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A CYTIDINE SPECIFIC ENDONUCLEASE ACTIVITY ASSOCIATED WITH PURIFIED REOVIRUS VIRIONS

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SUMMARY

Purified Virions of Reovirus degrade poly C in single or double-stranded form. The nuclease activity is present in subviral particles but not in cores.

INTRODUCTION

The reovirus virion contains a polymerase or transcriptase activity associated with the viral core which can transcribe messenger RNA from the genome in vitro (1,2). Associated with the core are 2 other enzyme activities: the phosphohydrolase which converts nucleotide triphosphates to diphosphates (3,4) and a methylase which appears to be involved in methylation of the blocked 5 end of the reovirus messenger RNA (5,6). The poly (A) polymerase, not detectable in cores but only in complete virions or infectious subviral particles (7), is probably responsible for the synthesis of the oligo (A) found in reovirus virions.

Ribonucleases which degrade both single-stranded and double-stranded RNA have been found associated with the frog virus 3 (8) and with some RNA tumour viruses (9) but the function of these

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enzymes remains obscure. We have recently detected an endonuclease in purified reovirus virions which degrades Poly C in both single-stranded and double-stranded form. We here describe some of the properties of the enzyme activity which can be detected in subviral particles, but not in cores.

MATERIALS & METHODS

Purification of Virus L cells infected with Reovirus (m.o.i. 1 - 2p.f.u./ml) were harvested 20 hours after infection, suspended in PBS and extracted 3 times with Freon. The aqeous virus suspension (I) was layered over a cesium chloride gradient (1.32 - 1.42 g./ml) and centrifuged in the Spinco SW 25 rotor for 120 mins at 53,000 g. The virus band (II) was dialyzed against TE buffer (Tris buffer pH 7.4 10 mM containing 0.1 mM EDTA) and virions sedimented by centrifugation at 66,000 g. for 80 mins. The re-suspended virus (III) was layered over a sucrose gradient (20-40%) and centrifuged at 54,000 g. in the Spinco SW 25 rotor for 80 mins. The virus band was dialyzed against TE buffer (IV) pelleted as above, re-suspended (V) and centrifuged again through a cesium chloride gradient. The virus band was harvested and dialyzed to give the final purified virus.

Production of cores and subviral particles Cores of reovirus were produced by incubating virions with chymotrypsin in TE buffer at 37°C and pH. 7.4. for 60 mins (11). Subviral particles (SVP) were obtained by incubating virus for 1 hour at 40° with chymotrypsin (200µg/ml) in 10mM Tris pH 8.0 containing 0.15 M Nacl (12). The SVP and cores were run on CsCl gradients and shown to have a density of 1.445 g /cm^3 and 1.382 g /cm^3 respectively.

RESULTS

The ability of purified suspensions of reovirus to split various single-stranded polynucleotides was tested at pH 7.6 in the presence of magnesium chloride and at pH 5.5 in EDTA. While there was no release of TCA-soluble material at acid pH, with any of the polymers tested using up to 65 µg of virus, at pH 7.6, in the presence of magnesium chloride, a significant breakdown of poly C was found to occur. Poly I, poly U, poly G and poly A were not degraded at either pH. As shown in figure 1, the release of

