Proteins of a Polyhedral Cytoplasmic Deoxyvirus

III. Structure of Frog Virus 3 and Location of Virus-Associated Adenosine Triphosphate Phosphohydrolase

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The adenosine triphosphatase associated with frog virus 3 shows high specificity for ATP or deoxyadenosine triphosphate and appears to be distinct from the corresponding activity in host cells, poxvirus, or reovirus. The enzyme activity is probably integrated into virus particles since it is firmly associated with subviral particles produced when approximately 50 to 60% of the outer viral protein is removed by detergent treatment. The occurrence of adenosine triphosphatase activity within three unrelated viruses suggests that adenosine triphosphatase might be a necessary function for most viruses that replicate in the cell cytoplasm.

The polyhedral cytoplasmic deoxyvirus frog virus 3 (FV₃) is complex in structure; it contains at least 16 different polypeptides (K. B. Tan and B. R. McAuslan, *Virology*, *in press*) and an adenosine triphosphatase activity [9; B. R. McAuslan, *In K. G. Wolstenholme (ed.), "Strategy of the Viral Genome," in press*]. Virus-associated adenosine triphosphatase activity has also been reported for poxviruses (7, 8) and reovirus (3, 5). The existence of adenosine triphosphatase in these unrelated viruses raises the possibility that this enzyme might be essential for viral replication [9; B. R. McAuslan, *In K. G. Wolstenholme (ed.), "Strategy of the Viral Genome," in press*].

The adenosine triphosphatase in reovirus and poxvirus is associated with viral cores (3, 4, 7). For purposes of comparison, it is important to establish whether FV₃-associated adenosine triphosphatase is located within the virus or merely adsorbed to the outer surface of the virus particle.

In this report we present evidence that adenosine triphosphatase is probably integrated within FV₃ particles. Furthermore, on the basis of the comparative specificities of the nucleotide phosphohydrolases of three unrelated viruses grown in the one host cell, it seems likely that such enzyme activities are virus coded.

MATERIALS AND METHODS

Cells and virus. Techniques for the preparation and assay of FV₃ have been described in detail recently (9; K. B. Tan and B. R. McAuslan, *Virology*, *in press*). The virus was purified essentially as described by K. B. Tan and B. R. McAuslan (*Virology*, *in press*).

In brief, virus particles in the cytoplasmic extract from infected baby hamster kidney (BHK) cells were separated from host material by rate zonal centrifugation in sucrose gradients (20 to 65%, w/w; 30,000 × *g*, 20 min). Virus was further purified by repeated (2×) centrifugation in sucrose density gradients (40 to 80%, w/v; 109,000 × *g*, 4 hr). The viral material was then collected and suspended in 0.1 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride, pH 7.8 [final titer 5 × 10⁷ plaque-forming units (PFU) per ml, 1.5 optical density (OD)₂₆₀ units per ml, 0.5 mg of protein/ml].

Radioactive FV₃ labeled either with ³H-thymidine or a mixture of ¹⁴C-labeled amino acids was prepared and purified as described by K. B. Tan and B. R. McAuslan (*Virology*, *in press*). Radioactivity was measured by liquid scintillation spectrometry. Reovirus was propagated in BHK cells before purification by the method of Banerjee and Shatkin (1). The purified virus banded at a density of 1.36 g/cm³, which is the same as that of reovirus grown in L cells.

Enzyme assay. Nucleotide phosphohydrolase activities were assayed by methods described recently (9). Reaction mixtures contained 250 nmoles of radioactive deoxy- or ribonucleotide triphosphates, 1.5 μmoles of MgCl₂, 20 μmoles of Tris-hydrochloride (pH 8.0), and 4 to 20 μg of viral protein in a total volume of 250 μliters. The mixture was incubated for 40 min at 37 C unless otherwise cited. Either ATP-³²P (0.34 μCi per assay) or tritiated deoxy- or ribonucleotides (2.5 μCi per assay) were used. The substrate concentrations were at saturating levels for maximal enzyme velocity. To measure nucleotide phosphohydrolase activity in reovirus, an amount of chymotrypsin (Miles Laboratories) equivalent to half the weight of viral protein was added to the reaction mixture.

Electron microscopy. A drop of sample was applied to a carbon film on a Formvar-coated grid for 1 min,

and excess material was removed with a piece of filter paper. After staining with a 2% solution of phosphotungstic acid (pH 7), the grid was examined in a Joel electron microscope.

Polyacrylamide gel electrophoresis. Purified virus was disrupted with 2.5% sodium dodecyl sulfate (SDS) and 0.15 M dithiothreitol and analyzed in an 8.5% polyacrylamide gel containing SDS as described by K. B. Tan and B. R. McAuslan (*Virology, in press*). About 25 μ g of protein was applied to gels 15 cm long and 4 mm in diameter. Electrophoresis was at 2 to 3 v/cm for 16 hr.

