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Regulation of Synthesis of Two Immunologically Distinct Nucleic Acid-Dependent Nucleoside Triphosphate Phosphohydrolases in Vaccinia Virus-Infected HeLa Cells

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The two nucleic acid-dependent nucleoside triphosphate phosphohydrolases, previously purified from vaccinia virus cores, were shown to be immunologically distinct enzymes. Antiserum prepared against purified phosphohydrolase I and antiserum prepared against purified phosphohydrolase II only neutralized the activity of that enzyme used as antigen. Both enzymes were induced in HeLa cells after vaccinia infection. DNA-cellulose chromatography was used to purify the two phosphohydrolases from the cytoplasms of infected cells. The enzymes were identified by their different substrate specificities, nucleic acid dependence, and neutralization with specific antiserum. A third chromatographically separable nucleic acid-dependent phosphohydrolase similar to phosphohydrolase I in substrate specificity but not neutralizable by antiserum to either phosphohydrolase I or II, was also isolated from infected cells. No nucleic acid-dependent nucleoside triphosphate phosphohydrolase activity was detected by similar methods from uninfected HeLa cells. Formation of these virus-induced enzymes was prevented by actinomycin D and cycloheximide, indicating a requirement for de novo RNA and protein synthesis, respectively. The kinetics of induction and inhibition by cytosine arabinoside, an inhibitor of DNA synthesis, suggested that synthesis of the phosphohydrolases is a late viral function. Rifampin, an inhibitor of vaccinia virus growth which prevents virion assembly, had no inhibitory effect on the induction of the phosphohydrolases. This result was consistent with the finding that these enzymes exist in a soluble as well as in a particulate form in the cytoplasm of infected cells. Addition of another specific anti-poxviral drug, isatin-β-thiosemicarbazone, to vaccinia-infected cells partially inhibited induction of the phosphohydrolases.

Vaccinia virus, a member of the poxvirus group, is a large, complex, double-stranded DNA virus that replicates within the cytoplasm of its host. Control of viral functions is mediated by the induction of a number of enzymatic activities at both early and late times after infection (6, 10, 16, 18, 19, 21, 23, 40). Several enzymes, including a DNA-dependent RNA polymerase (12, 28), polyadenylate polymerase (3, 11, 26), DNase (2, 36), protein kinase (5, 31), and a nucleoside triphosphate phosphohydrolase (7, 29), are packaged within the virus core possibly to function during the initial stages of infection. All of the enzymes, with the exception of the RNA polymerase, have been solubilized in our laboratory and some have been purified to near homogeneity (17, 26, 32-34, 37, 38). The present paper is concerned with the nucleoside triphosphate phosphohydrolases, enzymes which hydrolyze nucleoside triphosphates to diphosphates with the liberation of inorganic phosphate.

Recently we reported that the nucleoside triphosphate phosphohydrolase activity that is solubilized from the virus is dependent upon a nucleic acid cofactor (32-34). This phosphohydrolase activity was separated by DNA-cellulose chromatography into two components with different nucleoside triphosphate substrate and polynucleotide cofactor requirements (33, 34). Phosphohydrolase I hydrolyzes only ATP or dATP and utilizes denatured deoxyribonucleic acids, synthetic DNA-DNA hybrids, and some synthetic DNA-RNA hybrids as cofactors, but does not utilize synthetic homopolymers, synthetic RNA-RNA hybrids, denatured RNA, or native DNA or RNA. Phosphohydrolase II, on the other hand, hydrolyzes all four common

ribonucleoside triphosphates as well as dATP and TTP. Furthermore, phosphohydrolase II has a less stringent nucleic acid cofactor requirement and utilizes any polynucleotide containing single-stranded regions.

Important questions that remain to be answered concern whether phosphohydrolases I and II are completely different enzymes, whether they are coded for by the viral genome, the regulation of their synthesis after infection, and what viral function(s) is coupled to nucleotide hydrolysis. In this communication we show that phosphohydrolase I and II are immunologically distinct, that similar enzymes cannot be detected in uninfected cells, and that both enzymes are induced after the onset of viral DNA synthesis in vaccinia-infected HeLa cells.

MATERIALS AND METHODS

Cells and virus strains. Vaccinia virus (WR) was grown and purified from HeLa cells (27).

