

Ribonuclease Present in Myxoviruses

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Endoribonuclease splitting single-stranded poliovirus RNA is associated with virions of the myxoviruses fowl plague virus and Newcastle disease virus. These virions are devoid of deoxyribonuclease hydrolyzing single-stranded M13 phage DNA and ribonuclease hydrolyzing double-stranded poliovirus RNA. The latter enzyme, however, is present in allantoic fluids of infected chick embryos from which the virions are obtained.

Following the discovery of a DNA-dependent RNA polymerase in poxvirus^{1, 2}, or an RNA-dependent RNA polymerase in reovirus³ and of the RNA-dependent DNA polymerase^{4, 5} in oncogenic RNA-viruses, in the last few years an increasing number of enzymes synthesizing nucleic acids have been found to be associated with animal viruses.

Besides the synthesizing enzymes, enzymes degrading nucleic acids are also present in virions of different animal viruses. Endodeoxyribonuclease found in adenovirus type 2⁶ was recently localized in the virions' penton⁷. Endoribonucleases splitting single- or double-stranded RNA are associated with the oncogenic frog virus 3^{8, 9}. Endonuclease activity was demonstrated in preparations of avian myeloblastosis virus¹⁰ and of orthomyxoviruses and paramyxoviruses¹¹. Ribonuclease (RNase H) activity degrading RNA-DNA-hybrids, but not single- or double-stranded RNA or double-stranded DNA, was found in virions of avian myeloblastosis virus. This activity is associated with the reverse transcriptase of the virus particle^{12, 13}. We have communicated the existence of an endoribonuclease associated with virions of orthomyxoviruses and paramyxoviruses¹⁴. In this paper we present details demonstrating the association of this activity with virus particles, as well as some properties of the enzyme(s).

Materials and Methods

Virus preparations

Fowl plague virus (FPV), strain "Rostock", and Newcastle disease virus (NDV), strain "Italien", were propagated in 11 to 12-day-old chick embryos

at 37 °C. After 36 to 48 hours the infected eggs were chilled and the allantoic fluid was harvested. The viruses were concentrated and purified by a modification of the original high and low speed centrifugation^{15, 16} using the 4·500 ml rotor type 15 in a Beckman/Spinco ultracentrifuge. FPV and NDV were spun down at 15,000 rpm for 180 min at 4 °C. The sediment was dissolved in buffered saline and re-centrifuged at 80,000 × *g* after removal of insoluble material by low speed (2000 × *g*) centrifugation. The virus pellet dissolved in buffered saline, called "crude virus concentrate", usually contains between 30,000 and 120,000 hemagglutinating units (HAU) per ml of virus preparation. Details of the procedure and the purity of the material will be described (Wiegiers and Drzeniek, in preparation). The crude virus concentrate was further purified by sucrose gradient centrifugation, and the purity was checked using enzyme markers¹⁷. The purified virus preparation obtained is the subject for experiments described in this paper.

Poliovirus RNA

Single-stranded (ss) RNA was obtained by phenol extraction of purified poliovirus type 1, strain Mahoney, grown in HeLa cells in the presence of ³H-uridine. Double-stranded (ds) RNA (poliovirus replicative form) was prepared from HeLa cells infected with poliovirus type 1, strain Mahoney, in the presence of ³H-uridine¹⁸.

M13 phage DNA

Single-stranded M13 phage DNA was prepared from ³H-labelled M13 phages as described by Jaenisch *et al.*¹⁹.

Hemagglutinating activity

Hemagglutinating activity was measured by the agglutination of 0.5% chick erythrocytes in plastic trays as previously described²⁰.

