Ribonucleic Acid Polymerase Activity Associated with Purified Calf Rotavirus

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SUMMARY

The presence of an RNA-dependent RNA polymerase is demonstrated in purified rotavirus particles. Optimum polymerase activity was found between 45 to 50 °C, at pH 8, and in the presence of 10 mM-magnesium ions. The polymerase product was highly sensitive to pancreatic RNase (97%) in low or high salt concentration. The enzyme was activated by EDTA treatment of intact particles or heat shock. The similarities between reovirus, blue-tongue virus and rotavirus polymerases are discussed.

INTRODUCTION

A reovirus-like agent has been found to be commonly associated with acute non-bacterial gastroenteritis in infant animals of various mammalian species (Mebus et al. 1969; Much & Zajac, 1972; Flewett, Bryden & Davies, 1973; Middleton, Petric & Szymanski, 1975). This has led to the recognition of a new group of morphologically and serologically (Woode et al. 1976) related viruses, called rotaviruses (Flewett et al. 1973) or duoviruses (Davidson et al. 1975).

More recent studies have established that the genome of these viruses consists of 11 double-stranded RNA segments (Newman et al. 1975; Rodger, Schnagl & Holmes, 1975) justifying the tentative classification of these agents within the Reoviridae family. In spite of some differences in morphology, antigenicity and chemical composition between rotaviruses and reoviruses, the existence of an endogenous polymerase could be suspected. The aim of the present investigation was to demonstrate the existence of such an enzyme using the calf rotavirus and to determine in an in vitro system the optimal conditions for its activity. Special emphasis has been placed here on a critical comparison of the in vitro polymerase activities of calf rotavirus and reovirus showing that (i) no difference exists for the basic requirements of the two polymerases and (ii) conditions of activation of the two polymerase activities are significantly different.

METHODS

Virus. The calf rotavirus used in this study was isolated from a five-day-old calf with naturally occurring diarrhoea. The faeces that contained large amounts of rotavirus particles were clarified and a filtered sample used to serially inoculate two gnotobiotic, colostrum-deprived calves, at the Laboratoire de Microbiologie du C.R.V.Z. Theix (Dr Gouet). Each experimentally infected calf became diarrhoeic 2 to 3 days post-infection. The virus shed by both calves was characterized by electron microscopy and by a fluorescent antibody test as previously described (Scherrer et al. 1976). A single faecal sample collected from the second
gnotobiotic calf was used as a source of virus throughout this work. Reovirus type 3 was grown in BHK cells and used as a control.

**Virus purification.** One vol. of faecal sample was diluted with 1 vol. of PBS and 2 vol. of Freon 113 were added. Homogenization in a Polytron homogenizer, was carried out for 5 min at 0 °C. After centrifugation of the homogenate (at 6300 g, for 10 min, 4 °C) the aqueous phase was removed, a ½ vol. of fresh PBS was added and the extraction procedure carried out once more. This cycle was repeated until the aqueous phase was no longer sufficiently turbid to warrant further repetition (about 4 to 6 cycles). Usually the aqueous phases from the two first cycles of extraction were re-extracted with fresh Freon 113. Virus in the pooled aqueous phases was pelleted by centrifugation for 90 min at 100000 g. The pellet was resuspended in PBS and CsCl was added to achieve a density of 1.37 g/ml. This was then centrifuged for 17 h at 35000 rev/min in an SW 50 rotor at 4 °C in a Beckman L 50 ultracentrifuge. The resultant virus-containing band, visible near the middle of the tube, was collected by lateral puncturing, examined by electron microscopy and desalted by centrifuging through a column of Sephadex G25 (Neal & Florini, 1973). This partially purified virus was further purified by rate zonal centrifugation in a sucrose gradient (15 to 45 %, w/v). Gradients were spun for 90 min at 24000 rev/min in a Spinco SW 27 rotor, collected from the top and monitored for u.v. extinction at 254 nm with an ISCO UA 5 spectrometer. Then the virus was pelleted and purified again by isopycnic banding in CsCl as described above. For reovirus this last step was omitted.

The density of virus particles was determined by measuring the refractive index \( n_\text{r}^{25} \) with an Abbe refractometer. The density was calculated using the formula

\[
d = 10.8601 n_\text{r}^{25} - 13.4974.
\]

Virus concentrations were estimated from the relationship established for the reovirus (Lai & Joklik, 1973): \( 5.4E_{260} = 1 \text{ mg/ml of virus}. \)

