Mechanism of Inhibition of Vaccinia Virus Replication in Adenovirus-Infected HeLa Cells

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The ability of vaccinia virus to replicate in HeLa cells which had been previously infected with adenovirus type 2 (Ad2) was studied in order to gain insight into the mechanism by which adenovirus inhibits the expression of host cell functions. Vaccinia virus was employed in these studies because it replicates in the cytoplasm, whereas Ad2 replicates in the nucleus of the cell. It was found that vaccinia deoxyribonucleic acid (DNA) synthesis is greatly inhibited in adeno-preinfected HeLa cells provided that vaccinia superinfection does not occur before 18 hr after adeno infection. The inhibition of vaccinia DNA synthesis can be traced to an inhibition of vaccinia protein synthesis and viral uncoating. Vaccinia ribonucleic acid (RNA) synthesis is not inhibited in adeno-preinfected cells, but the vaccinia RNA does not become associated with polysomes.

Cellular deoxyribonucleic acid (DNA) synthesis has been reported (16) to be blocked extensively in mammalian cells 18 to 24 hr postinfection with adenovirus type 2 (Ad2). Host protein synthesis is also specifically inhibited 16 to 20 hr postinfection with Ad5 (2). However, there are difficulties associated with studying points of specific synthetic blockage in HeLa cells induced by adenovirus due to the fact that the host cell possesses a high degree of complexity. We resolved therefore to study the mechanism by which adenovirus blocks the replication of another DNA virus (vaccinia). It is possible that the inhibition of vaccinia replication will shed some light on the manner by which adenovirus inhibits the synthesis of host cell DNA. Vaccinia was chosen for this study because it replicates in the cytoplasm. This enables an easy distinction between vaccinia DNA synthesis and replication of adenovirus or host DNA in the nucleus. Furthermore, methods have been devised for assay of vaccinia-specific enzymes and for selectively isolating vaccinia messenger ribonucleic acid (RNA) in infected cells. Thus, one can monitor specifically vaccinia RNA, protein, and DNA synthesis.

MATERIALS AND METHODS

Cells and viruses. HeLa, S₃ cells, vaccinia virus strain WR, and Ad2 were used in the present study. Vaccinia was purified and infections were carried out as previously described (1, 8). Purification of adeno-

virus employed only cesium chloride (CsCl) instead of rubidium chloride but was otherwise identical to the method of Green and Pina (6). Infections of HeLa cells with purified adenovirus was carried out essentially by the procedure of Maizel et al. (15) except that the viral adsorption period was increased to 1 hr. After adsorption the cells were diluted to 5×10^6 per ml with Eagle's minimal spinner medium containing 5% calf serum. The multiplicity of infection was 300 to 500 particles per cell for vaccinia and 1,000 particles per cell for Ad2.

Preparation of cell extracts. Cells were harvested by centrifugation and the cytoplasmic fraction was prepared in hypotonic buffer as previously described (1). Polyribosome analysis of the latter cytoplasmic fraction was performed by sucrose gradient centrifugation (18). The crude cytoplasmic fraction was used directly for assays of thymidine kinase (4) and DNA polymerase (10).

DNA-RNA hybridization. Ad2 DNA was purified essentially by a Pronase digestion and phenol extraction procedure (14). Vaccinia DNA was purified as previously described (11). DNA-RNA hybridization was performed by the method of Bolle et al. (3). The annealing was carried out at 68°C with 25 µg of DNA and approximately 50,000 counts/min of purified RNA in about 0.5 ml of twofold concentrated standard saline citrate (NaCl, 0.3 M; Na citrate, 0.03 M).

RESULTS

Production of Ad2 in HeLa S₃ cells. The production of progeny virus particles in HeLa cells was measured by the purification and quantitation of virus which was labeled with ³H-thymidine

throughout the course of viral infection. Virus was prepared from samples of infected cells and purified as described. The amount of radioactivity and optical density were determined after banding the virus in gradients of CsCl. The results of this analysis are shown in Fig. 1. Production of mature virions commences at about 18 hr and continues linearly up to 30 hr postinfection.