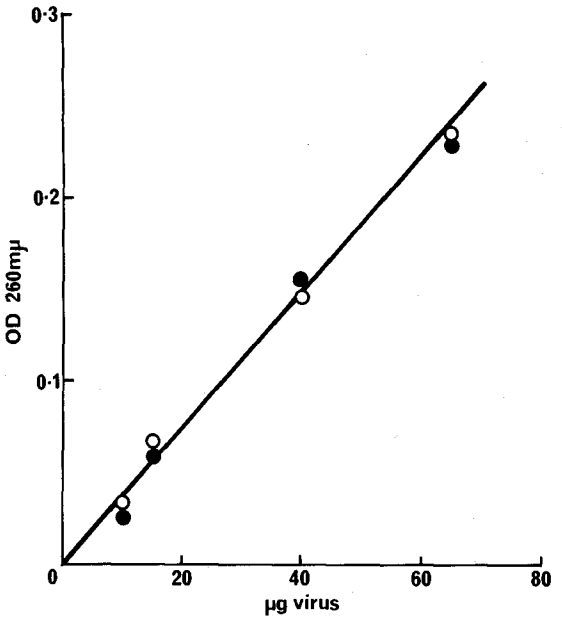

Figure 1. Breakdown of poly C and Poly I: C by Reovirus virions. Purified virus in the concentrations indicated was incubated at 37° in Tris chloride buffer 0.05M pH 7.6 containing 1mM $MgCl_2$ with 30 μg poly C (●—●) or 60 μg of poly 1:C (O——O) in a final volume of 1 ml. After 1 hour, 1 ml of 10% cold TCA was added, the precipitate removed by centrifugation and washed twice with 5% TCA. The TCA soluble material absorbing at 260 nm was estimated.

TCA-soluble material is proportional to the amount of reovirus added. The nuclease activity was found to be optimal at pH 7.0-7.6 in the presence of ImM magnesium chloride, neither calcium nor manganese could replace magnesium.

The activity detected by release of TCA-soluble material could be an exonclease or an endonuclease. The kinetics of the reaction shown in figure 2 suggest that the enzyme being measured is an endonuclease, since there is a lag before the appearance of TCA-soluble material. Furthermore, upon analyzing the products of the reaction by paper chromatography, the smallest product detected was a trinucleotide.

To test for nuclease activity of reovirus using double-stranded nucleic acid as substrate, poly I: C was prepared by annealing the single-stranded nucleotides and freed from any

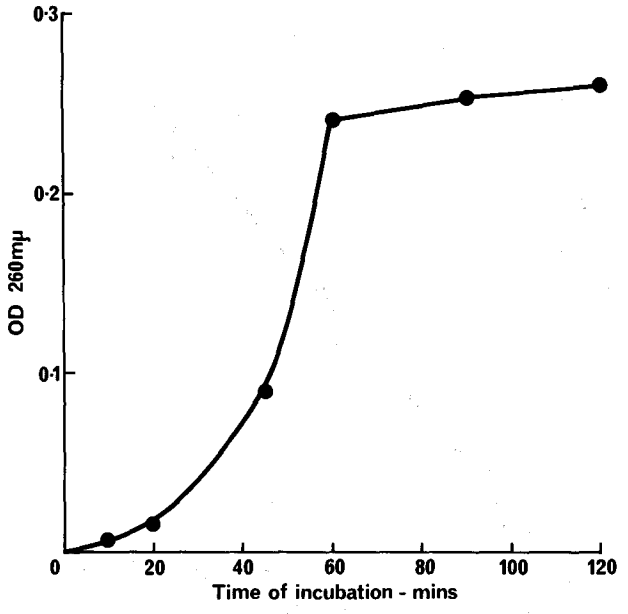

Figure 2. Time course of release of TCA-soluble material from poly C (30 μg) on incubation with reovirus (65 μg) under conditions described in figure 1.

