Virus-Associated Nucleases: Location and Properties of Deoxyribonucleases and Ribonucleases in Purified Frog Virus 3

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At least three nuclease activities are associated with purified frog virus 3. These activities are endodeoxyribonuclease (pH 7.5, double-stranded [DS] and single-stranded [SS] deoxyribonucleic acid [DNA]); endodeoxyribonuclease (pH 5.0, DS and SS DNA); endoribonuclease (DS and SS ribonucleic acid [RNA], pH 7.5). These activities are not adsorbed to the surface of the virion but are within the viral capsid and require detergent disruption of virions to unmask enzyme activity. Only one activity, deoxyribonuclease (pH 5.0, SS and DS DNA) appears to be core-associated after detergent disruption of virions. The ribonuclease degrades poliovirus replicative-form RNA, reovirus native RNA, and poly(I)·poly(C) to a product with a sedimentation coefficient of about 6S. Q β 6S DS RNA and 4S transfer RNA are not degraded. The ribonuclease appears to be a late function of the virus and is elicited in a soluble form as well as a virus-associated form.

Some complex viruses have enzyme activities, other than ribonucleic acid (RNA) polymerase, firmly associated with their virions. For example, poxvirus, reovirus, and frog virus 3 (FV₃), although quite unrelated biologically, have nucleotide phosphohydrolase activities (4, 6, 16). In each of these cases, the enzyme activity is associated with the subviral structures known as cores. The location of this enzyme activity in cores, together with circumstantial evidence for viral coding of such activity (2, 16), has led to the suggestion (8) that nucleotide phosphohydrolase activity is an integral part of the virion rather than a contaminant and might have an essential role in virus replication.

In addition to nucleotide phosphohydrolase activity, poxviruses have deoxyribonuclease activities within cores (1, 11). These also may have a function in viral deoxyribonucleic acid (DNA) replication. Although there is at present no practical way to prove this directly, the idea would be strengthened if other deoxyviruses were found to have the same complement of deoxyribonucleases with the same intraviral location. With this in mind, we have undertaken a survey of the nucleases in FV₃ which, like poxvirus, is a deoxyvirus that replicates in the cell cytoplasm.

This communication describes attempts to characterize FV₃-associated nucleases in some detail and to establish their location in the virion.

MATERIALS AND METHODS

Virus. FV₃ was produced and purified by using gradients of ribonuclease-free sucrose as described by Tan and McAuslan (15). Virus thus obtained had only trace amounts of host polypeptides associated with it (15) but was subjected to further purification by density gradient centrifugation in a linear potassium tartrate gradient [25 to 45% (w/w) tartrate in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.8, SW41 rotor, 36,000 rev/min, 4 hr at 15 C]. Virus was removed from tartrate by centrifugation and suspended in sterile phosphate-buffered saline at a concentration of 0.2 mg of viral protein (10⁸ plaque-forming units [PFU] per ml). As shown before (15), such virus preparations were free from any host membranes detectable by electron microscopy.

The virus preparation used was free of bacteria detectable by inoculation of blood-agar plates, thio-glycolate medium, or Trypticase soy broth. Mycoplasma have not been detectable in these purified virus stocks.

Detergent and buffered saline. The nonionic detergent Nonidet P-40 (NP40) was obtained from the Shell Oil Co. Phosphate-buffered saline (calcium- and magnesium-free) was obtained from Grand Island Biological Co., Grand Island, N.Y.

Preparation of cores. Cores were prepared from detergent-treated FV₃ as described recently (2). One milliliter of the detergent-treated (0.5% NP40, 0.05 M mercaptoethanol, room temperature for 15 min) virus was layered on 2 ml of 30% sucrose (0.01 M Tris, pH 7.4) and then centrifuged (60,000 rev/min,

60 min, SW65 rotor, 4 C). Cores sedimented to the bottom of the tube and were suspended to 1 ml with phosphate-buffered saline.

Polyribonucleotides. Native reovirus RNA (double-stranded [DS] RNA) labeled with ^3H -uridine was prepared by the method of Shatkin and Rada (13). Poliovirus replicative form (RF) RNA (^3H -uridine-labeled, specific activity approximately 5×10^3 counts per min per μg) and transfer RNA (tRNA) from *Escherichia coli* (^{14}C -uridine-labeled, specific activity 3×10^4 counts per min per μg) were provided by G. Koch and S. Pestka, Roche Institute of Molecular Biology.