Assay for nucleic acid-dependent nucleoside triphosphate phosphohydrolase. The reaction mixture (0.1 ml) contained 0.1 M morpholinopropane-sulfonic acid (pH 7.0); 1 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM [^{3}H]ATP, and, unless indicated, 5 μg of heat-denatured salmon sperm DNA. One enzyme unit hydrolyzes 1 nmol of ATP to ADP per min.

Purification of phosphohydrolase I and II and preparation of antisera. Phosphohydrolases I and II were purified through the DNA-cellulose chromatography step (34). Active fractions from several preparations were pooled and separately rechromatographed on columns of DNA-cellulose. The rechromatographed phosphohydrolases I and II from about 200 mg of virus were then concentrated 10-fold with a BioRad ultrafilter b/HFU-1 cellulose acetate C hollow fibers, and used as antigens for immunization of rabbits. Initial injections of antigen emulsified in an equal volume of complete Freund adjuvant were administered into the hind muscles of rabbits. This was followed by injections of antigen emulsified in incomplete Freund adjuvant 10 and 17 days after the initial injection. The rabbits were bled on day 21 and the serum was extracted. The gamma globulin fractions from both preimmune and immune sera were isolated by $(NH_4)_2SO_4$ precipitation (4) and dialyzed against 10 mM Tris (pH 8.4).

Neutralization of phosphohydrolase activity. Varying concentrations of gamma globulin derived from preimmune or immune sera were diluted in 10 mM Tris-hydrochloride (pH 8.4) containing 4 mg of bovine serum albumin per ml. An equal volume of enzyme was added and the mixture was incubated at 5 C for 60 min. These samples were then assayed at 37 C for phosphohydrolase activity.

Detection of phosphohydrolase activity in cytoplasmic extracts. HeLa cells (4×10^6 cells/ml) were incubated with approximately 30 PFU/cell (1,500 particles/cell) of purified vaccinia virus at 37 C for 30 min. Unadsorbed virus was removed by centrifugation, and the cells were resuspended at 4×10^5 per ml. Mock-infected cells were treated in an identical fashion except that no virus was used. The time that the cells were diluted after the 30-min adsorption period was called zero time. At various times after infection, 10^7 cells were removed and washed twice in isotonic Tris-saline. The pellets were resuspended in a final volume of 1 ml in buffer A (0.1 M Tris-hydrochloride [pH 8.4], 1 mM DTT, 1 mM trisodium EDTA) containing 50 mM NaCl and disrupted by Dounce homogenization. Nuclei were removed by sedimentation and sodium deoxycholate, NaCl, and DTT were added to the supernatant to final concentrations of 0.1%, 0.25 M, and 0.05 M, respectively. After 15 min at 4 C, the material was sonicated twice for 30 s by using the microprobe of the Branson sonifier at half-maximal setting. This material was then added to 0.5 ml of packed DEAE-cellulose (Whatman DE52) equilibrated with buffer A containing 0.25 M NaCl. The slurry was mixed periodically over a period of 15 min in the cold and then passed through a glass fiber-plugged Pasteur pipette. The eluate was recovered and assayed for nucleoside triphosphate phosphohydrolase activity in the presence or absence of nucleic acid.

Purification of nucleic acid-dependent nucleoside triphosphate phosphohydrolase activity from infected HeLa cells. Vaccinia virus-infected or mock-infected HeLa cells (2.7×10^8 total cells) were collected 10 h postinfection and disrupted. After the cytoplasm was treated with sodium-deoxycholate, NaCl, and DTT, the material was centrifuged at $100,000 \times g$ for 60 min. The supernatant was filtered through a column (0.9 by 7 cm) of DEAE-cellulose equilibrated with buffer A containing 0.25 M NaCl. The flow-through was collected and diluted with buffer A to reduce the NaCl concentration to 50 mM. This was then filtered through a second DEAE-cellulose column equilibrated with the latter buffer. The flow-through containing the phosphohydrolase activity was then adsorbed to a column (0.9 by 20 cm) of DNA-cellulose also equilibrated with the above buffer. The phosphohydrolases were eluted with 300-ml gradients of 0.05 to 0.25 M NaCl in buffer A. The column was then washed with 1 M NaCl in buffer A, and all fractions were assayed for nucleic acid-dependent-phosphohydrolase activity with either ATP or UTP as substrate.

