Synthesis of Polynucleotide 5'-Triphosphatase in Vaccinia Virus-Infected HeLa Cells

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Synthesis of polynucleotide 5'-triphosphatase, which is presumably involved in the initial modification in the series of reactions by which 5'-termini of vaccinia mRNA become capped and methylated, has been demonstrated in vaccinia virus-infected HeLa cells. Synthesis of the enzyme is prevented by actinomycin D and cycloheximide, suggesting that both de novo DNA-dependent RNA and protein syntheses are required. On the other hand, cytosine arabinoside, an inhibitor of viral DNA replication, does not prevent induction of the enzyme. The latter observation, together with the kinetics of synthesis of the enzyme in vaccinia virus-infected HeLa cells, suggests that polynucleotide 5'-triphosphatase is an "early" or prereplicative viral protein. Immunoglobulin produced against the purified virion-associated polynucleotide 5'-triphosphatase as antigen neutralized the activity of the induced polynucleotide 5'-triphosphatase, thus indicating the identity of the two enzymes.

Vaccinia virus has been shown to possess several core-associated enzymes, some with as-yet-undetermined biological functions. The latter group includes two nucleic acid-dependent nucleoside triphosphate phosphohydrolases (8, 21, 25, 27, 28), a protein kinase (6, 26), and DNase (1, 30-32) activities. A virion-associated DNA-dependent RNA polymerase (12, 20) is responsible for synthesizing viral mRNA, which may be polyadenylated by an endogenous polyadenylic acid [poly(A)]polymerase (4, 10, 17, 19).

Several core-associated enzymes have been assigned roles in a sequence of reactions in which the 5'-termini of viral mRNA is modified to produce a capped structure (7, 14-16, 33). The primary reaction in this sequence is postulated to be the hydrolysis of the γ phosphate from 5'-triphosphate-terminated mRNA catalyzed by a core-associated polynucleotide 5'-triphosphatase (33). The 5'-diphosphate-terminated polynucleotide is considered to be the substrate for the guanylyl- and methyltransferases (14).

As with other virion-associated enzymes (3, 11, 24, 29), it was of interest to study the synthesis of polynucleotide 5'-triphosphatase in vaccinia virus-infected cells. Such studies would give an insight into the regulation of synthesis of this protein and whether the information coding for this synthesis is specified by the virus.

MATERIALS AND METHODS

Virus. Stock vaccinia virus (WR) was produced in spinner cultures of HeLa cells as described by Joklik

(9), but without purification through sucrose gradients.

Assay for polynucleotide 5'-triphosphatase. The reaction mixture (50 μ l) contained 50 mM Tris-hydrochloride (pH 8.4), 2 mM $MgCl_2$, 2.5 mM dithiothreitol, and 1 to 4 pmol of 5'-[γ - ^{32}P]ATP-terminated poly(A), prepared as described by Maitra and Hurwitz (13), using *Escherichia coli* RNA polymerase and heat-denatured calf thymus DNA. Reactions were carried out as described by Tutas and Paoletti (33). One unit of activity is the amount of enzyme that produces 0.1 pmol of P_i in 5 min.

Assay for DNA-dependent nucleoside triphosphate phosphohydrolase. The procedure was that described by Paoletti et al. (28), using 1 mM [3H]-ATP.

Detection of polynucleotide 5'-triphosphatase in extracts of vaccinia virus-infected HeLa cells. HeLa cells at approximately 2×10^5 /ml were harvested by centrifugation at $1,000 \times g$ for 10 min and resuspended in 1% of the initial volume of fresh growth medium in the absence or presence of particular drugs. Vaccinia virus (10 PFU/cell) was added to the cells and adsorbed at 37°C for 30 min with constant agitation. Mock-infected cells were prepared in an identical fashion by substituting 1 mM Tris-hydrochloride (pH 9.0) for virus. Unadsorbed virus was removed by centrifugation, and the cells were resuspended at 2×10^5 /ml in fresh medium in the absence or presence of the drug of interest; this was considered zero time. At various times 50 to 100 ml of cells were harvested and washed twice with 0.01 M Tris-hydrochloride (pH 8.4) containing 0.15 M NaCl.