Q β 6S RNA (^3H -cytidine-labeled, specific activity 9×10^3 counts per min per μg) was synthesized by the method of Banerjee et al. (3) and donated by P. Trown, Hoffmann-La Roche, Inc. Poly(A) (^3H -A)·poly(U) duplex (Miles Laboratories) had a specific activity of 9,000 counts per min per μg . The polymer was layered onto a 5-ml linear sucrose gradient (5 to 20% [w/w] sucrose; 0.1 M NaCl; 0.001 M ethylenediaminetetraacetate [EDTA]; 0.01 M Tris-hydrochloride, pH 7.4) and centrifuged (SW65 rotor, 55,000 rev/min, 4 hr). Two fractions representing the peak of activity were pooled, and the duplex polyribonucleotide, isolated by ethanol precipitation, was used as substrate. Poly(I)·poly(C) (^3H -C) duplex (Miles Laboratories) (specific activity 10^4 counts per min per μg) was processed in the same way as poly(A)·poly(U) to provide substrate of comparable molecular weight. Only about 25% of the starting material banded in the gradient at the same point as the peak of poly(A)·poly(U) activity; this was collected and used as substrate in the assays described below. The sedimentation coefficient was not determined exactly but was of the order of 12 to 15S.

^3H -lambda DNA. Lambda phage (λc , 857 S7) was thermally induced in *E. coli* (W3110 *thy*⁻) growing in ^3H -thymidine-containing medium. After purification of lambda phage by the method of Yamamoto et al. (17), ^3H -DNA was extracted from this by the procedures of A. Skalka (*personal communication*). The specific activity of the DNA was approximately 60,000 counts per min per μg . When required, DNA was denatured (by adjustment to 0.05 M NaOH for 5 min at room temperature) and then readjusted to pH 8 with 1 M Tris-hydrochloride buffer before use. We determined the *S* value of the lambda DNA to be 31 and 40S in neutral and alkaline gradients, respectively.

Assay of ribonuclease activity. For assay of ribonuclease (DS RNA) the complete reaction mixture (150 μliters) contained: poliovirus RF RNA or reovirus RNA, 2 to 4 μg , 5,000 to 10,000 counts/min; NaCl, 0.2 M; NP40, 0.25% (v/v); Tris-hydrochloride buffer, pH 7.5, 0.12 M; MgCl_2 , 0.001 M; viral protein, 1.0 μg . The mixture was incubated usually at 37 C for 30 min, following which the mixture was adjusted to a final concentration of 1% sodium dodecyl sulfate and 0.001 M EDTA. The products of the reaction were separated by velocity sedimentation as described under each figure.

The gradients used were 5 to 20% (w/w) sucrose (in 0.1 M NaCl, 0.34% sodium dodecyl sulfate, 0.01 M Tris-hydrochloride, pH 7.5). For assay of ribo-

nuclease (single-stranded [SS]RNA), the procedures described recently (10) were used. This assay was essentially as described for DS ribonuclease except that SS poliovirus RNA was the substrate, and, where indicated, NaCl was omitted from the reaction.

Endodeoxyribonuclease. The assay measures the conversion of a unit length of lambda DNA (3×10^7 molecular weight) to acid-insoluble fragments. The reaction mixture (0.1 ml) contained 0.1 M Tris (pH 7.5), 2×10^{-3} M MgCl_2 , 1.0 μg of ^3H -labeled lambda DNA, 0.25% NP40, and 25 μliters of solubilized enzyme (1–2 μg of protein) or 20 μliters (4 μg of protein) of FV₃. After incubation (60 min at 37 C), the reaction was stopped by chilling in ice, and the reaction mixture was adjusted to 0.2 M NaOH for alkaline sucrose velocity analysis.

For sedimentation on neutral sucrose gradients, the reaction mixtures were adjusted to 0.01 M EDTA and 1 M NaCl before being layered onto gradients.

Sucrose gradient velocity sedimentation for deoxyribonuclease assay. Reaction mixtures (0.1 ml) were layered onto 3.6 ml of a 5 to 20% (w/v) linear sucrose gradient and centrifuged (SW56 rotor, 55,000 rev/min, 15 to 18 C) for various times as described in legends to figures. Either alkaline (0.3 M NaOH, 0.7 M NaCl, pH 12.5) or neutral (1.0 M NaCl, 0.001 M EDTA, 0.01 M Tris-hydrochloride, pH 7.4) sucrose solutions were used as indicated. Sedimentation values were obtained by the method of Studier (14).