Inhibitors. The effect of inhibitors on the synthesis of phosphohydrolases in vaccinia-infected HeLa cells was tested as follows. HeLa cells (6×10^7 total cells) were infected with 3 PFU per cell (1,500 particles per cell) of purified vaccinia virus, and after absorption at 37 C for 30 min were resuspended in media containing the indicated drug. Twenty hours postinfection, the cells were collected and washed twice in isotonic Tris-saline. The pellets were resuspended in a final volume of 2 ml of buffer A containing 0.25 M NaCl and 0.1% sodium deoxycholate. The samples were then sonicated. The sonication was repeated after 30 min at 4 C. The samples were centrifuged at 136,000 $\times$ g for 60 min, and the supernatants were filtered through a small column containing a 1.5-ml packed volume of DEAE-cellulose equilibrated with buffer A containing 0.25 M NaCl. The flow-through was col-

lected and diluted with buffer A containing 125 μg of bovine serum albumin per ml to reduce the NaCl concentration to 50 mM. This material was then adsorbed to columns of a 2-ml packed volume of DNA-cellulose equilibrated with buffer A containing 100 μg of bovine serum albumin per ml and 50 mM NaCl. When the sample had passed through, the columns were washed and eluted with 10-ml volumes of buffer A containing 100 μg of bovine serum albumin per ml and 0.05, 0.15, 0.30, and 1.0 M NaCl.

Source of drugs and chemicals. The drugs and chemicals used in the current study were obtained from the following sources: actinomycin D and 5-fluorodeoxyuridine, Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md.; cycloheximide and cytosine arabinoside, The Upjohn Co.; rifampin, Dow Chemical Co.; isatin-β-thiosemicarbazone (IBT), Mann Research Laboratories; [^{3}H]ATP and [^{3}H]UTP, New England Nuclear; and unlabeled ATP and UTP, P-L Biochemicals.

RESULTS

Neutralization of phosphohydrolase I and II by specific antisera. Our previous studies indicated that two nucleic acid-dependent nucleoside triphosphate phosphohydrolase activities isolated from vaccinia-virus cores had different substrate and nucleic acid cofactor specificities (33). The first experiments described here were designed to determine whether the enzymes are immunologically distinct. The phosphohydrolases were solubilized from vaccinia virus cores and purified as previously described (34). Figure 1A shows a typical separation of phosphohydrolases I and II achieved by DNA-cellulose chromatography. The separated enzymes obtained from several such DNA-cellulose column fractionations were pooled and rechromatographed to eliminate any cross-contamination (Fig. 1B, C). The enzymes were then concentrated and used to prepare antiserum in rabbits. The immune gamma globulin (IgG)-containing fractions were isolated and tested for neutralization of phosphohydrolase activity. The results indicate that IgG prepared against phosphohydrolase I neutralized the latter activity, but had little or no ability to neutralize phosphohydrolase II (Table 1). In a similar manner, IgG prepared against phosphohydrolase II neutralized the activity of that enzyme but failed to neutralize phosphohydrolase I. Thus, these two enzymes are immunologically distinct.

Induction of phosphohydrolase activities. The next experiments were designed to determine whether phosphohydrolases I and II are virus-induced enzymes. Infection of HeLa cells with vaccinia virus resulted in an increase in ATPase activity detectable at about 2 h, the time at which viral DNA synthesis begins (9, 39) (Fig. 2A). Significantly, this activity was stimulated by addition of DNA to the reaction mixture, whereas the base-line ATPase activity of uninfected HeLa cells exhibited no such stimulation (Fig. 2A). The induction of UTPase activity was also measured because UTP is a substrate for phosphohydrolase II but not for phosphohydrolase I, whereas ATP is a substrate for both enzymes (33). A rise in DNA-dependent UTPase activity paralleled that of DNA-dependent ATPase activity (Fig. 2B), suggesting that synthesis of both phosphohydrolases was induced by vaccinia virus.