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Ribonuclease activity

a. With ss RNA as substrate. RNase activity was determined as follows: 50 μ l of virus (FPV or NDV) were incubated with 0.5 μ g of 3 H-uridine labelled single-stranded poliovirus RNA (specific activity 24,000 cpm/ μ g RNA) in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.2, 0.001 M $MgCl_2$ in a total volume of 100 μ l. After 6 hours at 37 °C, 25 μ l of 3% yeast RNA solution were added and the RNA precipitated with 100 μ l 10% trichloroacetic acid. The samples were centrifuged at 10,000 $\times$ g for 10 min. The supernatant was counted in a toluene based scintillation fluid containing Triton X-100.

b. With ds RNA as substrate. 0.2 ml reaction mixture containing 100 μ l of purified NDV (1,600 HAU) or 100 μ l allantoic fluid (1,100 HAU) obtained from NDV-infected eggs were incubated in 0.2 M NaCl according to Palese and Koch⁸ with 2 μ g of poliovirus replicative form RNA for 2 hours, and analyzed in the same gradient as in Fig. 2, except that the centrifugation time was 3 hours.

Deoxyribonuclease activity

DNase-activity was tested by the method described by Palese and McAuslan²¹ using 3 H-labelled single-stranded DNA of the M13 phage. 100 μ l of purified myxovirus preparation were incubated at 37 °C with 50 μ l ss DNA, 20 μ l 1% Nonidet P40, 40 μ l 10^{-2} M $MgCl_2$, 40 μ l Tris-buffer, pH 7.4, and 150 μ l H_2O . After 60 min 10 μ l of 10% sodium dodecylsulfate 20 μ l salmon sperm DNA (5 mg/ml) and 20 μ l Pronase (2 mg/ml) were added to the sample and kept at 37 °C for 30 min. The mixture was layered on a neutral 5 to 20% sucrose gradient containing 1 M NaCl in 0.01 M Tris-buffer, pH 7.4, or on an alkaline sucrose gradient containing 0.1 M NaOH and 0.9 M NaCl. After centrifugation at 55,000 rpm for 150 min in the SW 56 rotor (Beckman), 0.2 ml fractions were collected and counted.

Results

Purified preparations of two different myxoviruses, fowl plague virus (FPV), and Newcastle disease virus (NDV), were examined for the presence of nuclease activities. The virus preparations were subjected to rate zonal centrifugation in sucrose gradients. The obtained fractions were tested for their ability to depolymerize poliovirus RNA after incubation at 37 °C. Fig. 1 demonstrates the coincidence of hemagglutinating (HA) and RNase activity. The HA activity represents FPV or NDV virions.

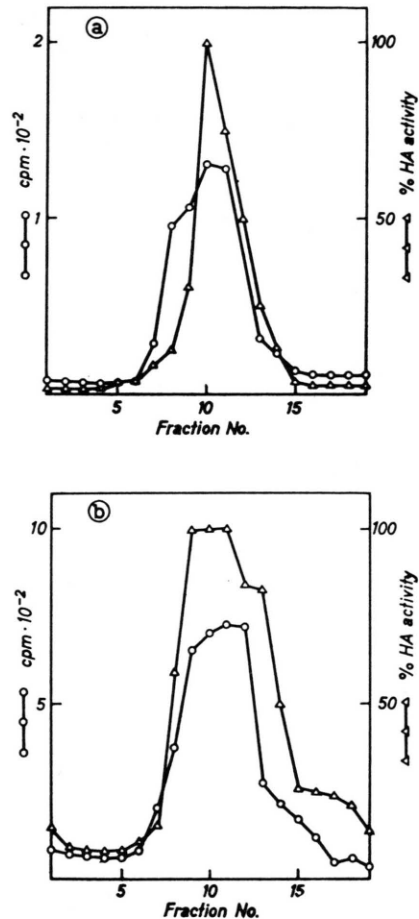


Fig. 1. Sucrose density gradient centrifugation of fowl plague virus (a) and Newcastle disease virus (b). 0.5 ml of a purified virus preparation containing 32,000 hemagglutinating units (HAU) of fowl plague virus or 8,000 HAU of Newcastle disease virus was layered on top of 5 to 20% sucrose gradient in 0.05 M Tris-HCl buffer, pH 7.2, containing 0.1 M NaCl. Centrifugation was carried out in a Beckman SW 40 rotor at 4 °C. Centrifugation conditions: (a) 35 min at 25,000 rpm; (b) 40 min at 15,000 rpm. 10-Drop fractions were collected and assayed for hemagglutinating and RNase activity. Δ — Δ , hemagglutinating activity; $\circ$ — $\circ$, RNase activity.