Solubilization of FV₃ endodeoxyribonuclease. Purified FV₃ was adjusted to a final concentration of 0.5% NP40 and 0.05 M 2-mercaptoethanol for 15 min at room temperature, and then this virus solution was centrifuged at $100,000 \times g$ for 60 min at 4 C. The supernatant (NP40-supernatant) was used as a source of solubilized endonuclease.

Infection of BHK cells with FV₃. To measure induction of enzymes, hamster kidney cells (BHK 21/13) were grown as monolayers in Dulbecco's modified Eagle medium. The cells were infected with FV₃ at an input multiplicity of 2 to 5 PFU per cell. To block FV₃ DNA synthesis in some experiments, the medium was adjusted to 10^{-4} M cytosine arabinoside immediately after adsorption of virus. To prepare extracts for enzyme assay, cells were harvested and disrupted in hypotonic buffer, and the cytoplasmic fraction was obtained (9). This fraction was then centrifuged ($100,000 \times g$, 60 min) to provide a soluble fraction for enzyme assay.

RESULTS

Degradation of linear DS DNA. Highly purified FV₃ was prepared from infected hamster kidney cells. The virus was assayed for endonuclease activity as described above. Using as substrate lambda DNA carefully prepared to minimize shear during isolation, and using centrifugation conditions that show the distribution of end product sizes, we found the following.

(i) FV₃ has endonuclease activity, but it is absolutely necessary to disrupt FV₃ with the detergent NP40 to unmask nucleases. This result

is essentially in accord with a recent preliminary communication (P. Palese and B. R. McAuslan, *Virology*, *in press*). NP40-disrupted FV_3 degraded DS lambda DNA endonucleolytically. A broad distribution of product sizes was apparent (Fig. 1A, B), but no significant amount of acid-soluble products, which under the conditions used would remain at the top of the gradient, was produced.

(ii) Measurement of the mean sedimentation velocity of the product in both alkaline and neutral sucrose gradients indicated that, after degradation of lambda DS DNA by FV_3 , the product had a greater sedimentation velocity (average approximately 25S) in neutral gradients than in alkaline gradients (average approximately 15S) (Fig. 1A, B). These *S* values correspond to molecular weights of 9.7×10^6 and 5×10^6 , re-

spectively, estimated as the DS form. The average DS cleavage product has, therefore, about two single-strand breaks. This indicates that under the conditions of assay, FV_3 -associated nuclease cleaved DS DNA primarily by single-strand scissions.

(iii) Activity was measurable over a broad pH range with both SS and DS DNA. With DS DNA, pH 7.5 was optimal; with SS DNA, pH 5.0 was optimal (Fig. 2A, B). We have not determined whether SS and DS activities are physically separable enzymes.

Degradation of DS RNA. The presence of ribonuclease activities (DS RNA and SS RNA) in FV_3 was reported recently (10). In our hands the sensitivity of the assay with radioactive poliovirus RF RNA substrate is of the same order as the biological assay described in the earlier report. In fact only by using very small amounts of virus (0.1 μ g of protein) at reduced incubation

FIG. 1. Comparison of the cleavage product of digestion of λ double-stranded (DS) linear DNA by purified FV_3 -associated or solubilized endonuclease (pH 7.5) in neutral and alkaline sucrose gradients. 3H -labeled, DS λ DNA was incubated with Nonidet P-40 (NP40)-disrupted FV_3 or the NP40 supernatant fraction as described in text. A, After 60 min of incubation at 37 C, the reaction was stopped by adding EDTA (final concentration 0.01 M) and NaCl (final concentration 1.0 M) and the mixture was centrifuged through a 5 to 20% neutral sucrose gradient (2.5 hr, 55,000 rev/min, 18 to 20 C, SW56 rotor). B, After 60 min of incubation at 37 C, the reaction was stopped by addition of 0.3 M NaOH, and the reaction mixture was centrifuged through a 5 to 20% alkaline sucrose gradient (2 hr, 55,000 rev/min, 18 to 20 C, SW56 rotor). 3H - λ DNA, no enzyme (Δ); 3H - λ DNA plus NP40-disrupted FV_3 ($\circ$); 3H - λ DNA plus solubilized enzyme ($\triangle$). Fractions (0.15 ml) were collected from the bottom of the tube, and radioactivity was assayed by liquid scintillation spectrometry.