Purification of the induced phosphohydrolases. The identities of the induced phosphohydrolases were established by chromatography on DNA-cellulose and determination of substrate specificity, nucleic acid dependence, and neutralization by specific antiserum. The basic scheme used to purify the enzymes from vaccinia virus cores was used to isolate the phosphohydrolases from vaccinia virus-infected HeLa cells. The results of the last step in the purification, DNA-cellulose chromatography, are shown in Fig. 3. Assays were made using

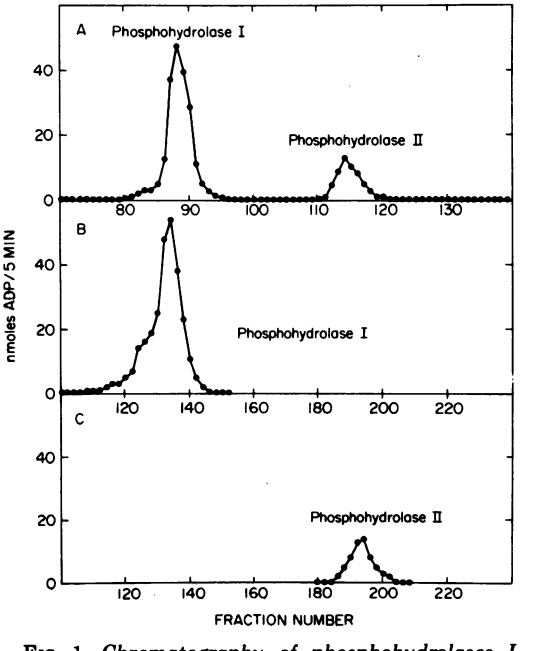

FIG. 1. *Chromatography of phosphohydrolases I and II on DNA-cellulose. (A) Typical separation of phosphohydrolases I and II achieved by chromatography on columns of DNA-cellulose. (B) and (C) Rechromatography of purified phosphohydrolases I and II, respectively, on columns of DNA-cellulose for use as antigens.*

TABLE 1. *Neutralization of phosphohydrolase activity by specific antisera*

Gamma globulin (μg)[a]	Phosphohydrolase I (nmol of ADP/5 min)	Phosphohydrolase II (nmol of ADP/5 min)
Anti-phosphohydrolase I		
3	22.6	19.9
6	16.6	21.4
13	6.9	20.5
25	2.1	19.9
50	0.6	22.4
100	0.4	22.1
Anti-phosphohydrolase II		
3	30.0	11.4
6	30.0	8.7
13	22.1	5.4
25	21.1	3.6
50	23.7	1.9
100	14.5	1.9
Preimmune		
50	22.7	23.3
100	17.4	22.9
Bovine serum albumin		
100	32.5	18.1

[a] Enzyme and gammaglobulin were held at 5 C for 60 min and then assayed at 37 C.

[^{3}H]ATP as substrate and denatured salmon sperm DNA as cofactor (Fig. 3A). The ATPase activity from uninfected cells did not adsorb to DNA cellulose. In contrast, two peaks of activity were eluted by a gradient of increasing salt when extracts of infected cells were applied. Additional activity was eluted with 1 M NaCl. Figure 3B illustrates the results obtained when the same column fractions were tested for UTP hydrolyzing activity. Again, all the activity from uninfected cell extracts passed through the DNA cellulose column without being adsorbed. A single peak of activity was eluted from the column when the infected cell extract was applied, and this coincided with the second peak of ATPase activity. The sequence of elution of the phosphohydrolase activities and their specificities for hydrolysis of ATP and for UTP suggested that the first peak in panel A is phosphohydrolase I and the second is phosphohydrolase II. The enzyme eluted with 1 M NaCl could not be phosphohydrolase II since UTP was not hydrolyzed, and it was therefore either residual phosphohydrolase I, or a third virus-induced phosphohydrolase activity. Essentially, similar results as those described in Fig. 3 were obtained when entire uninfected or infected HeLa cells were used instead of cytoplasmic extracts.

Stimulation of induced phosphohydrolases by nucleic acids. The following experiment was done to test whether the induced phosphohydrolases that bound to and were eluted from columns of DNA-cellulose were stimulated by nucleic acid cofactors. The individual phosphohydrolases eluted from DNA-cellulose were pooled and assayed in the absence or presence of denatured salmon sperm DNA. The hydrolysis of ATP by the induced phosphohydrolases I and II and by the phosphohydrolase activity eluted with 1.0 M NaCl, but not by the flow-through material, were greatly stimulated by the addition of nucleic acid (Table 2). The hydrolysis of

