Degradation of Single- and Double-Stranded RNA by Frog Virus 3

(virus-associated enzymes/DNA virus/nuclease)

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ABSTRACT Purified preparations of frog virus 3 possess ribonuclease activities directed against single- and double-stranded RNA. Double-stranded RNAs isolated from purified reovirus type 3 and from HeLa cells infected with poliovirus and single-stranded poliovirus RNA from purified virus are readily degraded by incubation with frog virus 3. The mode of action of the nucleasees is endonucleolytic. Under the assay conditions used for the viral enzymes, crude extracts of uninfected HeLa, L, and baby hamster kidney cells did not show enzyme activity against double-stranded RNA but exhibited activity against single-stranded RNA. The dependence of the viral nucleasees on divalent cations for optimal activity and the inhibition of the cleavage of single-stranded RNA by 0.2 M NaCl suggests that the enzymes are either virus-coded or virus-induced.

A number of enzymes capable of synthesizing and degrading polynucleotides have been found to be associated with viruses. Polymerases using ribonucleotide triphosphates (1-6) are most likely to mediate early transcription of viral genomes. There is also substantial evidence that an RNA-dependent DNA polymerase is essential for the replication of onogenic RNA viruses (7-9). Little is known about the function of nucleases associated with animal viruses although there is little doubt that these enzymes are integral components of the mature virus particle. These include the DNases in pox-virus (10), adenovirus (11), Rous sarcoma virus (12), and frog virus 3 (FV3) (Palese, P. & McAuslan, B. R., submitted to J. Virol).

We report here that purified preparations of FV3, a complex, DNA-containing virus that replicates in the cytoplasm of infected cells, contain ribonucleases capable of degrading both double- and single-stranded RNA. An enzyme activity against double-stranded RNA has not been found in any other virus.

MATERIALS AND METHODS

Growth and purification of virus

FV3 was grown in baby hamster kidney (BHK) cells and purified by sonal and density gradient centrifugation (13). The virus preparations contained 1.5 A260 units, which corresponded to 0.5 mg of protein and 5 × 10⁶ plaque-forming units (PFU) per ml. Purified reovirus type 3 (Dearing strain) grown in L-929 cells was kindly provided by A. Shatkin. Poliovirus type 1, strain Mahoney, was propagated in HeLa cells and purified as described (14). All virus preparations were checked for bacterial contamination by incubation of aliquots in thioglycolate media at 37°C for 72 hr.

Preparations of substrate RNA species

Single-stranded poliovirus RNA was obtained from purified ³²P-labeled virus by phenol extraction (14). The original specific activity was 60,000 cpm/μg RNA. Double-stranded ³²P-labeled poliovirus RNA (RF-RNA) (specific activity, 30,000 cpm/μg RNA) was prepared from virus-infected cells, and the purity was established as described (15). Double-stranded RNA labeled with [¹H]uridine (specific activity, 4000 cpm/μg) extracted from purified reovirus type 3 with phenol was a gift from A. J. Shatkin and A. K. Banerjee. All radioactivity samples were counted in a toluene-based scintillation fluid containing Triton-X-100.

Assay of ribonuclease

The standard assay mixture for ribonuclease activity against double-stranded RNA contained 10 μg of FV3 protein and 0.5 μg of RF-RNA in a final volume of 0.2 ml TS buffer [10 mM Tris-HCl (pH 7.5)-0.2 M NaCl-1 mM MgCl₂-0.2% Nonidet P40 (NP-40)]. Incubations were for 60 min at 37°C. NaCl was omitted in the ribonuclease assay for single-stranded RNA that contained 1 μg RNA in 0.2 ml. Assay conditions were modified as indicated in the text. After the reaction was terminated by the addition of 0.5% sodium dodecyl sulfate (SDS), the samples were layered onto 15-30% sucrose gradients containing 0.1 M NaCl-5 mM Tris-HCl (pH 7.4)-0.5% SDS (16). Double-stranded poliovirus RNA was centrifuged for 3 hr and single-stranded poliovirus RNA for 2 hr in an SW-56 rotor at 55,000 rpm and 18°C. Double-stranded reovirus RNA was analyzed by centrifugation for 5 hr in an SW-56 rotor at 55,000 rpm and 18°C in 15-30% sucrose gradients [0.1 M NaCl-10 mM sodium acetate (pH 5)] (17). Ribosomal and soluble RNA used as markers were extracted by phenol from L-929 cells (18).

Ribonuclease activities against polio RF-RNA and single-stranded RNA were also determined by measurement of the decrease in infectivity with the agar cell-suspension plaque assay in HeLa cells (19).

RESULTS

Degradation of double-stranded poliovirus RNA by ribonuclease associated with FV3 particles

The degradation of ³²P-labeled poliovirus RF-RNA in high salt, i.e., 0.2 M NaCl and 1 mM MgCl₂, by purified FV3 can

Abbreviations: FV3, frog virus 3; RF-RNA, replicative form of RNA; SDS, sodium dodecyl sulfate; BHK, baby hamster kidney cells; SSC, standard saline citrate [0.15 M NaCl-0.015 M sodium citrate (pH 7.5)]; NP-40, Nonidet P40.

