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Proteins of Polyhedral Cytoplasmic Deoxyvirus

II. Nucleotide Phosphohydrolase Activity Associated with Frog Virus 3

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A nucleotide phosphohydrolase is firmly associated with a purified polyhedral cytoplasmic deoxyvirus, frog virus 3. This adenosine triphosphatase is distinguishable from known mammalian cell adenosine triphosphatases and from adenosine triphosphatase of an unrelated cytoplasmic replicating virus grown in the same host cell. The enzyme activity has a high specificity for adenosine triphosphate; the product of the reaction is adenosine diphosphate. The presence of similar activities in reovirus and poxvirus indicates that adenosine triphosphatase might have a function in the replication of these viruses.

The discovery that some viruses carry polymerase activities capable of transcribing the viral genome in vivo and in vitro has led to a resurgence of interest in enzymes associated with viruses. The polymerases so far described include a deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase (poxvirus; references 7, 10), RNA-dependent RNA-polymerase (reovirus and vesicular stomatitis virus; references 1, 3, 14), and an RNA-dependent DNA polymerase (Rous sarcoma virus; reference 16). These polymerases are probably essential for initiation of the viral replication cycle. However, there are a number of other virus-associated enzymes to which, as yet, one can ascribe no obvious function. For example, there are two deoxyribonucleases induced by and located within poxvirus (8, 13) and adenosine triphosphatase associated with poxvirus and reovirus (4–6, 11). After a survey of enzyme activities that are integrated within deoxyviruses and therefore likely to participate in viral replication, we found a nucleotide phosphohydrolase activity firmly associated with the polyhedral cytoplasmic deoxyvirus frog virus 3 (FV_3). To provide some basis for speculation on the role of viral adenosine triphosphatases in general and the FV_3 adenosine triphosphatase in particular, we have determined some properties of its activity for comparison with those of reovirus and poxvirus. The association of adenosine triphosphatase activity with these distinctly different viruses suggests that it might have some general function in the initiation of viral replication.

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MATERIALS AND METHODS

Cells and virus. Monolayers of BHK-S cells permissive for FV_3 replication (18) were grown at 37 C in Hanks minimal essential medium (MEM; Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum. When confluent, the cells were infected with FV_3, 5 plaque-forming units (PFU) per cell. Adsorption was allowed to proceed for 1 hr at room temperature. Culture medium was added and, after 36 hr at 28 C, the infected cells were harvested and washed once with 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 7.8. Cells were then resuspended and disrupted in reticulocyte swelling buffer (RSB, 0.01 M Tris-hydrochloride, *p*H 7.4, 0.01 M KCl, 0.0015 M $MgCl_2$) with a Dounce homogenizer. Nuclei were removed by centrifugation ($500 \times g$ for 5 min), and the cytoplasmic fractions (crude virus: 10^8 PFU/ml; 3.7 mg of protein/ml) were layered onto 30-ml sucrose velocity gradients (20 to 65%, w/v, in RSB). The gradients were centrifuged ($30,000 \times g$ for 20 min at 10 C). Visible viral bands (10^8 PFU/ml; 1 mg of protein/ml) were collected and relayered onto sucrose density gradients (40 to 80%, w/v, in RSB). The gradients were centrifuged ($90,000 \times g$ for 4 hr at 10 C). The viral bands (10^7 PFU/ml; 500 μg of protein/ml) were collected and pooled. After dilution in Tris-hydrochloride (0.1 M, *p*H 7.8), the virus was pelleted ($200,000 \times g$ for 30 min at 5 C). The pellet of purified virus (density 1.27 g/cm^3 in CsCl) was resuspended in Tris-hydrochloride (0.1 M, *p*H 8) and stored at −20 C. The one lot of purified virus [10^7 PFU/ml; 1.4 optical density at 260 nm (OD_{260}) units/ml, 500 μg of protein/ml] was used for all experiments described.

Nucleotide phosphohydrolase assay. The reaction mixture, unless otherwise stated, consisted of 0.03 OD_{260} units of FV_3, 5 to 100 nmoles of ^{3}H-adenosine triphosphate (ATP) (specific activity, 450 μCi/μmole), 1.5 μmoles of $MgCl_2$, and 20 μmoles of Tris-hydrochloride (*p*H 8.0) in a total volume of 250 μliters.

