparate substrate concentra-

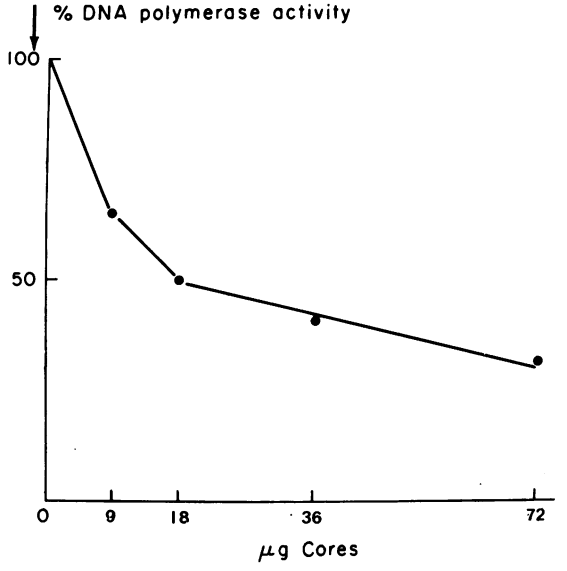

FIG. 4. Relationship between the amount of core material and DNA polymerase activity. Nuclei were isolated from uninfected cells in the logarithmic phase of growth and assayed for the polymerase activity after addition of suspensions of cores to the reaction mixture.

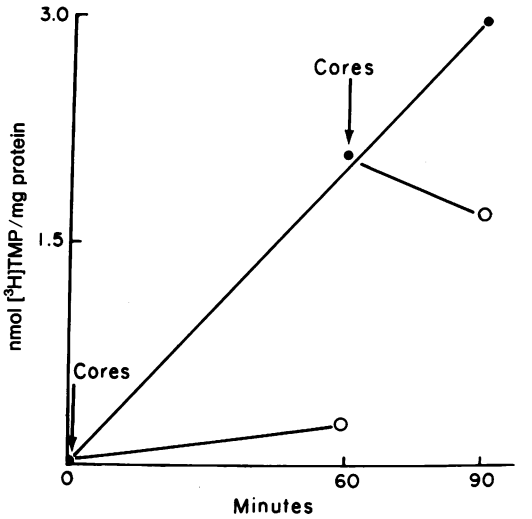

FIG. 5. Effect of addition of viral cores on the nuclear DNA polymerase activity. Nuclei were isolated from uninfected cells in logarithmic phase of growth and assayed for polymerase activity. Cores were added to the reaction mixture at the times indicated by the *arrow*.

TABLE 3. *Relationship between the amount of exogenous template in the reaction mixture and nuclear DNA polymerase activity after addition of vaccinia cores*

μg of DNA added	nmol [³H]TMP/mg of protein		
	Nuclei (alone)	Nuclei (10 μg cores)	% Inhibition
0	1.00	0.70	30
10	2.85	2.10	25
20	3.92	2.14	45
40	4.05	2.20	46
80	4.10	2.00	50

tions were used, the amount of DNA polymerase inhibition due to cores was the same, indicating that the nucleoside triphosphates were not the site of inhibition by the core factor (Table 4). Similar results were obtained when nuclei from infected cells were assayed in the presence of two concentrations of substrate.

To further characterize the site of action of the cores' inhibitory factor of the polymerase reaction, single-stranded DNA and transfer RNA were added to the reaction mixture, single-stranded DNA being the substrate for the cores' DNase and tRNA a competitive inhibitor of these DNases. The results of these experiments are summarized in Table 5. Heat-denatured DNA, added at different concentrations, decreased the inhibition caused by addition of viral cores on the nuclear DNA polymerase activity of rapidly growing cells. Similarly tRNA, added at the concentration that suppresses DNase activity in the cores by 50%, causes a decrease in the inhibition. Nuclei from virus-infected and control cells were also assayed for DNA polymerase activity in the presence of heat-denatured DNA and tRNA. Both additions released the inhibition in the infected cells.

DISCUSSION

The results presented in this communication suggest that a poxvirus-associated DNase activity is involved in the inhibition of host DNA synthesis. The main supporting evidence is the finding that after infection the nuclear DNA polymerase activity is inhibited and a DNase activity, resembling that present in virus cores, appears in the nuclei. Both phenomena occur in the absence of protein synthesis and with UV-inactivated virus, suggesting that incoming particles are responsible for the effects observed.

In vitro experiments in which virus cores are added to nuclear preparations reveal a similar inhibition of DNA polymerase activity. An explanation invoking an effect on the template, the substrate, the product, or the polymerase enzyme, itself, is conceivable. The experiments presented here indicate that neither template nor substrate is involved, but most probably the newly synthesized single-stranded DNA molecules are. This conclusion is supported by the experiments (Table 5) in which addition of single-stranded DNA and tRNA decreases the inhibition of the nuclear polymerase in the presence of cores. Although the DNA polymerase may itself be the target of some as yet unrecognized inhibitor originating from the virion, the combined observations described here make this possibility unlikely.

If the proposed explanation for the observed inhibition of nuclear DNA polymerase activity and DNA replication proves to be correct, our data may have revealed the first instance in virus cytopathology linking the inactivation of a specific host-cell function with an enzyme activity originating from the invading particle.

TABLE 4. *Effect of different concentrations of substrate on DNA polymerase activity of nuclei in vitro*

Nuclei obtained from	nmol of TMP per mg of protein per hr	
	Amount of nucleotides	
	0.3 μM	0.6 μM
Control cells	2.0	2.2
Virus-infected cells	0.8	0.9
Growing cells	5.0	4.0
Growing cells and 10 μg cores	0.6	0.5

Nuclei were isolated from uninfected cells, virus-infected cells, and rapidly growing L cells and assayed for DNA polymerase activity in the presence of two concentrations of nucleotides as indicated. The amounts of the other reactants are given in *Methods*.

TABLE 5. *Effect of single-stranded DNA and tRNA on DNA polymerase activity of nuclei in vitro*

Additions	nmol of TMP per mg of protein per hr			
	Growing cells	Growing cells + 10 μg cores	Control cells	Virus-infected cells
Native DNA (50 μg)	4.0	0.50	2.0	0.8
Native DNA (25 μg) + denatured DNA (12 μg)	4.0	1.24	2.8	2.0
Native DNA (25 μg) + denatured DNA (25 μg)	3.3	1.40	2.4	2.0
Native DNA (25 μg) + tRNA (20 μg)	3.3	1.70	2.3	2.6

Nuclei were isolated from uninfected cells, virus-infected cells, and rapidly growing L cells and assayed for DNA polymerase activity in the presence of different concentrations of heat-denatured calf thymus DNA and *Escherishia coli* tRNA (kindly supplied by Dr. Isaar Smith).

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