**Assay for polymerase activity.** Purified virus was pelleted at 100000 g for 90 min, resuspended in tris chloride 100 mM, pH 8, and added to the reaction mixture. The standard reaction mixture was adjusted to a final vol. of 0.25 ml which contained: tris chloride (pH 8) 25 μmol; adenosine triphosphate 0.6 μmol; cytidine triphosphate 0.6 μmol; guanosine triphosphate 0.6 μmol; phosphoenol pyruvate 2 μmol; pyruvate kinase 5 μg; magnesium acetate 25 μmol; actinomycin D 2.5 μg; \(^3\text{H}-\text{uridine triphosphate} 10 \mu\text{Ci (sp. act. 2 Ci/mmol).} \) This reaction mixture was incubated at 37 °C and the reaction was stopped by the addition of carrier yeast RNA and 3 ml of 5 % trichloroacetic acid containing 10 g/l sodium pyrophosphate. After 15 min at 0 °C the precipitates were collected by filtration on glass fibre filters (Whatman GF/C), washed with cold TCA, dried and counted in a liquid scintillation spectrometer Intertechnique SL 30.

**RNA-RNA hybridization technique.** After a 1 h incubation at 37 °C, the polymerase reaction mixture was centrifuged at 40000 rev/min in the SW 50 rotor of a Spinco ultracentrifuge to remove virus particles. The \(^3\text{H}-\text{labelled product in the supernatant was deproteinized with phenol-SDS, precipitated with 66% ethanol, desalted through a Sephadex G 25 column and finally precipitated again with ethanol.} \) Variable amounts of virus RNA (obtained by phenol extraction from purified virus preparations) in 10 mM-tris chloride, pH 7.9, 1 mM-EDTA, were mixed with 2500 ct/min of \(^3\text{H-RNA in the same buffer. The total assay mixture (50 μl) was heated at 98 °C for 5 min, then cooled in ice, made 0.3 M with NaCl and annealed for 2 h at 65 °C. For estimating ribonuclease resistance, ribonuclease A was added to a final concentration of 10 μg/ml and the solution was incubated for 20 min at 37 °C before precipitation with TCA and scintillation counting.} \)
RNA polymerase associated with rotavirus

Fig. 1. Isopycnic centrifugation of D and L rotavirus particles in CsCl gradient. (a) Untreated virus (L particles); (b) purified L particles treated for 20 min with 1 mM-EDTA at 37 °C; (c) mixture of standard (L particles, density = 1.359 g/ml) and EDTA treated particles. The latter were pelleted before isopycnic centrifugation in order to remove any trace of EDTA. Sedimentation is from left to right.

Chemicals. 

Purification of calf rotavirus and buoyant density determination

After the first isopycnic banding in CsCl, three light scattering bands were visible. Band no. 1 was at the top of the tube and had a density of 1.2 to 1.3 g/ml. Band no. 2 was located near the middle of the tube with a density of about 1.36 g/ml. The third band was always broad and diffuse with a density ranging from 1.39 to 1.41 g/ml. By electron microscopical examination of the different bands, it was found that the majority of the virus particles were located in band no. 2. A few particles could also be detected in bands no. 1 and 3 in association with some debris. Rate zonal sedimentation of the virus obtained from band no. 2 led to a single symmetrical peak.

In order to achieve a better purification and to determine its exact buoyant density, the virus was further purified in CsCl. It can be seen in Fig. 1 that the virus bands at a density of 1.359 ± 0.003 g/ml (mean value and standard deviation for six determinations). The purified virus was identified as calf rotavirus on the basis of examination in the electron microscope, specific immunofluorescence tests in tissue culture and polyacrylamide gel electrophoresis of the genome.

Characteristics of rotavirus RNA polymerase activity

Preliminary experiments indicated that EDTA pre-treatment of purified virus considerably enhances the polymerase activity. Fig. 1 shows that this treatment causes a definite shift of the virus buoyant density from 1.359 g/ml to 1.378 ± 0.007 g/ml (four determinations). Rotavirus having a density 1.378 g/ml will be referred to as rotavirus D (dense), and rotavirus having a density 1.359 g/ml as rotavirus L (light).

The requirements for in vitro uptake of labelled UTP into an acid insoluble product were examined using highly purified D virus (Table 1). The incorporation requires the presence of the four ribonucleoside triphosphates. The omission of CTP or CTP-GTP considerably...
Table 1. Characteristics of the in vitro synthesis of RNA by purified calf rotavirus D

<table>
<thead>
<tr>
<th>Reaction conditions*</th>
<th>Polymerase activity† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>− virus</td>
<td>0.9</td>
</tr>
<tr>
<td>+ actinomycin D 10 μg/ml</td>
<td>105</td>
</tr>
<tr>
<td>+ rifampicin 10 μg/ml</td>
<td>85</td>
</tr>
<tr>
<td>+ uridine 10 mg/ml</td>
<td>88</td>
</tr>
<tr>
<td>+ 2 mercaptoethanol 0.5 mM</td>
<td>102</td>
</tr>
<tr>
<td>− CTP</td>
<td>3</td>
</tr>
<tr>
<td>− CTP − GTP</td>
<td>0</td>
</tr>
<tr>
<td>− Mg²⁺</td>
<td>1</td>
</tr>
<tr>
<td>+ RNase 10 μg/ml‡</td>
<td>2</td>
</tr>
<tr>
<td>+ RNase 10 μg/ml§</td>
<td>3</td>
</tr>
</tbody>
</table>