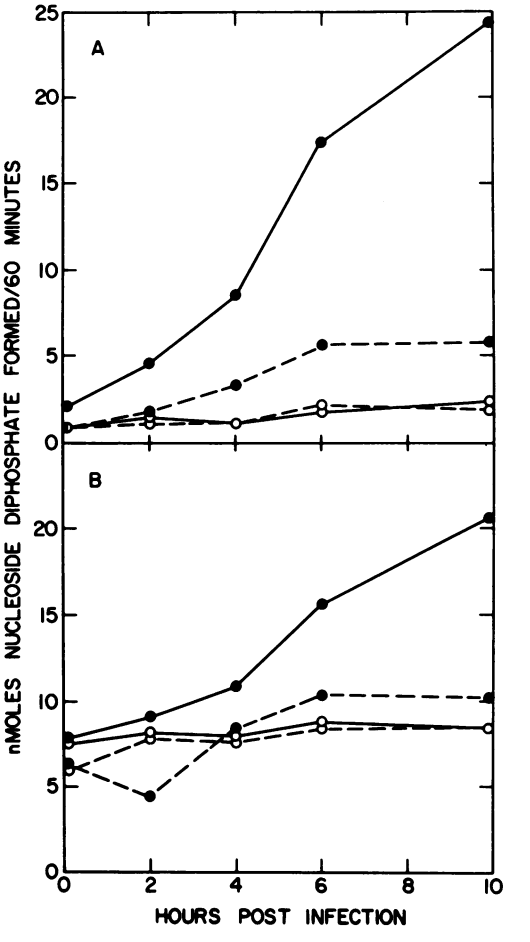

FIG. 2. *Induction of phosphohydrolase activity in vaccinia-infected HeLa cells. Cytoplasmic extracts from HeLa cells were assayed for DNA-stimulated ATPase (A) or UTPase activity (B). Uninfected-HeLa cell cytoplasmic extracts assayed in the absence of exogenous DNA* (---O---) *or in the presence of DNA* (—O—). *Infected HeLa cell cytoplasmic extracts assayed in the absence of exogenous DNA* (---●---) *or in the presence of DNA* (—●—).

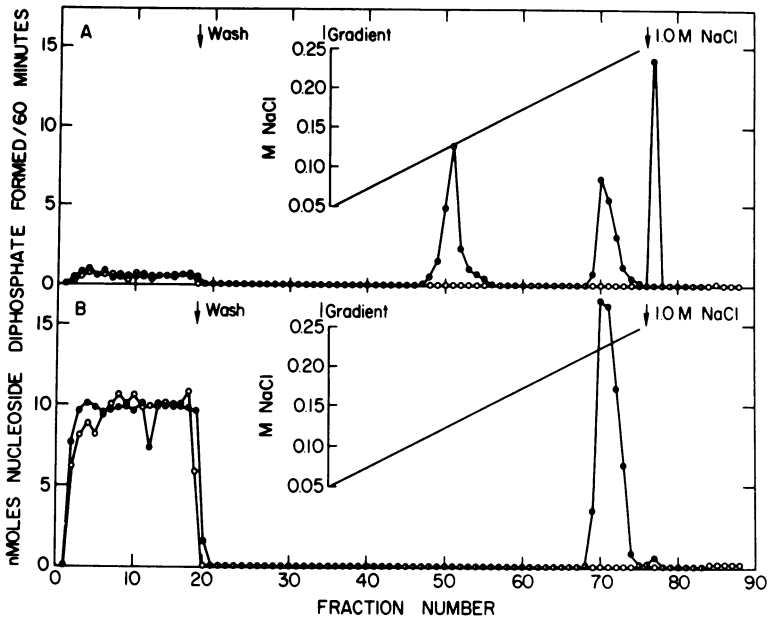

FIG. 3. *Purification of induced phosphohydrolase activity on columns of DNA-cellulose. (A) ATP hydrolysis from uninfected HeLa cells (O) or from vaccinia-virus-infected HeLa cells (●). (B) UTP hydrolysis from uninfected HeLa cells (O) or from vaccinia virus-infected HeLa cells (●).*

TABLE 2. *Stimulation of phosphohydrolase activities by denatured salmon sperm DNA*[a]

Column fraction	nmol of ADP/30 min	
	− DNA	+ DNA
Flow through	1.8	2.4
Phosphohydrolase I	3.8	27.8
Phosphohydrolase II	7.5	28.8
1.0 M NaCl eluate	1.1	50.5
	nmol of UDP/30 min	
	− DNA	+ DNA
Phosphohydrolase II	5.8	19.8

[a] The regions of phosphohydrolase activity from the experiment described in Fig. 3 were individually pooled and assayed for phosphohydrolase either in the absence or presence of denatured salmon sperm DNA.