FIG. 2. FV_3 endodeoxyribonuclease activity at different pH values. Purified FV_3 was assayed for deoxyribonuclease activity in the presence of NP40 as described in text except that, as indicated, the buffer used was either 0.1 M sodium acetate, pH 5.0; 0.1 M potassium phosphate, pH 6.0; 0.1 M Tris-hydrochloride, pH 7.5. Either double-stranded (DS) or single-stranded (SS) 3H - λ DNA was used as substrate. After incubating reacting mixtures at 37 C for 60 min, the reaction was stopped by adding of EDTA and adjusting the mixture to 0.2 M NaOH. Reaction mixtures were centrifuged on alkaline sucrose gradients as described in text, and the gradients were fractionated (0.15 ml fractions). Radioactivity in each sample was determined by liquid scintillation spectrometry with a mixture of Spectrofluor (Nuclear-Chicago)-toluene- and Triton X-100. Substitution of other buffers for those described indicated that the changes observed were due to pH rather than salt effects. A, DS DNA used; B, SS DNA used. Control, no enzyme (Δ); pH 7.5 ($\triangle$); pH 5 ($\circ$); pH 6.0 ($\bullet$).

temperatures (28 C) could we detect the intermediates formed during endonucleolytic degradation of DS RNA.

Reaction mixtures were prepared with poliovirus RF RNA as substrate. The amount of virus used was reduced to 0.1 μ g per assay, the temperature of incubation was reduced to 28 C, and the incubation times were as shown; otherwise the assay was as described in Materials and Methods. Samples were withdrawn at different times during incubation and analyzed on gradients. The mode of action appeared to be endonucleolytic; that is to say, there was a progressive reduction in molecular size with time of incubation (Fig. 3), and no release of acid-soluble products could be detected. The size of the end product was investigated by running replicates of the assay just described and centrifuging the mixtures on gradients under conditions where tRNA (sedimentation coefficient 4S) banded near the center of the gradient. The shoulder on the tRNA marker towards the upper part of the gradient was due to low-molecular-weight impurities in the tRNA used. The product obtained from poliovirus RF RNA after 10 or 30-min incubation times (Fig. 3) sedimented faster than tRNA. In fact, the end product of degradation at 28 C had a sedimentation coefficient of about 6S (Fig. 4). Contrary to the re-

FIG. 3. Decrease in sedimentation rate of double-stranded (DS) RNA with time of incubation. Poliovirus replicative-form RNA was incubated with RV_3 as described in text, but the temperature of incubation was reduced to 28 C. Samples were centrifuged after 0 (●); 4 (△); 10 (×), or 30 (○) min of incubation. After addition of ^{14}C -tRNA marker, the reaction mixture was layered over a 5 to 20% sucrose gradient and centrifuged (Spinco SW56 rotor, 55,000 rev/min, 150 min, 20 C). Fractions (0.2 ml) were collected from the bottom and radioactivity was determined in a mixture of Spectrofluor-toluene-Triton X-100. Position of the marker is indicated by arrow.

FIG. 4. Comparison of sedimentation rates of product and tRNA. Samples of the reaction mixtures containing poliovirus replicative-form RNA incubated under the conditions described by Fig. 3 were mixed with ^{14}C -tRNA and centrifuged on 5 to 20% sucrose gradients (Spinco SW56 rotor, 55,000 rev/min, 8 hr, 20 C). Product of 10-min incubation (●); 30-min incubation product (or 60-min incubation at 37 C) (○); ^{14}C -tRNA (×).

sults of Palese and Koch (10), no further degradation of the product was found on prolonged incubation (up to 3 hr) or at 37 C, even in the presence of a 10-fold excess of enzyme. Similar results were obtained with reovirus native RNA.

Action on other duplex polyribonucleotides. The duplex polyribonucleotides poly (A)·poly (U) and poly (I)·poly (C), Q β 6S RNA (which is double-stranded to a considerable degree), and *E. coli* tRNA (which has at least some paired regions) were each tested as substrates under essentially the same assay conditions as described in Materials and Methods. The amount of viral protein used was 1 μ g, and incubation was for 60 min at 37 C. Neither Q β 6S RNA nor tRNA underwent significant reduction in size (Fig. 5).