RESULTS

Preparation of subviral particles. Subviral particles ("cores") can be prepared from poxvirus and reovirus; such "cores" have adenosine triphosphatase activity associated with them (4, 5). We attempted the production of FV_3 "cores" by treatment of purified radioactive FV_3 with the nonionic detergent NP_{40} (Shell Oil Co.). 3H -thymidine-labeled FV_3 was treated for 15 min at room temperature with either 0.05% or 0.5% NP_{40} in 0.05 M Tris (pH 7.6) containing 0.05 M mercaptoethanol and then centrifuged in CsCl gradients. Virus particles normally banded at a position corresponding to a density of 1.275 g/cm³. After treatment with 0.05 or 0.5% NP_{40} , particles banded at densities of 1.365 and 1.385 g/cm³, respectively (Fig. 1). Detergent treatment of the virus resulted in 1,000-fold loss of infectivity as measured by standard plaque assay.

Polypeptides associated with subviral particles. NP_{40} treatment of virus produced a population, homogeneous with respect to density, of subviral particles. When FV_3 doubly labeled with 3H -thymidine and ^{14}C -amino acids was treated with NP_{40} (0.5%, 15 min, room temperature) before centrifugation on sucrose gradients, a large proportion of labeled protein (approximately 65% of the total) appeared at the top of the gradient (Fig. 2); all thymidine label remained with the subviral particles or "cores."

At least 16 polypeptides constitute the structural proteins of FV_3 (K. B. Tan and B. R. McAuslan, *Virology, in press*). The polypeptides associated with cores were determined by disrupting cores with SDS (K. B. Tan and B. R. McAuslan, *Virology, in press*) and fractionating the resultant polypeptides on polyacrylamide gels. It appears (Fig. 3) that NP_{40} treatment readily removes polypeptides 3, 4, 6, 13, 14, 15, and 16 as well as a large proportion of polypeptide 5, which is a major polypeptide found by isotope labeling. On the other hand, polypeptides 1, 2, 7, 8, and 9 are firmly associated with cores.

In other experiments, radioactive polypeptides liberated from virus labeled with 3H -leucine were found to be those which were absent from viral cores.

FIG. 1. Formation of FV_3 subviral particles by treatment with NP_{40} . Purified 3H -thymidine-labeled FV_3 was treated with 0.05 and 0.5% NP_{40} as described in the text. Untreated and treated virus (250 μ liters, 1 OD_{280} unit/ml) were layered onto preformed CsCl density gradients [20 to 40% CsCl (w/w) in 0.01 M Tris-hydrochloride, pH 7.6]. After centrifugation (SW41 rotor, 35,000 rev/min, 4 hr, 5 C), fractions (0.5 ml) were collected and refractive indexes were measured. Radioactivity in each fraction was determined after collecting acid-precipitable material on membrane filters. Purified FV_3 not treated with NP_{40} (●—●), treated with 0.05% NP_{40} (○—○), treated with 0.5% NP_{40} (●—●).

Electron microscopy. Purified FV_3 preparations and subviral particles prepared from these by treatment with 0.5% NP_{40} were examined by electron microscopy. Negatively stained purified FV_3 particles varied somewhat in shape, but most were hexagonal. Full particles and some empty particles were observed (Fig. 4). The subviral particles produced by NP_{40} did not respond well to negative staining. They lacked a defined membrane and appeared to be stripped virus particles. Typical particles are shown in Fig. 4. It is clear that NP_{40} treatment does remove the outer component at least.

Adenosine triphosphatase activity of virus and subviral particles. The preceding data show that NP_{40} treatment of virus removes certain polypeptides almost completely while others remain associated with subviral particles. The polypeptides so liberated probably are those on the outer protein layer(s) of the virus, although one cannot exclude the possibility that some could be internal proteins.

Adenosine triphosphatase activity remains associated with whole virus despite repeated banding of virus in sucrose, CsCl, and tartrate density gradients. After conversion of purified

FIG. 2. Release of FV_3 proteins after treatment with 0.5% NP_{40} . Purified, labeled (3H -thymidine and ^{14}C -amino acid) FV_3 (left panel) was treated with NP_{40} (right panel) as described in the text. Untreated virus and treated virus (250 μ liters each) were then subjected to equilibrium sedimentation (SW65 rotor, 60,000 rev/min, 2 hr, 5 C) in sucrose gradients (40 to 80% w/v, 0.01 M Tris, pH 7.6). Fractions were collected and refractive indexes were measured. Radioactivity of acid-precipitable material in each fraction was determined by liquid scintillation spectrometry. 3H -thymidine ($\circ$ — $\circ$), ^{14}C -amino acid ($\bullet$ — $\bullet$).