Incubation time was for 30 min at 37 C. Separation of nucleoside mono-, di-, and triphosphates was effected by the method of J. F. Morrison (9). Samples (20 μliters) were removed at specified times and applied with marker unlabeled nucleotides to diethylaminoethyl (DEAE) cellulose paper (Whatman paper, DE 81). The chromatograms were developed with 0.6 M ammonium formate, pH 3.1 (5 hr descending). After the spots had been located by ultraviolet (UV) light, the chromatogram was cut into 1.25-cm wide strips and the radioactivity of each strip determined by scintillation spectrometry in a liquid scintillation system by using toluene and Liquifluor (New England Nuclear Corp., Des Plaines, Ill.). Studies on the specificity of enzyme action and the response to inhibitors of activities were conducted at substrate concentrations (0.5 mM) that were saturating for the enzyme.

In particular cases requiring no separation of the mono- and dinucleotides formed, a method involving the specific adsorption of nucleosides and nucleotides onto charcoal (acid-washed Norit) was employed. In such cases, reaction mixtures contained 0.03 OD_{260} units of FV_3, 0.34 μCi of ATP-γ-^{32}P (specific activity, 3.88 mCi/μmoles), 10 to 100 mmoles of ATP, 18 μmoles of Tris-hydrochloride, pH 8.0, 1.5 μmole of $MgCl_2$ in a total volume of 250 μliter. After 30-min incubation at 37 C, the reaction was terminated by chilling and addition of 1 ml of 1 N HCl–0.1 M sodium pyrophosphate. A 0.1-ml amount of 25% acid-washed Norit was then added. Reaction mixtures were centrifuged (26,000 × g for 15 min at 5 C) and radioactivity in the supernatant was determined by liquid scintillation spectrometry with Brays solution.

Nucleoside mono-, di-, and triphosphates (NMP, NDP, NTP) and deoxynucleotides (dNMP, dNDP, dNTP) were purchased from Sigma Chemical Co., St. Louis, Mo. Labeled NTP and dNTP were purchased either from New England Nuclear Corp. (Boston, Mass.), Schwarz BioResearch Inc. (Orangeburg, N.Y.), or Amersham/Searle. They included adenosine 5′-triphosphate (^{3}H-ATP; 0.45), adenosine 5′-triphosphate-gamma-^{32}P (ATP-γ-^{32}P; 3.88), deoxyadenosine triphosphate (^{3}H-dATP; 18.5), guanosine triphosphate (^{3}H-GTP; 11.8), cytidine triphosphate (^{3}H-CTP; 0.5), deoxycytidine triphosphate (^{3}H-dCTP; 26.4), uridine triphosphate (^{3}H-UTP; 2.02), and thymidine triphosphate (^{3}H-TTP; 17.4) where numbers after semicolon indicate the specific activity of each compound in Ci/mmole. Nonidet P_{40} (NP40) was obtained from the Shell Oil Co., England. Ouabain (Strophantin-G) was purchased from Mann Fine Chemicals, Inc., New York, N.Y. The antibiotic rutamycin was obtained from the Eli Lilly & Co., Indianapolis, Ind.

RESULTS

FV_3 was purified from cytoplasmic extracts of infected BHK cells by a combination of velocity sedimentation and density gradient centrifugation as described above. At stages during purification, virus was incubated with ^{3}H-ATP and the products of the reaction were analyzed by paper chromatography. It is clear from the results (Fig. 1) that an adenosine triphosphatase activity remains associated with the virus. This activity like pox-virus and reovirus adenosine triphosphatase (4–6, 11) converts ATP to adenosine diphosphate (ADP) but not to adenosine monophosphate (AMP) or adenosine. Failure to react with ADP shows that there is no phosphatase activity in the purified virus.

After repeated banding of purified virus in density gradients, adenosine triphosphatase activity of fractions from the gradients always coincided with OD_{260} and infectivity (Fig. 2).