Poly (A)·poly (U) underwent breakdown in the presence of 0.2 M NaCl; the broad distribution of products in the gradient (Fig. 6) suggests that unpaired regions of the polymer might be undergoing degradation by ribonuclease active on SS RNA (see below). Under the conditions of the assay, the conversion of poly(A)·poly(U) to acid-soluble material was 10%. In contrast,

FIG. 5. Action of FV_3 ribonuclease on $Q\beta$ 6S RNA and tRNA. Either 3H - $Q\beta$ 6S RNA or ^{14}C -tRNA (1 μg of each) was used instead of replicative-form RNA as substrate in a standard ribonuclease assay mixture (see text). At the end of incubation period (60 min), the mixtures were centrifuged under the same conditions described for Fig. 4. $Q\beta$ 6S RNA, no virus ($\bullet$); $Q\beta$ 6S RNA plus virus ($\bullet$ -- $\bullet$); tRNA, no virus ($\times$); tRNA plus virus ($\times$ -- $\times$).

poly(I)·poly(C) (the material we used had a high degree of fidelity in base-pairing and was resistant to pancreatic ribonuclease) was degraded by FV_3 in the presence of 0.20 M NaCl to a relatively homogeneous product with a sedimentation coefficient very close to 6S (Fig. 6).

Location of nuclease activities in FV_3 . Poxvirus deoxyribonucleases and nucleotide phosphohydrolase are located virtually exclusively with viral cores (1, 6). Conditions for the preparation of FV_3 cores have been established (2), and it has been shown that all the FV_3 nucleotide phosphohydrolase activity is associated with such cores (2, 8). It was of interest to see whether the FV_3 deoxyribonuclease or ribonuclease activities were also associated with cores.

Highly purified FV_3 particles were treated with 0.5% NP40 to solubilize the outer capsid and release cores as described previously (2). The mixture was then centrifuged (see Materials and Methods) to provide a supernatant fraction and a

FIG. 6. Degradation of poly(A)·poly(U) and poly(I)·poly(C). Reaction conditions were as described in text. After incubation, mixtures were centrifuged on 5 to 20% sucrose gradients as described for the assay of ribonuclease activity (Spinco SW65 rotor, 60,000 rev/min, 6 hr, 20°C); fractions (0.2 ml) were collected from the bottom of the tube. The arrow indicates the position of $Q\beta$ 6S RNA in gradient. Poly(A)·poly(U), no virus ($\bullet$ — $\bullet$); poly(A)·poly(U) plus virus ($\bullet$ -- $\bullet$); poly(I)·poly(C), no virus ($\circ$ — $\circ$); poly(I)·poly(C) plus virus ($\circ$ -- $\circ$).

particulate fraction (core). The supernatant fraction was then further centrifuged ($100,000 \times g$, 60 min) to remove nonsoluble fragments and provide a soluble fraction (NP40-supernatant). Cores were then usually washed once by resuspending them in phosphate-buffered saline (pH 7.0) and recentrifuging. They were then resuspended in phosphate-buffered saline (pH 7.0) containing 0.5% NP40 so that a given volume of this core fraction contained an amount of cores equivalent to that in an equal volume of the original virus used as a source of cores. Core and supernatant fractions were tested for nuclease activities as follows.

Deoxyribonuclease activity (SS, DS DNA). Deoxyribonuclease activity (pH 7.5) was found only in the soluble supernatant fraction. No ac-

tivity was found associated with cores even when a 10-fold greater amount of cores was used for assay. Only those results obtained with DS DNA are depicted, although similar results were obtained with SS DNA. The pH 7.5 deoxyribonuclease activity is clearly solubilized by NP40 disruption of virions and is completely removed from cores. In contrast, deoxyribonuclease activity (assayed with DS DNA at pH 5.0) was detectable in both the supernatant and core fractions (Fig. 7A, B). Cores could not be freed from this activity by further treatment with NP40 or by repeated washing in buffer. The limitations to quantitative endonuclease assays and possible differences in activity between bound and solubilized activities make it difficult to assess the efficiency of recovery of enzyme activity.

Ribonuclease activity (SS and DS RNA). Using the same subviral fractions described above, we tested for ribonuclease (DS RNA or SS RNA) activities. Both ribonuclease activities were solubilized by NP40 disruption and completely removed from cores (Fig. 8, 10). Ribonuclease could have been on the surface of virions or within the capsid. When purified whole virus was assayed for activity in the absence of NP40, no activity could be detected. This suggested that ribonuclease (DS RNA) probably was not on the surface. Further, when purified whole virus

FIG. 7. Distribution of endodeoxyribonuclease (double-stranded [DS] DNA) activity. Cores and Nonidet P-40 (NP40)-supernatant fractions were prepared as described in text. ^3H - λ DS DNA was incubated at 37 C for 60 min with either fraction. The reaction mixtures were analyzed on alkaline sucrose gradients as described in Fig. 1. A, Reaction at pH 5.0. B, Reaction at pH 7.5. ^3H - λ DS DNA (Δ -- Δ); ^3H - λ DS DNA plus cores ($\blacktriangle$); ^3H - λ DS DNA plus NP40-supernatant fraction ($\circ$).