Cells used to prepare cytoplasmic extracts were resuspended in 1.0 ml of buffer A (0.15 M Tris-hydrochloride [pH 8.4]-2 mM dithiothreitol-1 mM trisodium-EDTA-10% glycerol-0.1% Triton X-100) con-

taining 50 mM NaCl and disrupted in a Dounce homogenizer after the cells were allowed to swell in ice water. Nuclei and large debris were removed by centrifugation at $3,000 \times g$ for 15 min. To the equivalent of 2.5×10^7 cells was added 5 ml of buffer A containing 0.1% sodium deoxycholate and 0.2 M NaCl. Samples were sonically treated in a Branson Sonifier at 20% maximum power for 30 s and then centrifuged at $114,000 \times g$ for 1 h. Samples to be assayed for DNA-dependent nucleoside triphosphate phosphohydrolase and polynucleotide 5'-triphosphatase were either used directly or passed through columns of DEAE-agarose equilibrated with buffer A containing 0.2 M NaCl.

Whole-cell extracts were prepared by suspending harvested, washed cells in 0.1 M Tris-hydrochloride (pH 8.4), 0.3 M NaCl, 2 mM dithiothreitol, and 0.1% sodium deoxycholate and sonically treating the suspension four times for 15-s intervals in a Branson Sonifier with a microtip probe. Samples were then adjusted to final salt, buffer, and detergent conditions equivalent to those of the cytoplasmic extracts and were centrifuged like those extracts.

Preparation and assay of soluble or particulate polynucleotide 5'-triphosphatase. Samples for quantitating the distribution of soluble and particulate forms of polynucleotide 5'-triphosphatase were prepared as described above, except that 0.1% sodium deoxycholate and 0.2 M NaCl were introduced into the samples to be examined for particulate enzyme after the soluble and particulate forms had been separated by centrifugation at $114,000 \times g$ for 1 h.

Purification of polynucleotide 5'-triphosphatase and preparation of antiserum. The purification and characterization of the polynucleotide 5'-triphosphatase associated with purified vaccinia virus cores has been reported (33). The enzyme was purified through the polyuridylic acid-agarose step and concentrated to 3.4×10^7 units/ml (approximately 150 μ g/ml) against Ficoll. Rabbits were inoculated at weekly intervals with this antigen emulsified in complete Freund adjuvant. The rabbit was bled 7 days after the third injection, and the serum was extracted. The immunoglobulin fractions from both preimmune and immune sera were isolated by $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialyzed against 10 mM Tris-hydrochloride (pH 8.4) (5).

Neutralization assay for polynucleotide 5'-triphosphatase. Purified polynucleotide 5'-triphosphatase or extracts from infected or uninfected HeLa cells were mixed with varying amounts of immunoglobulin appropriately diluted with bovine serum albumin and held at 4°C for 1 h before assay.

Sources of drugs and chemicals. Cycloheximide, cytosine arabinoside, actinomycin D, rifampin, and unlabeled ATP were obtained from P-L Biochemicals. Tritium or ^{32}P -labeled ATP was purchased from New England Nuclear. *E. coli* RNA polymerase used to prepare radiolabeled poly(A) substrate was obtained from Sigma Chemical Co.

RESULTS

Presence of polynucleotide 5'-triphosphatase in cytoplasmic extracts of infected HeLa cells and neutralization with immu-

noglobulin. Preliminary experiments indicated that levels of polynucleotide 5'-triphosphatase activity in vaccinia virus-infected HeLa cell extracts were sufficiently high, when compared with extracts derived from uninfected HeLa cells (Table 1), that reliable data could be gathered without further purification of the enzyme. These results also showed that the induced enzymatic activity is localized in the cell cytoplasm.

Further evidence that the induced enzymatic activity detected in crude extracts was identical to that purified from vaccinia viral cores (33) was obtained from immunoglobulin-enzyme neutralization studies. Polynucleotide 5'-triphosphatase activity in infected HeLa cell cytoplasm was inhibited more than 90% when reacted with immunoglobulin produced in rabbits against the polynucleotide 5'-triphosphatase purified from vaccinia viral cores (Table 2). Similar levels of neutralization were observed when purified enzyme from vaccinia virus was used. In other studies (data not presented) no significant neutralization of the base line activity in uninfected HeLa cells (Table 1) was detected. These findings demonstrated the validity of assaying the induced enzyme in crude extracts derived from infected HeLa cells, as well as the identity of the induced enzyme with that associated with purified virions.