UTP by induced phosphohydrolase II was also stimulated by the addition of nucleic acid. The endogenous activity observed in the absence of added nucleic acid may be due to the leaching of nucleic acid from the DNA-cellulose column along with the enzyme, or to some nucleic acid-independent activity.

Neutralization of induced phosphohydrolases by antisera. The adsorption and elution of the induced phosphohydrolases from columns of DNA-cellulose, their stimulation by nucleic acid, and their specificity for hydrolysis of ATP and UTP suggested that they were identical to phosphohydrolases I and II purified from vaccinia virus cores. To prove this we took advantage of the monospecific antiserum described in a previous section of this paper. The induced phosphohydrolase I activity was neutralized by IgG made against phosphohydrolase I purified from virus cores, whereas induced phosphohydrolase II was not neutralized (Table 3). Induced phosphohydrolase II, on the other hand, was neutralized by IgG made against the phosphohydrolase II purified from virus cores, whereas the induced phosphohydrolase I activity was not. The specific neutralization of the induced phosphohydrolases indicates that they are identical to the enzymes isolated from purified virus cores. It should also be noted (Table 3) that the phosphohydrolase activity that was eluted with 1.0 M NaCl was not neutralized by either of the specific IgG fractions. This was not due to high NaCl concentration because the fractions were dialyzed before incubation with IgG. This result suggests that it may be a third activity distinct from phosphohydrolase I or II.

Effect of inhibitors of virus growth on induction of phosphohydrolases. We next addressed ourselves to the question of whether viral RNA, protein, and DNA synthesis were required for the induction of the phosphohydrolase activities. The purification procedure for

the enzymes was modified by use of stepwise elution from columns of DNA-cellulose in place of a continuous gradient to facilitate the analysis of multiple samples. The endogenous HeLa cell phosphohydrolase was obtained in the flow-through and 0.05 M NaCl wash; vaccinia-induced phosphohydrolase I was eluted with 0.15 M NaCl; vaccinia-induced phosphohydrolase II was eluted with 0.3 M NaCl; and additional induced phosphohydrolase activity was eluted with 1.0 M NaCl.

Actinomycin D, an inhibitor of DNA-directed RNA synthesis, and cycloheximide, an inhibitor of protein synthesis, completely prevented the induction of the nucleic acid-dependent phosphohydrolase activities (Table 4). These results indicate a requirement for de novo RNA and protein synthesis after infection for induction of these enzymes. Two inhibitors of DNA synthesis, 5-fluorodeoxyuridine and cytosine arabinoside, were tested for their effects on induction of the phosphohydrolases. In experiment A, 5-fluorodeoxyuridine, added at a concentration of 10^{-5} M at 30 min after virus addition, inhibited synthesis of these virus-induced enzymes by about 50%, although no virus growth was detected (Table 4). In experiment B, more stringent conditions, including the use of dialyzed horse serum and addition of 5-fluorodeoxyuridine before infection, yielded similar results. In contrast, cytosine arabinoside produced greater than a 90% inhibition of synthesis of phosphohydrolase I and an 85% inhibition of phosphohydrolase II (Table 4). The latter result, together with the kinetics of enzyme induction, suggests that synthesis of the nucleic acid-dependent phosphohydrolases are essentially late viral functions, but does not rule out the early synthesis of small amounts of these enzymes.

Two specific inhibitors of poxvirus growth, rifampin and IBT, were used to obtain additional information concerning the induction of these enzymes. Rifampin did not inhibit the synthesis of the nucleic acid-dependent phosphohydrolases. In fact, higher than usual levels of

TABLE 3. *Neutralization of vaccinia-induced phosphohydrolases by specific antisera*[a]

Gammaglobulin (μg)	Phosphohydrolase I[b] (nmol of ADP/5 min)	Phosphohydrolase II[b] (nmol of ADP/5 min)	1.0 M NaCl eluate (nmol of ADP/5 min)
Antiphosphohydrolase I (50)	0.8	6.2	4.3
Antiphosphohydrolase II (50)	8.2	2.6	5.0
Preimmune (50)	7.9	6.1	4.6
Bovine serum albumin (50)	8.7	6.1	4.9

[a] The phosphohydrolases of interest from the experiment described in FIG. 3 were dialyzed against 0.1 M Tris (pH 8.4), 1 mM DTT, and 1 mM trisodium EDTA to reduce the NaCl concentration and then neutralized with specific antisera.