FIG. 8. Distribution of ribonuclease (double-stranded [DS] RNA) between core and Nonidet P-40 (NP40)-supernatant fractions of purified FV₃. Purified FV₃ was treated with NP40 to produce a core fraction and an NP40-supernatant fraction as described in text. Each was assayed for ribonuclease activity with reovirus DS RNA as substrate. The amount of each fraction taken for assay was equivalent to taking 30 μ liters of original FV₃ stock. Control, no enzyme (Δ -- Δ); plus cores ($\bullet$); plus NP40-supernatant fraction ($\triangle$); plus FV₃ ($\circ$).

was first incubated with trypsin and then centrifuged out and resuspended in trypsin-free phosphate-buffered saline, ribonuclease (DS RNA) was still detectable, although less active, in NP40-disrupted FV₃ (Fig. 9). The slightly lowered activity is probably due to incomplete removal of trypsin.

Our data, in contrast to other results (10), suggest that ribonuclease activities are not surface constituents and are not, like adenosine triphosphatase, associated with cores. Palese and Koch (10) reported that the FV₃ ribonuclease acting on SS RNA is distinguishable from a similar activity in uninfected cells since it was inhibited by high salt concentrations (0.20 M NaCl). This suggested that this virus-associated SS ribonuclease activity is virus-specified. We also find that the SS ribonuclease activity is inhibited by 0.2 M NaCl, but only if whole virions are used for assay. Although the assay mixture includes NP40, if the NP40-disrupted virions are physically separated into distinct fractions, releasing the ribonuclease activity into the supernatant fraction, the ribonuclease is no longer

FIG. 9. Localization of ribonuclease (double-stranded [DS] RNA) within FV_3 . Purified FV_3 was tested for ribonuclease (DS RNA) activity after the following treatments. (i) Virus was not exposed to any detergent treatment before or during the reaction ($\square$). (ii) Nonidet P-40 was included in the reaction mixture as described in text ($\circ$). (iii) FV_3 was resuspended in 0.05 M Tris-hydrochloride (pH 7.5) and trypsin was added to a concentration of 0.13%. The mixture was incubated at 37 C for 5 min, layered over 2 ml of 36% sucrose in 0.1 M Tris-hydrochloride, pH 7.5, and centrifuged (SW65, 60 min, 4 C at 60,000 rev/min). The virus pellet was resuspended to its original volume in phosphate-buffered saline and assayed for ribonuclease in the presence (Δ) or absence ($\bullet$) of detergent. In these experiments native (DS) reovirus RNA was used in place of poliovirus replicative-form RNA. The sedimentation of reovirus RNA is indicated ($\blacktriangle$).

inhibited by high salt concentration (Fig. 10). Apparently, the high salt acts only on the ribonuclease when it is associated with an NP40-disrupted viral aggregate. In any case, under conditions where the soluble forms of the host- and virus-associated ribonuclease (SS RNA) are compared, neither is inhibited by high salt concentration, and there is no evidence to support the idea that the activity is unique to the virus.

Induction of nucleases. To speculate on the significance of virus-associated enzymes, it would be useful to know if the enzyme activities were virus-specified or at least induced by virus infec-

FIG. 10. Distribution of ribonuclease (single-stranded [SS] RNA) activity between core and Nonidet P-40 (NP40)-supernatant fractions; effect of salt on the solubilized activity. Purified FV_3 was disrupted with NP40 to prepare core and NP40-supernatant fractions. These were tested for ribonuclease (SS activity) using poliovirus SS RNA as substrate. Reaction mixtures were incubated for 60 min at 37 C. The reaction was arrested by addition of sodium dodecyl sulfate to a final concentration of 0.3%. Mixtures were then layered over 3.6-ml 15 to 30% sucrose gradients and centrifuged (SW56 rotor, 55,000 rev/min, 18 to 20 C, 2 hr). Control SS RNA, no enzyme ($\blacktriangle$); NP40-supernatant fraction with or without 0.2 M NaCl in the reaction mixtures ($\bullet$); cores with or without 0.2 M NaCl in the reaction (Δ).