In order to determine if viral capsid protein synthesis paralleled the synthesis of mature virions, cells were pulse-labeled for 3-hr intervals, followed by a chase period, with ^3H -leucine between 20 and 30 hr postinfection. Virus was then purified in all samples at 34 hr postinfection, and the amount of radioactive leucine which was incorporated into viral capsid protein was determined. The results (Table 1) indicate that capsid proteins are synthesized at a high rate between 20 and 30 hr postinfection. The latter experiment establishes that viral protein synthesis occurs actively between 20 and 30 hr postinfection. Table 1 also shows that the same is true of total protein synthesized after adeno infection between 20 and 30 hr.

Inhibition of vaccinia DNA replication in Ad2 preinfected cells. The ability of superinfecting vaccinia virus to replicate its DNA at different times after adeno infection was tested by measur-

FIG. 1. Time course of Ad2 virion synthesis in HeLa S_3 cells in suspension culture. HeLa cells (2.4×10^8) were infected with Ad2 and were continuously labeled with $0.2 \mu\text{Ci/ml}$ of ^3H -thymidine from 5 hr postinfection. Samples were harvested at various times and virus was purified. The amount of radioactivity and of optical density in the purified virus was measured. ^3H -thymidine counts per minute virus (●); O.D. 260 nm in pure virus (○).

TABLE 1. Synthesis of Ad2 proteins in HeLa cells^a

Time of labeling (hr)	Counts per minute	
	Total cytoplasm	Purified virus
20 to 23	6.73×10^5	12.6×10^3
23 to 26	7.7×10^5	14.4×10^3
26 to 29	8.7×10^5	17.9×10^3
20 to 23 (uninfected)	8.6×10^5	1.2×10^2

^a HeLa cells were infected with Ad2. At 20, 23, and 26 hr postinfection, samples of 10^8 cells were resuspended in Eagle's medium containing one-tenth of the standard leucine concentration and $20 \mu\text{Ci}$ of ^3H -leucine in 100 ml. After the 3-hr labeling period, the cells were centrifuged and resuspended in regular Eagle's medium. All cultures were harvested at 34 hr postinfection. Total incorporation of leucine into acid insoluble material and incorporation of leucine into highly purified virus was determined. Mock-infected cells were also labeled between 20 and 23 hr after the mock infection.

ing the amount of cytoplasmic DNA synthesis (representing vaccinia DNA) by the incorporation of ^3H -thymidine into acid precipitable material. The results of a typical experiment are shown in Fig. 2, where it may be seen that no inhibition of vaccinia DNA replication occurs when vaccinia infection is carried out prior to 16 to 18 hr post-Ad2 infection. However, the ability of vaccinia DNA to replicate diminishes rapidly when infection occurs after 18 hr post-Ad2 infection.

Cell-association and uncoating of vaccinia virus in Ad2-infected cells. The observation presented above, that vaccinia DNA synthesis is inhibited in Ad2-preinfected cells may result indirectly by an inhibition of: (i) uptake or adsorption of vaccinia virus; (ii) protein synthesis inhibition which would inhibit uncoating of vaccinia and also the production of early enzymes; (iii) inhibition of vaccinia RNA synthesis which would inhibit uncoating and also the production of early enzymes. In order to test for inhibition of vaccinia virus adsorption and uncoating, virus labeled with ^{14}C -thymidine was used to infect cells. Both adeno-preinfected and mock-infected cells were infected with the radioactive vaccinia. Three hours after vaccinia infection, the cells were harvested and washed. The cells were disrupted by sonication. The amount of vaccinia DNA which was uncoated was measured by its deoxyribonuclease sensitivity (9), and the amount of cell-associated total viral DNA was also measured. The results (Table 2) indicate that cell

FIG. 2. Effect of Ad2 on vaccinia deoxyribonucleic acid (DNA) replication. HeLa cells were infected with Ad2. At various times after Ad2 infection the cells were superinfected with vaccinia. The ability of vaccinia to synthesize deoxyribonucleic acid (DNA) was determined by measuring the incorporation of ^3H -thymidine into cytoplasmic DNA for a period of 5 hr following vaccinia superinfection. The points shown in the graph represent the total amount of thymidine incorporated in the ensuing 5 hr postvaccinia when vaccinia infection was carried out at the time indicated on the abscissa.

association of vaccinia virus is not inhibited in adeno-infected cells but that the uncoating of vaccinia is reduced.