The distribution of both activities indicates the association of RNase activity with virus particles and the high degree of purity of these preparations, since infectious allantoic fluid and crude virus concentrates contain large amounts of nucleases beyond the virion fractions (Wiegiers and Drzeniek, in preparation).

To determine the mode of action of the virus-associated RNases, the sedimentation profile of poliovirus RNA incubated with FPV or NDV was ana-

lyzed by sucrose gradient centrifugation. Fig. 2 indicates the endonucleolytic activity of FPV and NDV virions.

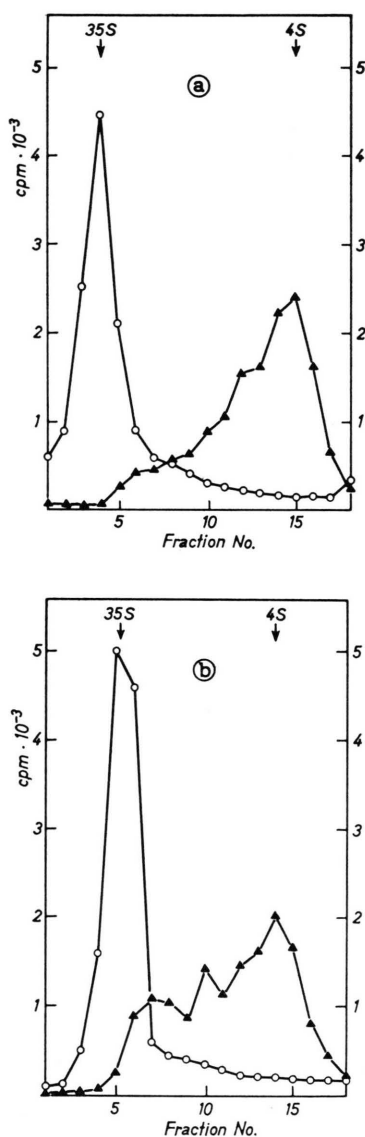


Fig. 2. Analysis of the ribonuclease product by sucrose density gradient centrifugation. 0.2 ml of the reaction mixture contained 0.1 ml purified virus: (a) fowl plague virus (6,400 HAU), (b) Newcastle disease virus (1,600 HAU) in 0.05 M Tris-HCl, pH 7.2, 0.1 M NaCl, 0.001 M MgCl₂, and 10 μ g of ³H-uridine labelled single-stranded poliovirus RNA. After incubation at 37 °C for 120 min, the reaction was terminated by the addition of 0.5% SDS. The reaction mixture was layered onto a 15 to 30% sucrose gradient containing 0.1 M NaCl, 5 mM Tris-HCl, pH 7.4, and 0.5% SDS. Centrifugation time was 2 hours in a SW 56 rotor at 55,000 rpm and 18 °C⁸. 0.2 ml fractions were collected and counted. ○—○, 35S poliovirus RNA; ▲—▲, 35S poliovirus RNA incubated with fowl plague virus (a) and Newcastle disease virus (b).

Fig. 3 elucidates the action of heat and the neutral detergents Nonidet P 40 and Triton N 101 on FPV and NDV RNases. Heating of the virions for 30 min at 60 °C increases the RNase activity. An increase of RNase activity is observed by treating FPV with Nonidet P 40 or Triton N 101. Larger amounts of these detergents, however, destroy the RNase activity of the NDV enzyme.