TABLE 4. *Effect of various drugs on the induction of phosphohydrolase activities in vaccinia-infected HeLa cells*

Expt	Drugs[a]	Column fraction (total enzyme units)				Infectivity[b] (%)
		Flow-through + 0.05 M wash	0.15 M NaCl	0.30 M NaCl	1.0 M NaCl	
A	Uninfected + no drugs	125 (95)[c]	7 (2)	32 (6)	3 (3)	
	Infected + no drugs	131 (100)	452 (100)	575 (100)	101 (100)	100
	Infected + actinomycin D	53 (40)	7 (2)	21 (4)	4 (4)	0
	Infected + cycloheximide	62 (47)	7 (2)	22 (4)	3 (3)	0
	Infected + 5-fluorodeoxyuridine	127 (97)	144 (32)	340 (59)	42 (42)	0
	Infected + rifampin	189 (144)	638 (141)	1,025 (178)	99 (98)	0
	Infected + IβT	94 (72)	71 (16)	183 (32)	65 (64)	5
B	Infected + no drugs	341 (100)	747 (100)	1,052 (100)	217 (100)	
	Infected + 5-fluorodeoxyuridine	308 (90)	258 (35)	491 (47)	153 (71)	
	Infected + cytosine arabinoside	299 (88)	56 (7)	158 (15)	36 (17)	

[a] The drugs in experiment A were added after the 30-min adsorption period. The concentration of actinomycin D, cycloheximide, 5-fluorodeoxyuridine, rifampin, and IβT was 5 μg/ml, 10 μM, 100 μg/ml, and 6.6 μg/ml, respectively. The drugs in experiment B were introduced before adsorption of virus and dialyzed horse serum was used. The concentration of 5-fluorodeoxyuridine and cytosine arabinoside was 10 μM and 20 μg/ml, respectively.

[b] Infectivity was calculated from the difference in plaque formation between samples taken at 3 and 19 h after infection and expressed as percentage of the infectivity obtained in the absence of drugs.

[c] Figures in parenthesis indicate per cent of activity of infected cell extracts in the absence of drugs.

activity were repeatedly observed. Whether this increase results from an increased rate of enzyme synthesis or from the fact that rifampin prevents virus assembly at a unique stage (8, 23, 25, 30) and may thus keep the enzyme in a soluble unpackaged form is not known.

IBT depressed the synthesis of each of the nucleic acid-dependent phosphohydrolases, consistent with some studies indicating decreased synthesis of certain other late proteins (1, 42).

Distribution of phosphohydrolases in particulate and soluble fractions of infected cells. In all previous experiments described here, 0.1% sodium deoxycholate, 0.25 M NaCl, and 0.05 M dithiothreitol were used to solubilize the phosphohydrolases from the cell extracts. We wished to determine next whether a substantial portion of the enzymatic activities were present in a nonparticulate form. Therefore, infected cells were disrupted by sonication and centrifuged at 38,000 × *g* for 30 min, conditions sufficient for the sedimentation of vaccinia virions. The pellet and supernatant fractions were then solubilized with sodium deoxycholate, salt, and reducing agent, and the phosphohydrolases were separated by stepwise elution from columns of DNA-cellulose. Table 5 shows that 36, 54, and 42% of phosphohydrolase I, phosphohydrolase II, and phosphohydrolase activity eluting with 1 M NaCl were found in the particulate fraction, respectively. The amount of phosphohydrolase activity in the soluble fraction was increased and the amount in the particulate fraction was correspondingly reduced when the infected cells were treated with rifampin (data not presented).

DISCUSSION

The two nucleic acid-dependent nucleoside triphosphate phosphohydrolases isolated from vaccinia virus cores were shown to be immunologically distinct. This was an important finding since previous studies had indicated that the molecular weights of the two enzymes were very similar (34). The immunological differences are consistent with the quite different enzymatic properties of phosphohydrolases I and II (33).

TABLE 5. *Distribution of phosphohydrolase activities in soluble and particulate fractions of infected HeLa cells*[a]

Phosphohydrolase	Particulate (enzyme units)	Soluble (enzyme units)	% Particulate
Phosphohydrolase I	320	573	36
Phosphohydrolase II	509	441	54
Phosphohydrolase eluting in 1 M NaCl	60	82	42

[a] Infected HeLa cells (4×10^7) were harvested 20 h postinfection and disrupted by sonication. The soluble and particulate fractions were solubilized and the phosphohydrolases were separated.