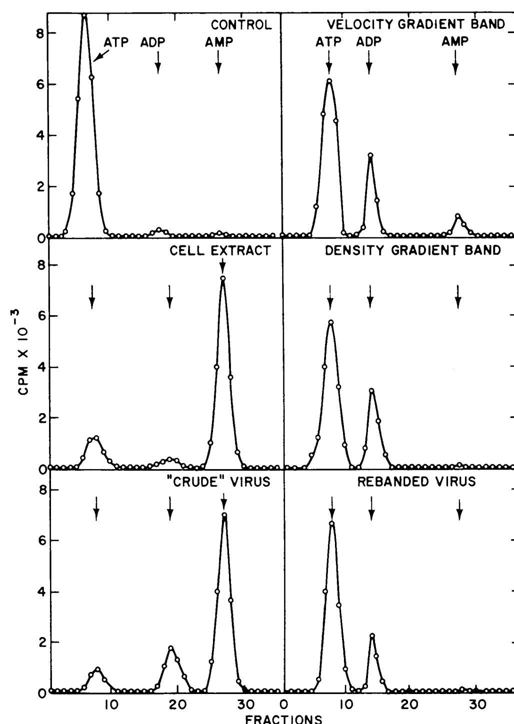

FIG. 1. *Adenosine triphosphatase activity of FV_3. Cell extracts and crude virus were prepared as described in Materials and Methods from uninfected and infected BHK cells, respectively. FV_3-associated nucleotide phosphohydrolase activity (with ^{3}H-ATP as substrate) was determined after banding by velocity sedimentation [80 to 20%, w/v, sucrose in K^+ reticulocyte swelling buffer (RSB), 30,000 × g for 20 min at 10 C in a SW 27 rotor], sucrose density gradient centrifugation (80 to 40%, w/v, sucrose in K^+ RSB, 90,000 × g, for 240 min at 5 C), or rebanding by sucrose density gradient. The control set indicated the purity of the substrate. Reaction mixtures were identical except that the total protein used in reactions was cell extract (200 μg), crude virus (200 μg), velocity gradient band (50 μg), density gradient band (25 μg), and rebanded virus (20 μg). Other conditions were as described in Materials and Methods.*

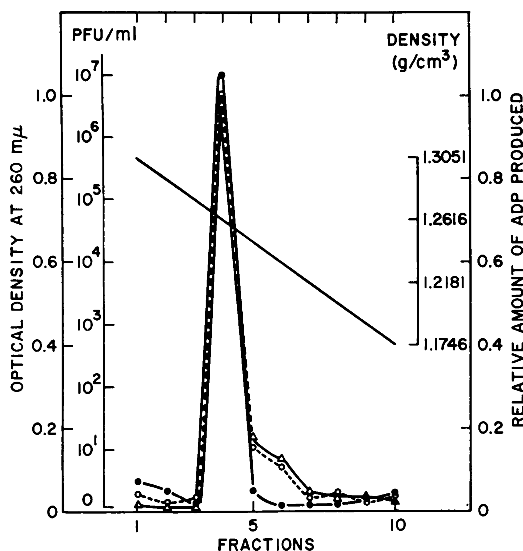

FIG. 2. *Coincidence of FV_3 nucleotide phosphohydrolase activity (^{3}H-ATP substrate) with optical density and infectivity. Purified FV_3 (0.4 ml, in 0.1 M Tris-hydrochloride, pH 8.0, 500 μg of protein/ml) was layered onto a K^+ tartrate density gradient (25 to 40%, w/w, K^+ tartrate in 0.1 M Tris-hydrochloride, pH 8.0). The gradient was centrifuged (200,000 × g for 120 min at 5 C). Fractions (0.5 ml) were collected from the bottom of the tube. Optical density (△) and infectivity (●) were determined on each fraction and enzymatic activity (○) was assayed on 50-μliter samples. Reaction mixtures were as described in Materials and Methods.*

Moreover, virus grown in cells prelabeled with isotopic amino acids and purified as described was found to be essentially free from any host polypeptides (McAuslan and Tan, *in press*). These data we interpret as indicating that the nucleotide phosphohydrolase activity is tightly associated with the virion and is not an adventitious contaminant. The kinetics of FV_3-associated adenosine triphosphatase activity are shown in Fig. 3A; the reaction is linear for the first 30 min. There is a linear relationship between reaction and concentration of virions (Fig. 3B).