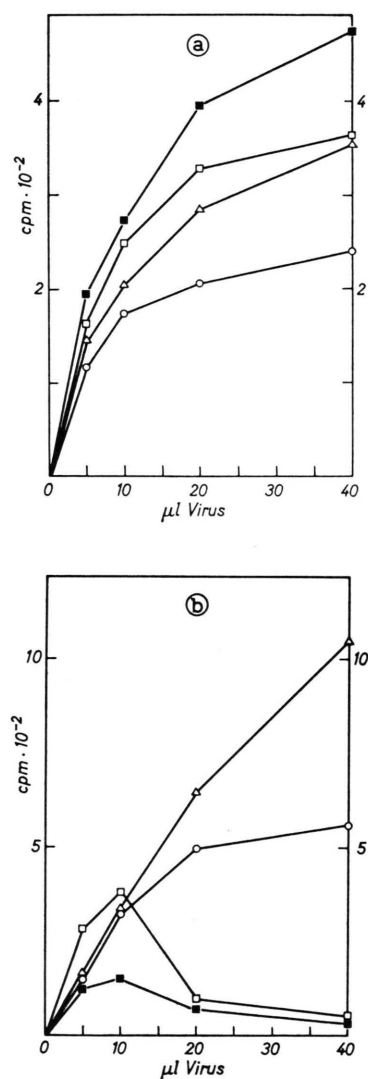


Fig. 3. RNase activity of virions treated with heat or detergents. Increasing amounts of purified virions were incubated with labelled poliovirus RNA as in Fig. 1. The incubation time was 120 min at 37 °C. (a) Fowl plague virus 64,000 HAU/ml; (b) Newcastle disease virus 16,000 HAU/ml. ○—○, untreated virus; △—△, virus treated for 30 min at 60 °C; □—□, virus incubated with 0.5% NP-40 at 37 °C for 30 min; ■—■, virus incubated with 0.5% Triton N 101 at 37 °C for 30 min.

No detectable depolymerizing activity towards double-stranded poliovirus RNA (replicative form) is found in FPV and NDV virions using the method of Palese and Koch⁸. Such activity, however, is present in the allantoic fluid of chick embryos infected with FPV or NDV (Wiegiers and Drzeniek, in preparation).

FPV and NDV are also devoid of deoxyribonuclease activity when tested with single-stranded DNA of the M 13 phage, since the distribution pattern of phage M 13 DNA was not effected by treatment with these virions.

Discussion

Our results demonstrate endoribonuclease activity associated with virions of FPV and NDV. At the moment we have no evidence whether this RNase is coded by the viral genome or a constituent derived from the cells in which the viruses were propagated. This activity does not seem to be bound accidentally to the virus particle, although it is present in allantoic fluid of infected chick embryos, since RNase hydrolyzing double-stranded poliovirus RNA — also present in the allantoic fluid — is absent in highly purified virus preparations. A similar situation was reported for the ATPase associated with virions of myxoviruses and for acid phosphatase absent in virus particles, although both enzymes were detected in the allantoic fluid of infected chick embryos¹⁷.

Endoribonuclease activity was found earlier in myxovirus preparations purified by equilibrium centrifugation in sucrose density gradients¹¹. No conclusive evidence was presented as to whether this activity is associated with virus particles. When starting with crude virus concentrates, we observed RNase activity in non-viral fractions of the gradient beyond the endoribonuclease associated with virus particles. To separate these activities, it is necessary

to use two different sucrose density centrifugation steps: One step which discriminates according to size and the other according to the density of the sample. Since crude preparations of myxoviruses contain non-viral particles, which have almost the same density as virus particles, the co-sedimentation of virions with the endoribonuclease activity in rate zonal centrifugation of highly purified preparations is the only appropriate measure of the association of this enzyme with virus particles.

At the moment the role of the endoribonuclease associated with myxoviruses can only be subject to speculations. Thus, the presence of a specific RNase in orthomyxoviruses could be responsible for the observed occurrence of this genome in six distinct pieces^{22, 23}. Since we found endoribonuclease activity both in orthomyxoviruses and in paramyxoviruses, the occurrence of RNA in one single piece in Newcastle disease virus²⁴ is in opposition to this suggestion. However, differences exist between the ortho- and paramyxovirus endoribonuclease, at least in their stability towards detergents (Fig. 3). Furthermore, the ribonucleoprotein of orthomyxoviruses is sensitive to pancreatic ribonuclease^{25–27}, whereas the NDV ribonucleoprotein is RNase-resistant²⁸.

It would be desirable to know, if the endoribonuclease is associated with the RNA polymerase found in the virus particle^{29–31} in analogy to the situation observed in avian myeloblastosis virus^{12, 13}. Experiments are underway to answer this and further questions concerning the localization of the enzyme in the virus particle and its function in the multiplication process of myxoviruses.

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