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be followed by analyses of sucrose gradients as shown in Fig. 1. The sharp peak of RF-RNA seen in the control sample broadens with increasing time of exposure to FV3 and is degraded to a smaller size. No accumulation of radioactivity is detectable at the top of the gradients and all RNA remains acid precipitable, indicating an endonuclease activity. The recovery of RNA from the gradients was 80-90%. Detergent treatment of the virus is not a prerequisite for eliciting ribonuclease activity directed against double-stranded RNAs, suggesting that the enzyme is either located near the surface of the virus particle or does not require the removal of the outer part of the virus in order to be active. Nevertheless, NP-40 was always added to the reaction mixture to obtain maximal activity.

The FV3 ribonuclease shows a strict dependence on Mg++. No activity could be detected in SSC [0.15 M NaCl-15 mM sodium citrate (pH 7.5)]. At low (5 x 10^-4 M) and high (5 x 10^-2 M) Mg++ concentrations, poliovirus RF-RNA is degraded to RNA fragments sedimenting at 4-10 S, whereas at 10^-4 M and 5 x 10^-4 M Mg++ all RF-RNA is cleaved to 4 S products by FV3. The FV3 enzyme is most active between pH 7.5 and 9. No activity was detected at pH values of 5 and 6. Under conditions described in Fig. 1, pancreatic RNase at concentrations up to 60 μg/ml does not degrade poliovirus RF-RNA as analysed by sucrose gradients, but more than 98% of the infectivity of poliovirus RF-RNA is inactivated.

Crude cell extracts of HeLa, BHK, and L-929 cells assayed under the conditions of high salt (0.2 M NaCl and 1 mM MgCl2) did not exhibit any detectable ribonuclease activity against poliovirus RF-RNA (Table 1). In addition, purified reovirus and poliovirus preparations did not degrade poliovirus RF-RNA. To exclude the possibility that this RNase is present in the host cell but occluded by an inhibitor, we added an extract of uninfected BHK cells to the standard reaction mixture containing poliovirus RF-RNA and purified FV3. The activity directed against poliovirus RF-RNA was not altered by cell extracts, indicating that there was no inhibitor present in uninfected cells that might have masked the ribonuclease activity of normal cells. Crude extracts of BHK cells infected with FV3, however, showed an RNase activity with respect to RF-RNA (Table 1). We conclude, therefore, that the ribonuclease acting on poliovirus RF-RNA is virus-coded or is induced by virus infection. Purified FV3 also degrades double-stranded reovirus RNA, whereas extracts of uninfected BHK cells do not degrade reovirus RNA (Fig. 2).

We followed the loss of infectivity of poliovirus RF-RNA after incubation with FV3 by measuring the reduction of induced infective centers by agar plate assay. The biological activity of RF-RNA decreases with increasing time of incubation with FV3 in high salt (0.2 M NaCl, 1 mM Mg++)

**Table 1. Ribonuclease activity on single- and double-stranded (RF) poliovirus RNA**

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Size of poliovirus RNA</th>
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<tbody>
<tr>
<td></td>
<td>Single-stranded</td>
</tr>
<tr>
<td>Purified FV3*</td>
<td>&lt;4 S</td>
</tr>
<tr>
<td>BHK cells‡</td>
<td>&lt;4 S</td>
</tr>
<tr>
<td>HeLa cells†</td>
<td>&lt;4 S</td>
</tr>
<tr>
<td>L-929 cells†</td>
<td>&lt;4 S</td>
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<tr>
<td>FV3-infected BHK cells</td>
<td>&lt;4 S</td>
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After incubation with FV3 preparations or cell extracts the size of poliovirus RNA was determined by sucrose density gradient centrifugation as described for Fig. 1. All s values are estimated with ribosomal and transfer RNA of L cells as markers.

* Standard reaction mixture as described under Materials and Methods.
† HeLa, BHK, and L-929 cells (10⁷ cells of each culture) grown in monolayers were washed with phosphate buffered saline (0.12 M NaCl-0.02 M sodium phosphate (pH 7.2)), scraped off the T-flask, and suspended in 0.5 ml 10 mM Tris-HCl (pH 7.5), containing 0.5% NP-40. After freezing and thawing, 50 μl of each cell extract was added to the standard reaction mixture containing either single- or double-stranded poliovirus RNA.
The degree of inactivation is dependent on the amount of virus present (Table 2) and on the salt composition in the reaction mixture. FVs does not alter the infectivity of RF-RNA in SSC, which makes the Mg++ unavailable. The infectivity of poliovirus RF-RNA increases by incubation with small amounts of FVs. This effect was consistently found but cannot be explained at the present time.

**Degradation of single-stranded poliovirus RNA by ribonuclease associated with FVs**

Purified preparations of FVs also possess a ribonuclease activity against single-stranded RNA. As can be seen in Fig. 3, single-stranded RNA from poliovirus is degraded to a smaller size when incubated with FVs.