Effect of cations. FV_3 adenosine triphosphatase activity was not stimulated by Na^+ or K^+ ions (Fig. 4A). This is in contrast to the response of mammalian microsomal adenosine triphosphatase (15). Unlike reovirus adenosine triphosphatase (4), Li^+ ions had no stimulating effect on the enzyme. The FV_3 nucleotide phosphohydrolase activity is dependent upon Mg^{2+} or Mn^{2+} ions, Mg^{2+} being slightly preferred to Mn^{2+} (Fig. 4B). Addition of ethylenediaminetetraacetic acid (EDTA), 2 mM (+EDTA), inhibits the residual activity observed in the absence of any added

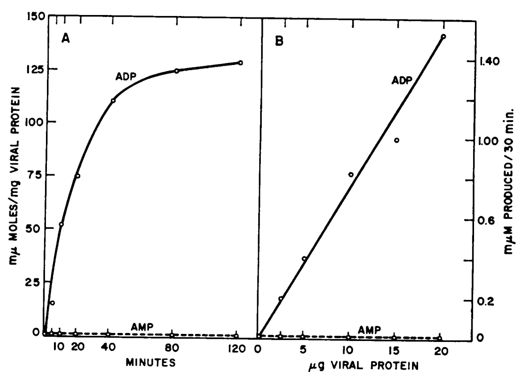

FIG. 3. *Kinetics of hydrolysis of ^{3}H-ATP by FV_3. (A) Rate of ^{3}H-ADP formation with respect to time. Only 25% of the substrate was hydrolyzed after 120 min. (B) Rate of ^{3}H-ADP formation with respect to virus concentration. Reaction mixtures were prepared as described in Materials and Methods.*

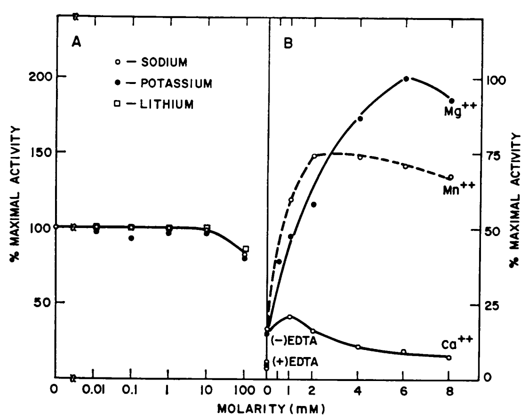

FIG. 4. *Effect of cations on adenosine triphosphatase activity. (A) Effect of Na^+ K^+, and Li^+ in the presence of Mg^{2+}. (B) Effect of Mg^{2+}, Mn^{2+}, and Ca^{2+} individually. Reaction mixtures were prepared as described in Materials and Methods, with ^{3}H-ATP as substrate. The molarity refers to the final concentration of mono- or divalent metal chlorides in the reaction mixture.*

divalent cations (−EDTA). Under the conditions used, the optimal $MgCl_2$ concentration was 6 mM. Unlike mammalian mitochondrial adenosine triphosphatase (18), viral adenosine triphosphatase was not stimulated by calcium ions.

pH and temperature optima. The *p*H optimum was approximately 8.0 in Tris-hydrochloride buffer (Fig. 5A). No other buffers were tested. In view of the fact that the temperature optimum for FV_3 replication is about 28 C, we studied the effect of temperature on the enzyme (Fig. 5B).

Maximal enzyme activity was obtained at about the temperature optimum for virus replication.

Inhibitors and activators. In Table 1, we have listed the effect of diverse agents on FV_3-associated nucleotide phosphohydrolase activity. The microsomal adenosine triphosphatase inhibitor Ouabain (15) was without effect as were sodium azide (2) and rutamycin, an inhibitor of mammalian cell mitochondrial adenosine triphosphatase (13). Neither mercaptoethanol nor NP_{40} are essential for the reaction, but their presence increased the activity slightly.