Inhibition of vaccinia early enzyme synthesis in Ad2-infected cells. Since the early enzymes of vaccinia are likely to be essential for viral DNA replication, their synthesis in Ad2-infected cells was determined. Thymidine kinase and DNA polymerase were chosen as representative vaccinia early proteins. As shown in Fig. 3, the synthesis of both these enzymes was considerably inhibited in adeno-preinfected cells. The data presented (Fig. 3 a) are corrected for background thymidine kinase activity which is known to increase after Ad2 infection (17).

Synthesis of vaccinia RNA in Ad2-infected cells. As mentioned previously, vaccinia protein synthesis could be inhibited as a result of a primary inhibition of vaccinia messenger RNA synthesis. The following experiment was performed to determine if there was an inhibition of vaccinia RNA synthesis. Cells were superinfected with vaccinia, the cultures were labeled with ^3H -uridine for 10 min, and the cytoplasmic fraction was prepared and counted. This labeling

TABLE 2. Uptake and uncoating of ^{14}C -vaccinia in Ad2-infected cells^a

Infection	Counts per minute		
	Before deoxyribonuclease	After deoxyribonuclease	Per cent uncoated
Vaccinia alone.	4918	2401	51.0
Ad2 then vaccinia.	4725	4150	12.4

^a Two sets of 5×10^6 HeLa cells were infected or mock-infected with Ad2. After 24 hr, they were infected with ^{14}C -thymidine labeled vaccinia virus (4.5×10^4 counts per min per 10^{10} virions). Three hours after infection with the labeled vaccinia, the cultures were harvested and the cells were sonicated in 1.2 ml of 0.05 M tris(hydroxymethyl)-aminomethane-hydrochloride-HCl (pH 7.5) containing 6×10^{-3} M MgCl_2 . Half of the cell extract was treated with 300 $\mu\text{g}/\text{ml}$ of pancreatic deoxyribonuclease for 20 min at 37 C. Both halves were acid precipitated and counted by liquid scintillation spectrometry.

procedure is known to selectively label vaccinia RNA which is synthesized in the cytoplasm (1). Cells not superinfected with vaccinia were also labeled and served as controls. RNA was also purified after labeling the cells for 30 min with ^3H -uridine. The RNA prepared from the cytoplasmic fraction was tested for its ability to anneal with Ad2 DNA or with vaccinia DNA. The results (Table 3) clearly indicate that vaccinia RNA is synthesized in adeno-preinfected cells at nearly the normal rate. Thus, the inhibition of enzyme synthesis and uncoating observed cannot be attributed to an inhibition at the level of transcription.

Does vaccinia RNA associate with ribosomes in Ad2-infected cells? Since vaccinia RNA is synthesized without the concomitant synthesis of vaccinia proteins, we analyzed the cellular polysome fraction for its content of vaccinia RNA. In cells preinfected with Ad2 for 24 hr, little if any vaccinia RNA was found associated with polysomes after a 10-min pulse (Fig. 4b). In mock-infected vaccinia infected controls, most of the cytoplasmic vaccinia RNA was found in the polysome fraction (Fig. 4a). Thus, it appears that the primary event in the inhibition of vaccinia reproduction by Ad2 is an inhibition at the level of translation which results in a failure to find vaccinia messenger RNA associated with ribosomes.

DISCUSSION

The observed inhibition of vaccinia replication in Ad2-infected HeLa cells could occur by at

FIG. 3. Induction of vaccinia deoxyribonucleic acid (DNA) polymerase and thymidine kinase in cells preinfected with Ad2. HeLa cells were either infected with Ad2 or mock infected. After 24 hr they were infected with vaccinia virus. Samples were collected at various times and assayed for thymidine kinase (A) and DNA polymerase activity (B) in the cytoplasmic fraction. Enzyme activity in vaccinia infected cells (●); enzyme activity in Ad2-infected and vaccinia superinfected cells (○). A background correction of approximately 10^3 counts/min was subtracted from each point of the thymidine kinase curve for Ad2-preinfected cells (see text).