tion. Considering the activities in question, namely ribonuclease (SS RNA), ribonuclease (DS RNA), deoxyribonuclease (DS, SS, pH 7.5), and deoxyribonuclease (DS, SS, pH 5.0), it was easiest to determine if ribonuclease (DS RNA) was induced by infection, since this latter activity was not detectable in extracts of uninfected cells. Monolayers of BHK cells were mock-infected or infected with FV_3 at an input multiplicity of 2 to 5 PFU/cell. Cells were harvested at 0, 3, 12, or 24 hr postinfection, and cytoplasmic extracts were prepared as described in Materials and Methods. These extracts were centrifuged ($100,000 \times g$, 60 min), and the soluble fraction (2.0 μ liters, 5 μ g of protein) was assayed for ribonuclease (DS RNA) activity. From Fig. 11 it can be seen that ribonuclease activity was not detectable at 0 hr postinfection; slight activity was evident at 3 hr postinfection, i.e., about the time that FV_3 replication begins. At 12 hr postinfection, a time when production of

FIG. 11. Induction of ribonuclease in FV_3 -infected cells. BHK monolayers were infected with FV_3 (see text). Replicates were treated with cytosine arabinoside (ara-C). Samples of infected cell extracts were assayed for ribonuclease (using polio replicative-form RNA) at 0, 3, 12, or 24 hr postinfection. For all time points, 2 μ liters (5 μ g of protein) of soluble cytoplasmic extract was used for assay. No ribonuclease activity was detectable at zero time even after increasing the amount of extract 10-fold. Left, Control, no extract added ($\blacktriangle$); time zero ($\triangle$); 3 hr ($\bullet$), 12 or 24 hr ($\circ$) postinfection with FV_3 . Right, Control, no extract ($\blacktriangle$); time zero, 3, 12, or 24 hr postinfection with FV_3 in the presence of ara-C ($\triangle$).

FV_3 is maximal (9), the ribonuclease activity was high. However, if cytosine arabinoside (10^{-4} M final concentration) was added at the time of infection to block FV_3 DNA synthesis (7), no ribonuclease (DS RNA) was elicited. From this we conclude that ribonuclease is induced in a soluble form by FV_3 replication and is probably a late function since FV_3 DNA synthesis is necessary for its induction.

Because of the limitations of quantitative endonuclease assays and the high background of ribonuclease (SS RNA) or endo- and exodeoxyribonuclease, we have not yet devised a satisfactory assay to detect the induction of these activities after FV_3 infection.

DISCUSSION

Based on the data presented we conclude that: (i) purified FV_3 has endodeoxyribonuclease and endoribonuclease activities within virions. Activity is not detectable unless the virus is disrupted with NP40.

(ii) Endodeoxyribonuclease (DS, SS DNA, pH 7.5) is located within the outer capsid and is completely solubilized by treating virus with the nonionic detergent NP40. We do not know if this activity represents one enzyme operating over the range pH 7.5 to 5.0 or an enzyme operating at pH 7.5 but contaminated with that activity described below (iii). The activity cleaves double-strand molecules primarily by introducing single-strand breaks.

(iii) An endodeoxyribonuclease (DS, SS DNA, pH 5.0) remains associated with the viral core after NP40 treatment. We cannot exclude the possibility that some activity may be liberated from cores by the NP40 treatment employed. Partial release of poxvirus core nuclease by NP40 has been reported (1).

(iv) Endoribonuclease activities (DS and SS RNA) are located within the outer capsid and are solubilized by NP40 treatment of virions. We think that the different results for distribution of activity found by others (10) might be due to poor purification of virus and its conversion to cores in their hands.

It is now evident that poxvirus and FV_3 have completely different spectra of associated nuclease activities (1, 11). Poxvirus has an exonuclease (pH 5, SS DNA) and an endonuclease (pH 7, SS DNA), both of which are firmly bound to cores. FV_3 has at least two separable endonucleases. One (pH 7.5, DS, SS DNA) is clearly not a core component, and the other (pH 5.0, SS, DS DNA) is bound to cores. If one considers only core-associated enzymes on the assumption that these are more likely to be integral parts of the virus, it seems that endodeoxyribonuclease activity is common to both viruses although the specific properties of the nuclease might be different in each case.

The fact that poxvirus, FV_3 , and adenovirus have endodeoxyribonuclease activity either as a core component (or as a capsid component in the

case of adenovirus [5]) indicates that such activity might be involved early in viral DNA replication. On this basis a search for endodeoxyribonuclease activity in the structural components of other DNA viruses is warranted. Preliminary data indicate endodeoxyribonuclease activity in purified equine herpesvirus (McAuslan, unpublished data).