It is interesting to note that although the phosphohydrolase activities, while still core-associated, were stimulated by exogenous nucleic acid (33, 34), only the solubilized enzymes could be neutralized by our antisera (data not presented). For this reason, we could not use the antisera to determine whether the phosphohydrolases are necessary for the synthesis or extrusion of RNA by intact cores.

Significantly, no nucleic acid-dependent nucleoside triphosphate phosphohydrolase activity in cytoplasmic or whole cell extracts from uninfected HeLa cells was detected, nor could any activity of this type be bound to and eluted from columns of DNA-cellulose. The absence of such activities made it feasible to study the induction of the vaccinia virus phosphohydrolases. Enzymes identified as phosphohydrolases I and II on the basis of DNA-cellulose chromatography, requirement for nucleic acid cofactor, specificity for nucleoside triphosphate substrate, and neutralization of enzymatic activity by specific antisera were isolated from vaccinia virus-infected cells. An additional DNA-dependent nucleoside triphosphate phosphohydrolase was eluted from DNA-cellulose with 1.0 M NaCl. This latter activity hydrolyzed ATP but not UTP, a property shared with phosphohydrolase I, but was not neutralized by IgG to either phosphohydrolase I or II. In some previous experiments, a third very minor ATPase activity was detected by DNA-cellulose chromatography of solubilized cores. It is not known whether this corresponds to the third virus-induced enzyme described here.

The failure of the nucleic acid-dependent phosphohydrolases to be induced when RNA and protein synthesis was prevented with actinomycin D and cycloheximide was consistent with our previous demonstration that phosphohydrolase I could be labeled with [^{3}H]leucine added after virus infection (34).

The synthesis of the phosphohydrolase activities in vaccinia-infected HeLa cells was largely prevented by cytosine arabinoside, an inhibitor of DNA synthesis. This finding suggests that these induced phosphohydrolases belong to the late class of viral proteins which are translated from mRNA transcribed from progeny viral

DNA. Even though no virus growth was detected in the presence of 5-fluorodeoxyuridine, another inhibitor of DNA synthesis, the synthesis of the induced phosphohydrolase activities was inhibited by only about 50%. Such a difference could result if 5-fluorodeoxyuridine permitted a very low level of DNA synthesis.

IBT, which inhibits poxvirus replication, has been reported to reduce the stability of polyribosomes formed with late viral mRNA (42) and the amount of late viral proteins (1, 42). More recently, Katz et al. (14) showed that many late virus structural proteins are made in the presence of IBT. Our data show that IBT decreases the levels of the induced phosphohydrolase activities in vaccinia-infected HeLa cells. It should be noted that in our studies virus growth was reduced only by 95% with IBT, and synthesis of the phosphohydrolases was not completely prevented.

The effect of rifampin on the induction of the phosphohydrolases was of particular interest. Previous studies had indicated that assembly of virus particles is blocked (8, 23, 25, 30) despite the synthesis of viral DNA and many or all structural proteins, or their higher-molecular-weight precursors (15, 23-25, 35, 41). We found that the levels of induced phosphohydrolases were even higher in the presence of rifampin than in its absence. Whether this results from a greater synthesis or from a more facile extraction of enzymes not packaged into virions is not known. Rifampin was shown to increase the total amount of phosphohydrolases in the nonparticulate fraction of infected HeLa cells. Nagayama et al. (30) had reported that rifampin inhibits the induction of ATPase activity in vaccinia-infected L cells; however, this result should no longer be considered seriously because at the time of that study the nucleic acid dependence of the ATPase activity was not known, and moreover, only a particulate fraction of the cytoplasm was examined. There is general agreement that rifampin prevents the formation of the virion-associated RNA polymerase activity (13, 20, 30); however, this activity has not yet been obtained in a nonparticulate form. Whether rifampin prevents the synthesis or assembly of the RNA polymerase from component polypeptides remains to be determined.

The effects of rifampin are readily reversible, and the drug has been used to synchronize vaccinia virus assembly for studies of morphogenesis (8). It may be possible to conjugate ferritin or peroxide to the monospecific antisera described here to follow the incorporation of the phosphohydrolases into subviral particles. It will be of interest to know at what stage of assembly these enzymes are packaged and their structural relationship to DNA and other viral components.

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LITERATURE CITED

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