TABLE 3. Hybridization of cytoplasmic RNA with vaccinia and Ad2 DNA^a

Infection from which RNA was purified	³ H-uridine incorporated into cytoplasm	RNA per cent of hybridized	
		Vaccinia DNA	Ad2 DNA
Vaccinia alone...	4914	18.0	0.2
Ad2-vaccinia.....	5922	19.2	1.6
Ad2 alone.....	1776	0.2	3.3

^a Three sets of 10^8 HeLa cells were infected or mock-infected with Ad2. At 24 hr these cultures were infected or mock-infected with vaccinia virus. Thirty minutes after vaccinia infection the cells were pulsed for 10 min with ³H-uridine (200 μ Ci in 100 ml of medium). The cytoplasmic fraction from one-half of each culture was prepared, and the amount of acid-precipitable radioactivity was determined. The ribonucleic acid (RNA) purified from the cytoplasm after a 30-min labeling period was tested for its ability to hybridize with an excess of either vaccinia or Ad2 deoxyribonucleic acid (DNA).

least three different mechanisms: (i) direct inhibition of DNA synthesis; (ii) inhibition of vaccinia messenger RNA synthesis; and (iii) inhibition of translation into protein of vaccinia messenger RNA.

The experimental results presented above show that vaccinia RNA synthesis is unaltered early after vaccinia infection of Ad2-preinfected cells.

Thus vaccinia transcription does not appear to be the primary site of inhibition.

The inhibition by Ad2 apparently occurs at the level of translation of vaccinia messenger ribonucleic acid (mRNA) since: (i) the uncoating of vaccinia cores, a process requiring protein synthesis (9), is inhibited. Uncoating is also a prerequisite to viral DNA replication. (ii) The synthesis of vaccinia-induced early enzymes is inhibited. (iii) Vaccinia mRNA fails to become associated with polyribosomes.

In summary then, the following sequence of events is likely to cause the inhibition of vaccinia DNA synthesis. Vaccinia protein synthesis is inhibited resulting in an inhibition of uncoating and early enzyme synthesis. Since the latter two processes are required for DNA synthesis, viral DNA replication is blocked indirectly.

The interesting feature of this translational inhibition of vaccinia RNA is its selectivity because Ad2 mRNA is actively translated, whereas vaccinia RNA is not. The inhibition of translation of vaccinia mRNAs could be achieved if an Ad2-induced repressor associated itself with ribosomes, altering them in such a way that they would accept only Ad2 mRNA. Alternatively, an Ad2 repressor could form a complex with vaccinia mRNA thus rendering the message incapable of attachment to ribosomes. Alteration in initiation factors are also possible causes of translational repression.

The inhibition of protein synthesis reported

FIG. 4. Lack of association of vaccinia ribonucleic acid (RNA) with polyribosomes in Ad2-infected cells. HeLa cells were infected with Ad2 or mock infected and were then superinfected with vaccinia at 24 hr. The cells were pulse labeled for 10 min with ³H-uridine 40 min after vaccinia infection, and the polyribosomes were analyzed as described in Materials and Methods. (A) Vaccinia infected only: counts per minute in RNA (●); OD, 260 nm (○). (B) Ad2 infected and vaccinia superinfected: counts per minute in RNA (●); OD, 260 nm (○).

here is selective for vaccinia proteins. This mechanism is therefore different from that observed with Ad5 fiber antigen (13, 14, 16). Ad5 fiber antigen appears to inhibit DNA-dependent RNA polymerase activity in Ad5-infected cells, possibly by binding to DNA. The latter antigen is non-specific in its inhibition since it affects even the replication of adeno, uninfected cells, and other viruses. The Ad2 function discussed here is specific and inhibits at the level of translation rather than at the level of transcription. It seems plausible that the observed inhibition of vaccinia virus protein synthesis is mediated by the same mechanism that is responsible for the specific inhibition of host cell protein synthesis in cells infected with Ad5 (2). In the latter case host protein synthesis was not inhibited until late in the Ad5 infection cycle, and it was probably mediated by a viral late function (perhaps a viral capsid protein) since the inhibition did not occur in cells treated with 5-fluoro-deoxyuridine to inhibit viral DNA synthesis. The authors of the above study (2) suggested that the observed inhibition of host protein synthesis occurs at a level other than transcription of host cell RNA.

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