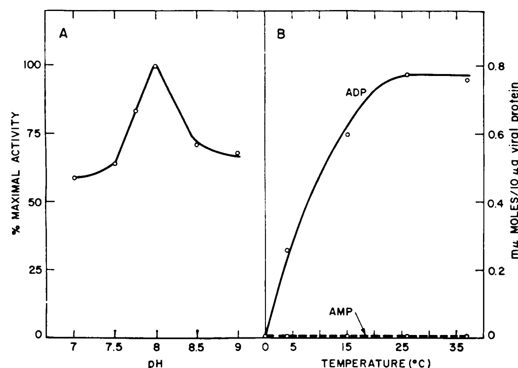

FIG. 5. *(A) pH dependence of FV_3 adenosine triphosphatase activity with ATP-γ-^{32}P as substrate. (B) Effect of temperature on FV_3 adenosine triphosphatase activity with ^{3}H-ATP as substrate. Reaction mixtures were prepared as described in Materials and Methods and incubated for 30 min at 37 C.*

TABLE 1. *Effect of different agents on FV_3 nucleotide phosphohydrolase activity*[a]

Treatment	Relative activity
None	1.00
Ouabain	
10^{-4} M	1.05
5×10^{-4} M	1.08
10^{-3} M	1.05
Rutamycin	
5 μg/ml	1.02
20 μg/ml	1.02
Sodium azide	
10^{-4} M	1.00
10^{-1} M	0.94
Mercaptoethanol	
2 mM	1.04
4 mM	1.24
16 mM	1.36
Nonidet P40	
0.10%	1.35
0.25%	1.37
0.50%	1.41
1%	1.45

[a] Reaction mixtures with ATP-γ-^{32}P as substrate were as described in Materials and Methods. Final concentration of inhibitors or activators was as indicated.

Substrate specificity. The specificity of the reaction was determined for comparison with other viral adenosine triphosphatases. FV_3 adenosine triphosphatase showed negligible activity towards isotopic GTP, CTP, or UTP under the conditions of the assay (Table 2). An alternative approach was to carry out the experiment in which labeled ^{3}H-ATP was incubated in a standard reaction mixture with a tenfold excess of either of the other unlabeled triphosphates. The data clearly demonstrate that GTP, CTP, or UTP do not interfere with the utilization of ATP in the phosphohydrolase reaction. The specificity of FV_3 nucleotide phosphohydrolase is in contrast to that exhibited by poxvirus or reovirus adenosine triphosphatases (4–6, 11).

Effects of inorganic orthophosphate and pyrophosphate. Nucleotide phosphohydrolase activity as a function of orthophosphate (Pi) and pyrophosphate (PPi) concentration in the reaction mixture is shown in Fig. 6. High concentrations of either Pi or PPi are inhibitory as they are for reovirus adenosine triphosphatase activity (4). This is probably due to a stoichiometric interaction of pyrophosphate (or phosphate) and magnesium ions rather than a direct effect on the enzyme.

Comparison of FV_3 and rabbit pox adenosine

TABLE 2. *Specificity of enzyme action*[a]

Substrate	Relative activity
^{3}H-ATP[b]	1.00
^{3}H-CTP[b]	0.04
^{3}H-GTP[b]	0.00
^{3}H-UTP[b]	0.00
^{3}H-dATP[b]	0.59
^{3}H-dCTP[b]	0.03
^{3}H-TTP[b]	0.08
^{3}H-ATP[b] plus CTP[c]	0.98
^{3}H-ATP[b] plus GTP[c]	0.92
^{3}H-ATP[b] plus UTP[c]	0.91

[a] Reaction mixtures and incubation time were as described in Materials and Methods except that substrate concentrations were adjusted as indicated. There was no significant change in the relative activities listed when the concentration of radioactive substrate was increased 20-fold.

[b] Where present, 5 nmoles.

[c] Where present, 50 nmoles.

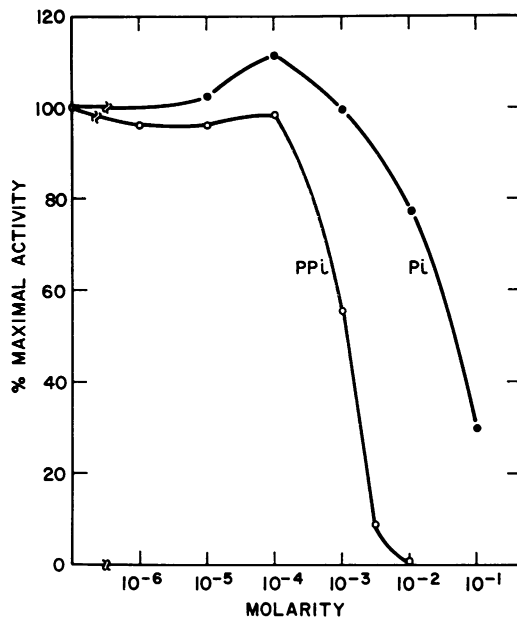

FIG. 6. *Effects of inorganic orthophosphate (Pi) or pyrophosphate on FV_3 adenosine triphosphatase activity. Molarity refers to the final concentration of Pi (Na_2HPO_4) or pyrophosphate ($Na_4P_2O_7$) in the reaction mixture.*