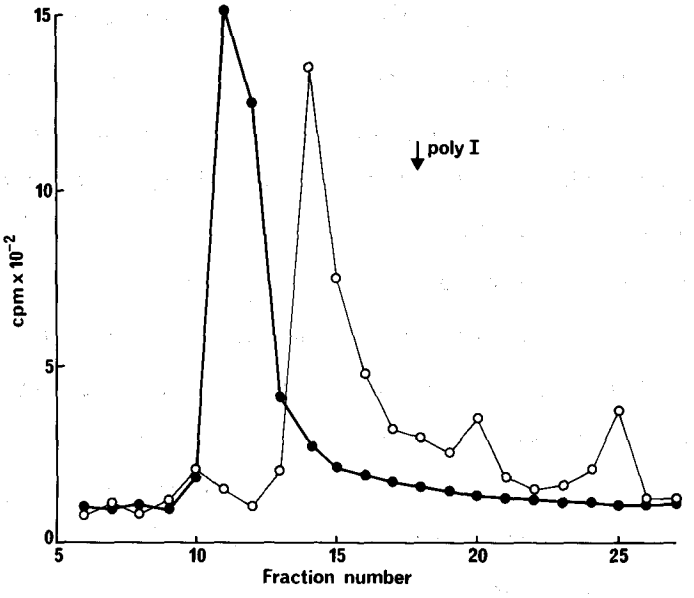

Figure 3. Chromatography of poly 1:C (prepared using tritium-labelled poly C) on Sephadex 100 after incubation with (O—O) and without (●—●) reovirus (65 μg) for 1 hour under conditions described in figure 1. The small molecular weight material, eluting later is not shown. Arrow indicates point of elution of poly I.

single-stranded tails by incubation with pancreative RNAase (1μg/ml) and passage through a column of Sephadex G100. Figure 1 shows that significant breakdown of the double-stranded polymer occured during incubation with the virions. Paper chromatography of the products showed the presence of only cytidine nucleotides indicating that only the poly C strand was degraded. Furthermore, the large molecular weight material remaining after incubation with reovirus was smaller than poly I: C but larger than poly I (figure 3). Thus, it would appear that the undegraded oligo C remained attached to the poly I.

Association of the nuclease with the viral particle. The nuclease activity, found to be associated with the viral particle could be an integral part of the virion or possibly a cellular enzyme which remained attached to the virion during purification. Table 1 shows that further purification of virions beyond the sucrose gradient does not change the specific activity of the enzyme. Furthermore, no enzyme activity splitting poly C could be found in uninfected L cell extracts carried through the same procedure as the infected cells (Table 1), nor in the foetal calf serum used to grow the cells.

Chmyotrypsin digestion of virions can be used to produce "cores" from which σ_1, σ_3 and σ_2 are missing (2,11) or infectious subviral particles (SVP) from which protein σ_3 is missing and a piece of σ_2 (12). Temperature, concentration of chymotrypsin and ionic composition of the buffer determine which are formed (7,13). We have found that core particles, produced by chymotrypsin digestion in tris saline at 37^{o} do not contain nuclease activity (data not shown), whereas SVP, produced after limited digestion at 40^{o} in tris buffered saline retain the activity (figure 4). It would appear that the nuclease activity behaves like the poly (A)

TABLE 1

Behaviour of endonuclease activity during purification of Reovirus Virions from infected L cells

		Uninfected Cells	Infected Cells		
		Units of Enzyme activity/ml	Units of Enzyme activity/ml	p.f.u./m. x 10^8	Units of Enzymeper 10^8 p.f.u.
I.	Freon Extract	7.0	11.03	3.2	3.6
II.	Virus band from 1st $CsCl_2$ gradient	0.8	1.05	5.8	0.18
III.	Pellet from sedimentation of virus band	-	1.45	10.1	0.14
IV.	Virus band from sucrose gradient centrifugation	-	3.95	41.0	0.07
V.	Pellet from sedimentation of virus band	-	3.13	43.0	0.075
VI	Virus band from 2nd $CsCl_2$ gradient	-	3.02	44.0	0.075

Reovirus was prepared in L cells and purified as described in Methods. Each fraction was assayed for nuclease activity as described in the legend to figure 1 (using 30 µg of poly C) and for infectious virus. One unit of enzyme activity is defined as that amount which releases 1 OD at 260 mm from 30 µg of poly C after incubation for 1 hour at 37° under the conditions described in Figure 1.

polymerase activity of reovirus during protetolytic digestion of the virus (7) and association of the nuclease activity with the SVP suggests that the enzyme is an integral part of the virion.