FV_3 to subviral particles by NP_{40} treatment, adenosine triphosphatase activity which originally coincided with the virus band in sucrose density gradients was now found to coincide with the position of subviral particles (Fig. 5). About 75% of the original adenosine triphosphatase activity was recovered in the core fractions, and no activity was found elsewhere in the gradient.

Separation of FV_3 -associated adenosine triphosphatase from adenosine triphosphatase of uninfected cells. Cytoplasmic extracts from uninfected cells and FV_3 -infected BHK cells were subjected to rate zonal sedimentation in sucrose gradients. The virus band and the corresponding fractions in the gradient from uninfected cells were collected and centrifuged to equilibrium in sucrose gradients under conditions where virus banded (Fig. 2) according to density. The gradients were fractionated, and adenosine triphosphatase activity was tested (Fig. 6). Clearly, particulate cellular adenosine triphosphatase was separated from virus-associated adenosine triphosphatase; gross contamination of virus with the cellular material is unlikely. Guanosine triphosphatase activity (relative level of adenosine triphosphatase : guanosine triphosphatase = 1 : 0.9) in infected cells was found in fractions which sedimented in sucrose gradient more slowly than virus and which were coincidental with fractions of uninfected cells containing maximal adenosine triphosphatase activity. After further purification, the virus banded in $CsCl$ at a position corresponding to a density of 1.275 g/cm³ (Fig. 7). Adenosine triphosphatase coincided with infec-

FIG. 3. Electropherogram of purified FV_3 before (a) and after (b) treatment with 0.5% NP_{40} and isolation of cores. Gels stained with Coomassie Blue were scanned at 620 nm in a glass cuvette in a Gilford spectrophotometer equipped with a linear transport mechanism. (a) and (b) were not scanned at the same intensity scale. The polypeptides are numbered according to the scheme of Tan and McAuslan (*Virology*, in press).

FIG. 4. Electron micrographs of negatively stained virus particles (top panel) and particles purified after treatment with 0.5% NP_{40} (bottom panel). $\times 42,000$.

tivity and optical density. No guanosine triphosphatase activity was detected in purified virus. If we assume that guanosine triphosphatase and adenosine triphosphatase activities are in the one host enzyme (and this remains to be proved), then the absence of guanosine triphosphatase activity in virus particles is a further demonstra-

FIG. 5. Adenosine triphosphatase activity and FV_3 subviral particles in density gradients. Untreated virus and detergent-treated virus (1 ml each) were subjected to equilibrium sedimentation in sucrose gradients (Fig. 2). Fractions (0.2 ml) were collected and OD_{260} and refractive indexes were measured. Adenosine triphosphatase activity of 20- μ l samples was assayed with ATP- γ - ^{32}P as substrate. The peaks of adenosine triphosphatase activity coincided with the peaks of optical density, which are not shown. Purified FV_3 not treated ($\bullet$ — $\bullet$), treated with 0.05% NP_{40} ($\circ$ — $\circ$), treated with 0.5% NP_{40} ($\bullet$ — $\bullet$).

FIG. 6. Adenosine triphosphatase activity of cytoplasmic extracts from uninfected and FV_3 -infected BHK cells in sucrose density gradient. Cytoplasmic extracts (from 4×10^7 cells) of infected and uninfected cultures were first submitted to a zonal sedimentation in sucrose gradients (K. B. Tan and B. R. McAuslan, *Virology*, in press). Visible virus band and the corresponding fractions in the gradient from uninfected cells were then re-banded in sucrose density gradients by equilibrium sedimentation (Fig. 2). Optical density for uninfected cells ($\circ$ — $\circ$) and infected cells ($\bullet$ — $\bullet$), adenosine triphosphatase activity for uninfected cells ($\circ$ — $\circ$) and infected cells ($\bullet$ — $\bullet$), assayed with 3H -ATP as substrate.

FIG. 7. Adenosine triphosphatase activity in $CsCl$ density gradient of purified FV_3 . The virus band from sucrose gradients (Fig. 6) was collected and further purified in a second density gradient. Purified FV_3 [1 ml in 0.01 M Tris (pH 8) and 0.7 mg of protein/ml] was centrifuged in a $CsCl$ density gradient (conditions as in Fig. 1). Fractions (0.4 ml) were collected from the bottom of the tube and refractive indexes were measured. Optical density ($\circ$ — $\circ$) of each fraction was determined. After dialyzing each fraction against 0.01 M Tris (pH 7.4), adenosine triphosphatase activity ($\bullet$ — $\bullet$) of 20- μ l samples was measured using ATP- γ - ^{32}P as substrate. Infectivity (not plotted) coincided sharply with optical density and adenosine triphosphatase activity.

tion of separation of virus from cellular adenosine triphosphatase.