The enzyme present in FVs preparations is active in the pH range 6-9. Addition of magnesium ions to the reaction mixture is not required, but EDTA at 10 mM is inhibitory. Apparently sufficient divalent cations are present in the RNA or virus preparation. In the presence of salt concentrations (0.2 M NaCl, 10 mM MgCl2) that are required for maximal enzyme activity against double-stranded RNA, no degradation of single-stranded RNA is observed. Extracts of both uninfected BHK cells and BHK cells infected with FVs also showed RNase activity against single-stranded RNA (Table 1). However, purified preparations of poliovirus and reovirus did not show any ribonuclease activity with respect to single- or double-stranded RNA under the assay conditions used with FVs.

Ribonuclease activity of FVs directed against single-stranded RNA can also be analyzed by reduction in infectivity. The degree of inactivation of viral RNA infectivity by FVs is dose and time dependent. In confirmation of the results obtained by sucrose gradient analysis, inactivation of single-stranded RNA infectivity by the FVs enzyme does not require added Mg++, although, as observed by gradient analysis, the inactivation of the infectivity of single-stranded RNA by FVs in SSC is strongly inhibited (Table 2). In contrast to all other known ribonucleases of animal cells that have been described (20) the FV enzyme acting on single-stranded RNA is inhibited by salt and by EDTA suggesting that it is virus-coded or virus-induced.

In an effort to determine whether both RNase activities are internal components of FVs, we treated virus particles with 0.5% NP-40. After 30 min at 37°C, the virus was centrifuged at 30,000 × g for 30 min. Supernatant and pellet were tested for RNases directed towards single- and double-stranded RNA. 50% of both enzyme activities were found in the supernatant fluid suggesting that these enzyme activities are surface constituents and that they are less firmly bound than other viral proteins including an adenosine triphosphate phosphohydrolase that has been described earlier (21). The remaining 50% of the enzyme activities were still associated with the pellet.

**DISCUSSION**

The present data show that extensively purified FVs possesses two RNase activities. One enzyme activity that is associated with FVs splits double-stranded RNA. This enzyme is not found in crude extracts of BHK, HeLa, and L-929 cells, but it is detected in crude extracts of BHK cells infected with FVs. The enzyme activity was consistently found in virus preparations of various passage numbers throughout the year, and no bacterial contamination was detected at any time. The activity of the enzyme that degrades double-stranded RNA is completely dependent on added Mg++ and thus seems to be different from the nuclease in serum described by Stern (22) and Friedman et al. (23). Based on these findings it is concluded that this enzyme is virus-coded or at least induced by the virus. The enzyme is probably located near the surface of the virion since it can be partly released by treatment with

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Time of incubation (min)</th>
<th>Double-stranded (RF) RNA</th>
<th>Single-stranded RNA</th>
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<tbody>
<tr>
<td>I</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>15</td>
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</tr>
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</table>

I. Poliovirus RF-RNA (4 ng) in SSC containing 20 mM Mg++ and single-stranded poliovirus RNA (8 ng) in 1 mM Mg++-20 mM Tris-HCl (pH 7.4) were incubated each with 0.5 µg of purified FVs in a total volume of 20 µl at 25°C. Aliquots of 5 µl were withdrawn at the times indicated, and the number of infective centers was determined by the agar cell suspension assay.

II. Reaction mixture as in I, but 100 ng of virus used.

III. Reaction mixture as in I, but 10 ng of virus used.

IV. Reaction mixture as in I, but in SSC.
the detergent, NP-40. This agrees with the observation that the undisrupted virus particle displays RNase activity without removal of external viral proteins. The FV1-associated RNase is active on double-stranded reovirus and poliovirus RF-RNA, and its mode of action appears to be endonucleolytic like ribonuclease III (24) found in bacteria.

The other enzyme activity associated with FV1 is an RNase that degrades single-stranded RNA. It is active at neutral and alkaline pH values and requires no additional Mg++. It is inhibited by high salt, but otherwise it is not distinguishable from RNase present in uninfected cells. Therefore, this is only suggestive evidence that this enzyme is virus specific.

Sucrose density analysis of RNA digests has been used for measurement of ribonuclease activity. The results correspond well with data obtained on the reduction of infectivity of poliovirus RNAs after incubation with FV1 preparations under different salt concentrations. Important advantages of the plaque assay are its greater sensitivity, especially in detecting RNase resistance under defined experimental conditions, and the convenience of using small quantities of RNA and virus.

We cannot determine at present if both RNase activities are due to one or different protein molecules. Both ribonuclease activities of FV1 show comparable activity directed against single- and double-stranded RNA, as determined by the infectivity assay, while bull semen ribonuclease (25) and pancreatic ribonuclease (26) are about 500 and 5000 times, respectively, more active against single-stranded RNA than against double-stranded RNA. The FV1 ribonucleases seem also to be different from ribonuclease H that is specific for RNA–DNA hybrids and that shows no activity with respect to double-stranded RNA (27). In view of recent findings that double-stranded RNA is present in cells infected by RNA (28) and DNA viruses (29), as well as in uninfected cells (30), and that double-stranded RNA is responsible for viral interference (30) we suggest that the FV1 RNase activities play an essential role in frog virus replication and in the metabolism of infected cells.

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