* Standard assay conditions were used as described in Methods except where noted.
† The value indicated represents the mean of duplicate determinations and is expressed as the percentage of complete system (8415 ct/min) incubated for 30 min. A background incorporation of radioactivity of 130 ct/min was obtained for 0 °C incubated control and was subtracted to obtain the values listed above.
‡ RNase was added after incubation in the standard conditions and the reaction mixture was further incubated at 37 °C for 15 min.
§ Same as ‡ except that the reaction mixture was adjusted to 0.3 M-NaCl before addition of RNase.

diminishes the RNA synthesis. The extent of incorporation is not significantly affected by the presence of actinomycin D (10 μg/ml) or rifampicin (10 μg/ml). The product is highly sensitive to ribonuclease A either at low or high (0.3 M-NaCl) ionic strength.

The influence of pH on the polymerase activity is shown in Fig. 2. Although incorporation occurs within a range from pH 7.5 to pH 8.5, optimum is clearly reached at pH 8.

Divalent ion concentration

As already noted in Table 1 the polymerase activity is completely dependent on the presence of divalent metal ions. The requirement is satisfied by Mg²⁺ at an optimum concentration of 8 to 10 mM (Fig. 3). When Mg²⁺ was replaced by Mn²⁺, the enzyme activity is reduced 10- to 20-fold. For Mn²⁺ there is no clear optimum concentration.

Kinetics of RNA synthesis

Fig. 4 illustrates the time course of the reaction and shows that there is a linear increase of incorporation as a function of time of incubation for at least 9 h at which time this particular experiment was terminated.

Temperature

The kinetics of RNA synthesis at 7 different temperatures are illustrated in Fig. 5 and show that the optimum temperature for polymerase activity is about 50 °C. At 20 °C the rate of synthesis is negligible.

Influence of various pre-treatment conditions on the activity of the polymerase

To detect polymerase activity in purified rotavirus, six pre-treatment conditions were employed: (1) None (virus kept at 0 °C); (2) heat shock at 60 °C for 30 s followed by immersion in ice; (3) incubation at 37 °C for 20 min in the presence of 250 μg/ml of chymotrypsin; (4) incubation at 37 °C for 30 min in the presence of 200 μg/ml pronase (pronase was pre-incubated for 2 h to eliminate any traces of nucleases); (5) incubation at 37 °C for
Fig. 2. Dependence of D rotavirus RNA polymerase activity on pH. The same conditions of standard assay were employed except that 25 μmol tris acetate was used instead of tris chloride up to pH 7.0.

Fig. 3. Effect of magnesium (●–●) and manganese (○—○) ions on D rotavirus polymerase activity. Except for Mg²⁺ and Mn²⁺ the standard reaction mixture was used.

Fig. 4. Time course of RNA synthesis. D rotavirus was incubated for the indicated intervals in the standard assay mixture.

Fig. 5. Kinetics of RNA synthesis by D rotavirus polymerase at 7 different temperatures.

20 min in the presence of 1 mM-EDTA; (6) incubation with Triton X-100 (0.1%, 37 °C, 20 min). Under all pre-treatment conditions the virus was kept in 100 mM-tris chloride, pH 8. These treatments were applied to both rotavirus D and rotavirus L. Additionally, heat shock and chymotrypsin digestion were applied to reovirus which served as a control.
Table 2. Effect of various pre-treatments of purified calf rotavirus particles on RNA polymerase activity

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>D virus density 1.378 g/ml</th>
<th>L virus density 1.359 g/ml</th>
<th>Reovirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>140,000</td>
<td>3,400</td>
<td>72</td>
</tr>
<tr>
<td>Heat shock 60 °C, 30 s then immersion in ice</td>
<td>135,000</td>
<td>98,000</td>
<td>45,900</td>
</tr>
<tr>
<td>Chymotrypsin 250 µg/ml, 37 °C, 20 min</td>
<td>155,000</td>
<td>4,900</td>
<td>52,000</td>
</tr>
<tr>
<td>Pronase 200 µg/ml, 37 °C, 30 min</td>
<td>161,000</td>
<td>5,100</td>
<td>ND†</td>
</tr>
<tr>
<td>EDTA 1 mM, 37 °C, 20 min</td>
<td>133,000</td>
<td>152,000</td>
<td>ND</td>
</tr>
<tr>
<td>Triton X-100 0.1 %, 37 °C, 20 min</td>
<td>143,700</td>
<td>3,800</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Each assay mixture contains 0.03 E60 unit of purified virus. Standard assay conditions as described in Methods. Values listed represent the means of duplicate determination. A background of 112 ct/min was subtracted in each case. The relatively low activity in rotavirus L is interpreted as being contaminating rotavirus D in the preparation.
† Not done.