Comparison of nucleotide phosphohydrolases in unrelated viruses. Preliminary measurements (unpublished data) have indicated that the K_m for FV_3 -associated adenosine triphosphatase lies in the range of those reported for reovirus and poxvirus (3, 7). It would not, therefore, be a useful criterion to distinguish it from other viral adenosine triphosphatases. Accordingly, the substrate specificities of reovirus, rabbitpox, and FV_3 were compared. The results (Table 1) indicate that each virus type exhibits different substrate specificities for the various nucleotides tested. Saturating levels of substrates were used. As compared to the activities found in FV_3 , reovirus grown in L cells or BHK or rabbitpox from BHK cells shows substantial activity for GTP and higher activity for uridine triphosphate. Further, the specificity of the FV_3 -associated nucleotide phosphohydrolase activity was not altered by conversion of particles to subviral entities.

DISCUSSION

We assume that those polypeptides readily removed from virus by NP_{40} represent the outer

TABLE 1. Nucleotide phosphohydrolase activity expressed relative to adenosine triphosphate (100%)^a

Nucleotide triphosphate	FV ₃ grown in BHK-cell	FV ₃ grown in BHK cells subviral particles	Reovirus grown in BHK cells	Reovirus grown in L cells	Rabbit-pox grown in BHK cells
Adenosine triphosphate	100	100	100	100	100
Guanosine triphosphate	0	0	30	30	56
Cytidine triphosphate	4	4	25	19	64
Uridine triphosphate	1	1	15	12	53
Deoxyadenosine triphosphate	70	65	94	98	

^a Nucleotide phosphohydrolase activity was measured as described previously (9). Purified frog virus 3 (FV₃, 10 μ g) was incubated for different times (15 to 90 min) with the indicated substrates. Initial velocities of the splitting rate for all nucleotide triphosphates are expressed relative to ATP (100%). The same method was used for poxvirus grown in baby hamster kidney (BHK) cells, reovirus from both L and BHK cells, and FV₃ subviral particles. A 10- μ g amount of reovirus was employed for each reaction mixture. The amount of purified FV₃ subviral particles used for the assay was obtained from an equivalent of 20 μ g of untreated virus.

layer(s) of the viral particles and that the resultant substructures are analogous to poxvirus or reovirus "cores." Certain polypeptides are firmly associated with viral deoxyribonucleic acid (DNA) after detergent treatment (Fig. 3). Of these, polypeptides 1 and 9 have been shown to be arginine-rich (8).

The subviral particles produced by detergent treatment are a homogeneous population with respect to density and do not appear to be merely collapsed particles (Fig. 1 and 4).

Nucleotide phosphohydrolase activity of purified FV₃ remains tightly associated with subviral particles produced by NP₄₀ treatment. Preliminary attempts to solubilize the enzyme by treatment with nonionic detergents, SDS, or *n*-butanol have not been successful. A mixture of mercaptoethanol plus sodium deoxycholate which solubilizes adenosine triphosphatase from vaccinia virus particles (Munyon and Paoletti, *personal communication*) did not result in the liberation of any significant amount of nucleotide phosphohydrolase from FV₃. Gentle digestion with

proteolytic enzymes did not achieve solubilization of the FV₃ adenosine triphosphatase. Therefore, the enzyme activity is unlikely to be merely adsorbed to the outer surface of virus particles but is probably an integral viral component.

When rabbitpox, reovirus, and FV₃ are grown in the one cell type and purified extensively, they exhibit different specificities with respect to nucleotide phosphohydrolase activity (Table 1; reference 5). If we accept that either rabbitpox or reovirus has one enzyme activity rather than four different activities, as has been shown by Munyon et al. (7) and Borsa et al. (3), then each of the three viruses has a different nucleotide phosphohydrolase, which is probably virus-specified. It seems improbable that each virus type would acquire adventitiously a different adenosine triphosphatase when maturing in the cytoplasm of a BHK cell.

The available evidence indicates that adenosine triphosphatase is an integral component of each of three unrelated viruses. These viruses all are comparatively large, have a double-stranded genome, and replicate entirely in the cell cytoplasm. In considering the possible function of reovirus adenosine triphosphatase, it has been suggested that is responsible for suppressing DNA synthesis by degrading dATP (3). This is an unlikely function in the case of the deoxyviruses, FV₃, and pox; however, in view of the recent findings of Banerjee et al. (2) that reovirus messenger RNA terminates with GDP, a possible role of nucleotide phosphohydrolase in this virus would be to convert the GTP at the 5' terminus to its corresponding GDP. It is conceivable that in FV₃ and pox replication viral adenosine triphosphatase might modify the appropriate terminal nucleotide of messenger ribonucleic acid. Early poxvirus messengers have terminal poly A sequences (6); whether FV₃ messengers have a corresponding sequence is not yet known.

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