TABLE 3. *Minimum conditions for rabbit pox nucleotide phosphohydrolase activity and the effect of different agents on this activity*[a]

Condition	Relative activity
Complete (^{3}H-ATP substrate)	1.00
Complete plus NaCl (1 mM)	0.97
Complete plus KCl (1 mM)	0.98
Complete minus mercaptoethanol	0.55
Complete plus Ouabain (10^{-3} M)	0.98
Complete plus sodium azide (10^{-1} M)	0.98
Complete plus rutamycin (20 μg/ml)	0.86
Complete but ^{3}H-CTP[b] instead of ^{3}H-ATP	0.64
Complete but ^{3}H-GTP[b] instead of ^{3}H-ATP	0.56
Complete but ^{3}H-UTP[b] instead of ^{3}H-ATP	0.53

[a] The complete reaction mixture contained ^{3}H-ATP (5 or 100 nmoles), $MgCl_2$ (6 mM), mercaptoethanol (16 mM), Tris-hydrochloride, *p*H 8.0 (20 μmoles), rabbit pox (0.09 OD) in a final volume of 250 μliters; the mixture was incubated for 1 hr at 37 C.

[b] Where present, 5 or 100 nmoles.

triphosphatase activities. Rabbit pox (RP) was purified from cytoplasmic extracts of infected BHK cells (12). Nucleotide phosphohydrolase activity of RP, like vaccinia (11), FV_3, and reovirus (4) adenosine triphosphatases, converts ATP to ADP but not to AMP or adenosine. Comparison of RP adenosine triphosphatase (Table 3) with FV_3 adenosine triphosphatase indicates one major difference. The RP adenosine triphosphatase is nonspecific, the relative rates of hydrolysis being ATP:CTP:GTP:UTP = 1:0.64:0.56:0.53.

DISCUSSION

An adenosine triphosphatase activity remains firmly associated with infective FV_3 during virus purification. Since such virus is free from significant contamination by host protein and since a significant portion (40%) of the outer viral structural protein can be removed by detergent treatment without loss of adenosine triphosphatase from the remaining substructure (McAuslan, Aubertin, and Palese, *unpublished data*), we believe that the adenosine triphosphatase activity is an integral part of the virion. When prepared from the cytoplasmic fraction of infected cells, FV_3 does not possess an envelope acquired from cell plasma membranes which themselves might contribute adenosine triphosphatase activity. The enzyme activity is not latent and the failure of NP_{40} and mercaptoethanol to activate this enzyme as they do for poxvirus polymerase (7, 10) probably reflects the ease with which ATP can enter the particle. Because an enzyme is not latent, it does not exclude the possibility that it is within a virus particle; poxvirus adenosine triphosphatase is not a latent activity yet is located within virions (5).

Thus, there are now three examples of adenosine triphosphatase activity in completely unrelated viruses that replicate in the cell cytoplasm: reovirus, poxvirus, and FV_3. Therefore, it is likely that structural adenosine triphosphatases provide some function essential for replication of these viruses.

For each virus type the associated adenosine triphosphatase activity cleaves ATP to ADP; no adenosine diphosphatase activity or phosphatase activity has been found. A striking difference between FV_3 adenosine triphosphatase activity and the other two in question is the high specificity of the former. FV_3 acts only on ATP (or dATP), whereas reovirus shows high activity with GTP, CTP, and UTP as substrates (4, 6); RP shows significant activity towards GTP, CTP, and UTP (Table 3).