Bond cleaved by nuclease. To determine whether the nuclease splits the sugar link at the 3' or 5' position, poly C was incubated under optimal conditions for 1 hour with reovirus and the reaction products were further degraded with alkali (0.3N

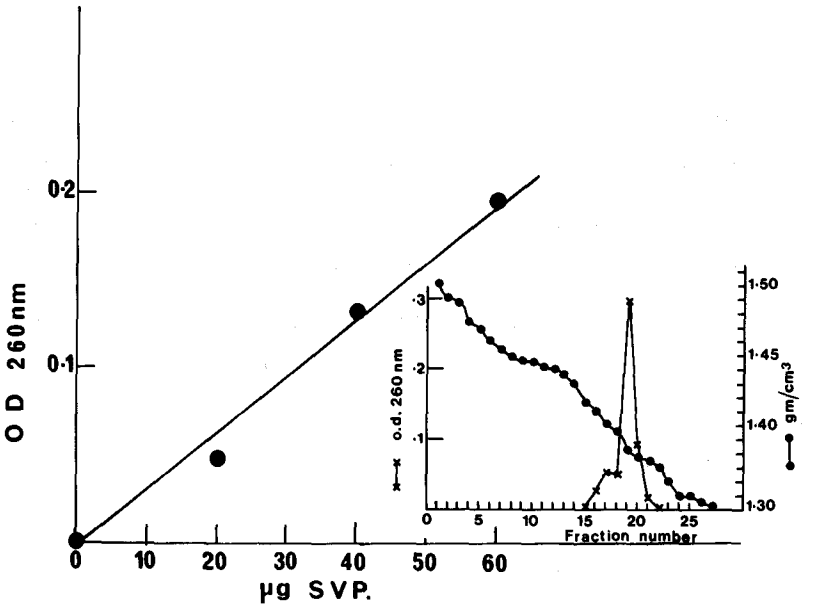

Figure 4. Breakdown of poly C by subviral particles. Virions were incubated with chymotrypsin (200 µg) for 1 hour at 40°C in 10mM Tris pH 8.0 with 0.15 M NaCl and the SVP so formed were (a) pelleted and re-suspended for assay of nuclease activity, and (b) layered onto a cesium chloride gradient as described in methods. Different volumes of the re-suspended SVP were incubated with 30 µg of poly C under the conditions described in figure 1, and TCA-soluble material absorbing at 260 nm was estimated. The inset shows the sedimentation profile of the SVP in a cesium chloride gradient.

NaOH) for 16 hours. After neutralisation with perchloric acid, the mixture was subjected to paper chromatography in ethanol: IM ammonium acetate pH 7.5 (75:30 to separate hydrolysis products. If the nuclease was splitting the bond between the phosphate and the 3' OH, then further degradation with alkali would yield mononucleotides, a nucleoside and a nucleoside diphosphate. If, however, the monesterified phosphate remained on the 3' OH group following digestion, the further degradation by alkali would yield only mononucleotides. In our experiments, only the mononucleotide was formed indicating that the reovirus nuclease forms products with the monoesterified phosphate on the 3' position and not with a 3' OH group.

DISCUSSION

Our data demonstrate an enzyme activity associated with reovirus virions which is not destroyed or released by limited digestion with chymotrypsin. Whether the enzyme is a virus-coded protein and plays a role in the processing of the reovirus genome remains to be seen. That the nuclease can split naturally-occuring RNA's was shown using the double-stranded RNA from the mould virus penicillium chrysogenum which appeared to be degraded specifically giving defined products (data not shown).

The ten reovirus genome segments contain cytosine in the 3' terminal position (14). While all available evidence indicates that the ten segments are synthesised and packaged as 10 separate units (14,15) and are not produced by the specific breakdown of a large molecule, some processing of the genome occurs (6,16). The nuclease activity we have found to be associated with the reovirus virion can split the bond between two cytosine residues in double-stranded RNA and it is conceivable that it could have a biological role in processing the genome.

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