Effect of cytosine arabinoside on the synthesis of polynucleotide 5'-triphosphatase and nucleic acid-dependent nucleoside triphosphate phosphohydrolase activity in infected cells. A distinction between some of the events occurring in vaccinia-infected cells can be made on the basis of viral DNA replication. Events occurring before, and not dependent on, viral DNA replication are considered "early," or prereplicative, whereas events dependent on viral DNA replication are considered "late," or

TABLE 1. Presence of polynucleotide 5'-triphosphatase activity in vaccinia virus-infected HeLa cells^a

Source of enzyme extract	Activity (pmol of P_i / 25- μ l extract per 5 min)
Uninfected whole cell	0.138
Infected whole cell	1.168
Uninfected cytoplasm	0.100
Infected cytoplasm	1.178

^a Extracts were prepared as described in Materials and Methods at 4 h postinfection, and each assay contained 4 pmol of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ -terminated poly(A) with a specific activity of 500 cpm/pmol. Infected cell extracts, whether derived from whole cells or cytoplasm, were diluted sixfold before assay. Uninfected cell extracts were not diluted.

TABLE 2. *Presence of polynucleotide 5'-triphosphatase activity in extracts of vaccinia virus-infected HeLa cells and neutralization with specific immunoglobulin^a*

Source of enzyme	Activity (pmol of P _i /25-μl extract per 5 min)		
	Pre-immune globulin	Immunoglobulin	Inhibition (%)
Infected HeLa cell cytoplasm (10 h postinfection)	0.277	0.020	93
Virion-associated purified enzyme	0.229	0.018	92

^a Extracts from infected cells were prepared and assayed as described in Materials and Methods. For reaction with immunoglobulin, 10 μl of extract or 10 μl (4 enzyme units) of purified enzyme was combined with 40 μl of preimmune or immunoglobulin (14 mg/ml) and maintained at ice temperature for 1 h before assay. Specific activity of [γ -³²P]ATP-terminated poly(A) was approximately 1,100 cpm/pmol.

postreplicative (23). By these criteria the two nucleic acid-dependent nucleoside triphosphate phosphohydrolases (24) also synthesized in vaccinia virus-infected cells are considered late viral proteins.

A useful reagent for categorizing viral functions in vaccinia-infected cells is cytosine arabinoside, a potent inhibitor of viral DNA replication. Synthesis of polynucleotide 5'-triphosphatase was not inhibited by cytosine arabinoside when the drug was present throughout the infectious cycle (Table 3). On the other hand, synthesis of nucleic acid-dependent ATPase activity, a late viral function, is effectively inhibited by the drug. In addition, significant levels of polynucleotide 5'-triphosphatase activity are observed by 2 h postinfection (Table 3), whereas no significant level of nucleic acid-dependent ATPase activity is observed until 4 h postinfection (Table 3). In other experiments, measurable levels of polynucleotide 5'-triphosphatase were detected by 1 h postinfection. The time of appearance and lack of inhibition by cytosine arabinoside strongly suggest that polynucleotide 5'-triphosphatase is an early viral function.

Inhibition of synthesis by cycloheximide and actinomycin D. Both cycloheximide, an inhibitor of protein synthesis, and actinomycin D, an inhibitor of DNA-dependent RNA synthesis, significantly inhibited the synthesis of polynucleotide 5'-triphosphatase in infected cells (Table 4). This suggests that both de novo RNA and protein synthesis are required for induction of this enzyme by vaccinia virus.