The enzyme activities in the outer capsid of FV₃ (including protein kinase [H. Silberstein, B. R. McAuslan, and J. T. August, Fed. Proc. 31: 1061, 1972]) might simply be entrapped during encapsidation due to their affinity for viral lipoproteins. Until it can be shown that they are induced by FV₃ infection and their properties compared to the host activities, it would be premature to speculate on their significance. Ideally one needs viral mutants to establish if the enzymes are viral functions. The ribonuclease (DS RNA) that is found in virions is probably a late viral function since its induction requires viral DNA synthesis and it is produced as a soluble enzyme in excess of that incorporated into virions. This ribonuclease activity is distinguishable from *E. coli* ribonuclease III (12) in that the latter can degrade both reovirus RNA and Q β DS RNA to acid-soluble products. We have not been able to detect this activity in cells infected with poxvirus, pseudorabies virus, or even African swine fever virus (McAuslan, Polatnick, and Hess, unpublished data), another polyhedral cytoplasmic deoxyvirus. Based on studies with detergent-disrupted virus, Fig. 12 summarizes the current state of our knowledge of the localization enzymes associated with poxvirus and FV₃.

FIG. 12. Diagrammatic representation of the location of viral enzymes. The physically separable subviral particle containing the viral nucleic acid is labeled "core." In both cases the core is surrounded by a lipid-containing capsid. The diagrams are not drawn to scale. NTPase indicates nucleotide phosphohydrolase activity.

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LITERATURE CITED

1. Aubertin, A., and B. R. McAuslan. 1972. Virus-associated nucleases: evidence for endonuclease and exonuclease activity in rabbitpox and vaccinia viruses. *J. Virol.* 9:554-556.
2. Aubertin, A., P. Palese, K. B. Tan, R. Vilagines, and B. R. McAuslan. 1971. Proteins of polyhedral cytoplasmic deoxyvirus. III. Further studies on the structure of frog virus 3 and location of virus-associated adenosine triphosphate phosphohydrolase. *J. Virol.* 8:643-648.
3. Banerjee, A. K., U. Rensing, and J. T. August. 1969. Replication of RNA viruses. X. Replication of a natural 6S RNA by the Q-beta RNA polymerase. *J. Mol. Biol.* 45:181-193.
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. Burlingame, B. T., W. Doerfler, U. Pettersson, and L. Philipson. 1971. Adeno-virus endonuclease: association with purified adenovirus type 2. *J. Mol. Biol.* 60:45-55.
6. Gold, P., and S. Dales. 1968. Localization of nucleoside phosphohydrolase activity within vaccinia. *Proc. Nat. Acad. Sci. U.S.A.* 60:845-852.
7. Kucera, L. S., and A. Granoff. 1968. Viruses and renal carcinoma of *Rana pipiens*. VI. Interrelationships of macromolecular syntheses and infectious virus production in frog virus-infected BHK 21/13 cells. *Virology* 34:240-249.
8. McAuslan, B. R. 1971. Virus-specified enzymes, p. 25-44. In K. G. Wolstenholme (ed.), *Strategy of the viral genome*. CIBA Foundation Symposium, London.
9. McAuslan, B. R., and W. R. Smith. 1968. Deoxyribonucleic acid synthesis in FV-3-infected mammalian cells. *J. Virol.* 2:1006-1015.
10. Palese, P., and G. Koch. 1972. Degradation of single- and double-stranded RNA by frog virus 3. *Proc. Nat. Acad. Sci. U.S.A.* 69:698-701.
11. Pogo, B. G. T., and S. Dales. 1969. Two deoxyribonuclease activities within purified vaccinia virus. *Proc. Nat. Acad. Sci. U.S.A.* 63:820-827.
12. Robertson, H. D., R. E. Webster, and N. D. Zinder. 1968. Purification and properties of ribonuclease III from *Escherichia coli*. *J. Biol. Chem.* 234:82-91.
13. Shatkin, A. J., and B. Rada. 1967. Reovirus-directed ribonucleic acid synthesis in infected L cells. *J. Virol.* 1:24-35.
14. Studier, F. W. 1965. Sedimentation studies of the size and shape of DNA. *J. Mol. Biol.* 11:373-390.
15. Tan, K. B., and B. R. McAuslan. 1971. Proteins of polyhedral cytoplasmic deoxyvirus. I. The structural polypeptides of FV₃. *Virology* 45:200-207.
16. Vilagines, R., and B. R. McAuslan. 1971. Proteins of polyhedral cytoplasmic deoxyvirus. II. Nucleotide phosphohydrolase activity associated with frog virus 3. *J. Virol.* 7:619-624.
17. Yamamoto, K. R., B. M. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large scale virus purification. *Virology* 40:734-744.