As shown in Table 2, reovirus exhibited a transcriptase activity only after heat shock or chymotrypsin digestion (Borsa & Graham, 1968; Skehel & Joklik, 1969). Rotavirus D incorporated 3H-UTP with a high specific activity under all experimental conditions. Chymotrypsin, pronase, EDTA, Triton X-100 and heat shock did not enhance the enzyme activity. Rotavirus L on the other hand had little polymerase activity compared with D virus. Chymotrypsin, pronase and Triton X-100 had no effect on L virus but a 20-fold enhancement of the polymerase activity was observed after heat shock or EDTA pre-treatment.

Characterization of the 3H-labelled product

On the basis of the above observations, rotavirus contains an RNA polymerase. Moreover it is highly probable that such an enzyme would operate as a transcriptase. To confirm this hypothesis 3H-labelled polymerase product was annealed with virus RNA. The extent of hybridization was obtained by calculating the percentage of the total single stranded RNA counts recovered as TCA precipitable counts in the RNase-resistant hybridization.
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product. The results are shown in Fig. 6. If double stranded RNA is omitted from the hybridization mixture less than 1% of the single stranded RNA can be recovered as RNase-resistant material. An increase in the amount of double stranded RNA results in a corresponding increase in percentage hybridization. The minimum amount of double stranded RNA required for optimum hybridization is in the order of 2 µg of double stranded RNA/2500 ct/min of 3H-labelled product.

DISCUSSION

The above results show clearly that calf rotavirus, a virus known to possess a segmented double stranded RNA (Welch & Thompson, 1973; Newman et al. 1975; Rodger, Schnagl & Holmes, 1975) contains a polymerase capable of synthesizing single stranded RNA in vitro. This polymerase transcribes the virus genome with a high degree of base sequence fidelity since 100% hybridization is reached after annealing with double stranded RNA extracted from purified virus.

Comparing reovirus and rotavirus polymerases it appears that both enzymes have very similar requirements for magnesium (concentration of about 10 mM for maximum activity), and pH (optimum at 8). Additionally, both enzymes have the same temperature dependence with a maximal activity at about 50 °C. The major difference between reovirus and calf rotavirus polymerase concerns the activation process. In the case of reovirus, the polymerase is known to be latent in intact particles, and becomes fully active only after the virus capsid has been altered by means of chymotrypsin treatment (Skehel & Joklik, 1969) or heat shock (Borsa & Graham, 1968).

When calf rotavirus was purified using PBS as dilution buffer, L particles were obtained: these had a low polymerase activity even after they had been exposed to chymotrypsin. Moreover, neither pronase nor Triton X-100 were able to activate the enzyme. Apparently these treatments do not alter the L particles significantly since it was found that their buoyant density remained constant (results not shown). In contrast, heat shock or treatment of L particles with 1 mM-EDTA occasions a considerable stimulation of the polymerase activity.

To our knowledge, rotavirus is the only virus that can be activated by this treatment. Since EDTA is known to solubilize several proteins or glycoproteins of the cell membrane (Singer, 1974) it can be hypothesized that the activation process involves a loss of external components. Interestingly enough Bridger & Woode (1976) have found that some faecal samples contain two particle types of rotavirus, characterized by the presence (smooth particles) or the absence (rough particles) of an extra capsid layer. Subsequently, rough particles were shown not to be infectious in culture, and to sediment at a density of 1.38 g/ml. Smooth particles had a density of 1.36 g/ml and were fully infectious. Our results demonstrate that particles having a density of 1.36 g/ml can be converted by chemical treatment to particles having a density of 1.38 g/ml, and that such a conversion corresponds to a considerable unmasking of the virus associated polymerase. Since our particles exhibit the same density as those described by Bridger & Woode (1976), it is possible that they may carry other common properties.

In the case of blue-tongue virus (BTV), the prototype of the orbiviruses, it was also demonstrated (Verwoerd et al. 1972; Martin & Zweerink, 1972) that a slight alteration of the particles, shifting the density from 1.36 g/ml to 1.38 g/ml, is sufficient to activate the endogenous polymerase. However, in contrast to rotavirus, the BTV polymerase enzyme is not activated by heat shock (Verwoerd & Huismans, 1972). Furthermore, its optimum temperature is 28 °C and not 50 °C.
Finally, when we compare the present results with those obtained by others with reovirus and BTV, it clearly appears that rotavirus polymerase has unique requirements (temperature, when compared to BTV; activation process when compared to both BTV and reovirus). These data may further justify provisional classification of rotavirus in a distinct genus of the Reoviridae family.

REFERENCES


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