Distribution of soluble and particulate

polynucleotide 5'-triphosphatase activity in infected cells. In the experiments described to this point, sodium deoxycholate was utilized in the preparation of the enzyme extracts from HeLa cells. Since this detergent effectively solubilizes the enzyme from purified virus (33) and presumably immature viral particles, we wished to determine whether the enzyme was present in a soluble or particulate form in infected HeLa cells. Cytoplasmic extracts were fractionated into soluble and particulate fractions by high-speed centrifugation and then treated with sodium deoxycholate to solubilize the enzymatic activity. Approximately 80% of the activity present at 10 h was in a soluble form (Table 5). These results also demonstrated the efficient solubilization of the enzymatic activity from the particulate fraction by sodium deoxycholate. Enzyme neutralization studies indicated that the

TABLE 3. *Synthesis of polynucleotide 5'-triphosphatase and DNA-dependent nucleoside triphosphate phosphohydrolase in vaccinia-infected HeLa cells in the presence of cytosine arabinoside^a*

Time after infection (h)	Polynucleotide 5'-triphosphatase (pmol of P _i /5-μl extract per 5 min)	DNA-dependent nucleoside triphosphate phosphohydrolase (pmol of ADP/10-μl extract per 60 min)
0	0.065	<1
2	0.545	<1
4	0.645	620
10	0.710	1,000
10 + cytosine arabinoside (20 μg/ml)	0.735	<1

^a Extracts were prepared through DEAE-agarose as described in Materials and Methods. Specific activities of [γ -³²P]ATP-terminated poly(A) and [³H]ATP were 600 cpm/pmol and 45,000 cpm/nmol, respectively. Activity in uninfected cells was essentially similar to zero time above.

TABLE 4. *Inhibition by cycloheximide and actinomycin D of the synthesis of polynucleotide 5'-triphosphatase activity in vaccinia virus-infected HeLa cells*

Source of extract ^a	Activity (pmol of P _i /5-μl extract per 5 min)
Uninfected HeLa cells	0.023
Virus-infected HeLa cells	0.710
Virus infected + cycloheximide (130 μg/ml)	0.052
Virus infected + actinomycin (6 μg/ml)	0.037

^a Infected cells were harvested and extracts were prepared at 10 h postinfection.

TABLE 5. *Soluble and particulate distribution of polynucleotide 5'-triphosphatase activity in vaccinia virus-infected HeLa cells^a*

Treatment of cytoplasmic extract	Activity (enzyme units)		
	Total cytoplasm	Particulate	Soluble
No sodium deoxycholate	3,080	530	2,360
With sodium deoxycholate	3,100	64	3,360

^a Extracts from infected HeLa cells were prepared at 10 h postinfection and solubilized with 0.1% sodium deoxycholate either immediately or after fractionation of the cytoplasmic extract into soluble and particulate fractions by centrifugation at $114,000 \times g$ for 60 min.

activities in the soluble and particulate fractions were inhibited to the same degree with specific immunoglobulin (data not presented).

Rifampin, a specific inhibitor of viral morphogenesis (18, 22), did not prevent the appearance of the particulate form of the enzyme when the drug was present at 100 $\mu\text{g/ml}$ (data not presented).

DISCUSSION

Polynucleotide 5'-triphosphatase, an enzyme associated with purified vaccinia virus cores, was shown to be induced in HeLa cells after infection with vaccinia virus and localized within the cytoplasm. That the induced enzymatic activity was identical to the purified polynucleotide 5'-triphosphatase obtained from virions was demonstrated by neutralization of the induced activity with immunoglobulin prepared against the purified enzyme obtained from viral cores. Synthesis of the enzyme was prevented by cycloheximide and actinomycin D. The requirement for de novo RNA and protein synthesis, together with radiolabeling of the enzyme after infection (33), suggests that it is synthesized after vaccinia infection. On the basis of the enzyme's appearance time (1 h postinfection) and the failure of cytosine arabinoside, an inhibitor of DNA synthesis, to prevent its induction, this protein is ascribed to the class of viral functions that are early or prereplicative.

It has been suggested (33) that polynucleotide 5'-triphosphatase, which cleaves the gamma phosphate from RNA and poly(A), is involved in the primary modification of mRNA 5'-termini, which are subsequently blocked and methylated. After this work was completed, experiments on the synthesis of mRNA guanylyltransferase and mRNA methyltransferases in HeLa cells infected with vaccinia virus were reported by Boone et al. (2). These authors presented

data showing that the mRNA guanylyltransferase and mRNA methyltransferases are "early" or prereplicative viral proteins. From this latter report and our data on the synthesis of polynucleotide 5'-triphosphatase reported here, it appears that all of the enzymes involved in the modification of the 5'-termini of vaccinia mRNA can be classified as "early" viral products that are subsequently encapsidated within the mature virion.

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