In view of the plethora of adenosine triphosphatases described for mammalian cells, it is difficult to prove that FV_3 does not acquire a host adenosine triphosphatase during maturation. However, it is unlikely that RP and FV_3 each acquire a different type of adenosine triphos-

phatase when grown in the one cell type. We therefore postulate tentatively that the structural adenosine triphosphatases of FV_3, poxvirus, and reovirus are virus specified and offer the following speculation regarding the role of adenosine triphosphatase in each of the three viruses in question: by analogy with the function of mammalian adenosine triphosphatase, cleavage of ATP by viral adenosine triphosphatase might be coupled to the transport of ions into the virion. This "vectorial" property of the enzyme would thus change the state of the virus from a dormant to an active intracellular form either by providing the appropriate ionic environment for viral polymerase to operate or by facilitating release of the viral genome from its protective protein coat.

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LITERATURE CITED

1. Baltimore, D., A. S. Huang, and M. Stampfer, 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. Proc. Nat. Acad. Sci. U.S.A. **66**:572–576.
2. Boegman, R. J., J. F. Manery, and L. Pinteric. 1970. The separation and partial purification of membrane-bound ($Na^+ + K^+$)-dependent Mg^{2+}-ATPase and ($Na^+ + K^+$)-independent Mg^{2+}-ATPase from frog skeletal muscle. Biochim. Biophys. Acta **203**:506–530.
3. Borsa, J., and A. F. Graham, 1968. Reovirus: RNA polymerase activity in purified virions. Biochem. Biophys. Res. Commun. **33**:896–901.
4. Borsa, J., J. Grover, and J. D. Chapman. 1970. Presence of nucleoside triphosphate phosphohydrolase activity in purified virions of reovirus. J. Virol. **6**:295–302.
5. Gold, P., and S. Dales. 1968. Localization of nucleoside phosphohydrolase activity within vaccinia. Proc. Nat. Acad. Sci. U.S.A. **60**:845–852.
6. Kapuler, A. M., N. Mendelsohn, H. Klett, and G. Acs. 1970. Four base-specific nucleoside 5′-triphosphatases in the subviral core of reovirus. Nature (London) **225**:1209–1213.
7. Kates, J. R., and B. R. McAuslan, 1967. Poxvirus DNA dependent RNA polymerase. Proc. Nat. Acad. Sci. U.S.A. **58**:134–141.
8. McAuslan, B. R., and J. R. Kates. 1967. Poxvirus-induced deoxyribonuclease: regulation of synthesis; control of activity in vivo; purification and properties of the enzyme. Virology **33**:709–716.
9. Morrison, J. F., 1968. Chromatographic separation of nucleoside phosphates on diethylaminoethylcellulose paper. Anal. Biochem. **24**:106–111.
10. Munyon, W., E. Paoletti, and J. T. Grace, Jr. 1967. RNA polymerase activity in purified infectious vaccinia virus. Proc. Nat. Acad. Sci. U.S.A. **58**:2280–2287.
11. Munyon, W. E., E. Paoletti, J. Ospina, and J. T. Grace, Jr. 1968. Nucleotide phosphohydrolase in purified vaccinia virus. J. Virol. **2**:167–172.
12. Planterose, D. N., C. Nishimura, and N. P. Salzman. 1962. The purification of vaccinia virus from cell cultures. Virology **18**:294–301.
13. Pogo, B. G., and S. Dales. 1969. Regulation of the synthesis of nucleotide phosphohydrolase and neutral deoxyribonuclease: two activities present within purified vaccinia virus. Proc. Nat. Acad. Sci. U.S.A. **63**:1297–1303.
14. Shatkin, A. J., and J. D. Sipe. 1968. RNA polymerase activity in purified reoviruses. Proc. Nat. Acad. Sci. U.S.A. **61**: 1462–1469.
15. Skou, J. C. 1957. The influence of some cations on an adenosine triphosphatase from peripheral nerves. Biochem. Biophys. Acta **23**:394–301.
16. Temin, H. M., and S. Mizutani. 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature (London) **226**:1211–1213.
17. Ulrich, F. 1965. The activation of mitochondrial adenosine triphosphatase by calcium. Biochim. Biophys. Acta **105**: 460–471.
18. Vilaginès, R., and B. R. McAuslan. 1970. Restricted replication of frog virus 3 in selected variants of BHK cells. J. Virol. **6**:303–309.