In Vivo and In Vitro Synthesis of Human Rhinovirus Type 2 Ribonucleic Acid¹

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HeLa cells infected with human rhinovirus type 2 synthesize a mixture of single- and double-stranded ribonucleic acid (RNA). The RNA synthesized by the membrane-bound RNA polymerase complex in vitro is also a mixture of single- and double-stranded RNA, whereas the deoxycholate-treated RNA polymerase complex synthesized only double-stranded RNA. Although twice as much cell-associated viral RNA is synthesized in vivo at 34 C than at 37 C, there is no difference in the rate of RNA synthesized in vitro at 34 C and 37 C by the polymerase complex. The RNA polymerase complex, after treatment with deoxycholate, sediments as a broad peak with an average sedimentation value of 120S.

The infection of a host cell with a picornavirus results in the appearance of a ribonucleic acid (RNA)-dependent RNA polymerase complex in the cytoplasm of the host cell. Poliovirus has been the prototype of this group of viruses. The viral polymerase is attached to the particulate membrane structures and can be released by treatment of these structures with an anionic-nonionic detergent mixture (3). The polio RNA polymerase complex solubilized by detergent has a sedimentation coefficient of 70S, and the complex synthesizes poliovirus-specific RNA in vitro.

Human rhinoviruses contain single-stranded RNA of 30 to 32S (8). The virions are acid-sensitive, and their buoyant densities in CsCl are higher than the pH 3 stable poliovirus (4, 12).

In this report we describe the in vivo RNA synthesis of the human rhinovirus type 2 (HRV-2) and the isolation of a RNA polymerase complex from HeLa cells infected with the same virus. This complex synthesizes viral RNA in vitro.

MATERIALS AND METHODS

Cells and virus. A continuous line of HeLa cells, "HeLa-O cells," was obtained from Flow Laboratory, Rockville, Md. They were propagated in monolayer culture at 37 C in McCoy's 5A medium (6) containing 10% calf serum, 100 µg of neomycin per ml, 100 µg of penicillin per ml, 100 µg of streptomycin per ml, and 25 µg of fungizone per ml.

The virus used was HRV-2, strain HGP, obtained from R. R. Grunert of Stine Laboratory, E. I. du Pont de Nemours and Co., Newark, Del.

Virus infection. Monolayers of HeLa cells were grown to about 80% confluency. They were washed once with calcium- and magnesium-free phosphate-buffered saline, then infected with virus at a multiplicity of infection of 40 plaque-forming units (PFU)/cell. Virus was adsorbed for 30 min at 34 C. McCoy's 5A medium containing 5% heat-inactivated fetal calf serum and antibiotics at concentrations identical to that used for cells was then added to the monolayers. Virus multiplication was carried out at 34 C. Actinomycin D was added to a concentration of 5 µg/ml in experiments where it was desirable to inhibit host RNA synthesis.

Polyacrylamide gel electrophoresis of viral RNA. Cylindrical gels (0.8 by 10 cm) were prepared with recrystallized acrylamide by using the base-soluble ethylene diacrylate in the buffer system described by Loening (6). Electrophoresis was carried out in a buffer containing 0.04 tris(hydroxymethyl)-aminomethane (Tris), 0.2 M sodium acetate, 0.002 M ethylenediaminetetraacetic acid (EDTA), and 0.5% sodium dodecyl sulfate (SDS), pH 7.7. The gels were fractionated into 2-mm fractions with a mechanical cutter (5) in a hexane-dry ice bath maintained at -20 C. The fractions were first solubilized with 0.5 ml of concentrated NH₄OH, then counted in Bray's counting solution in a liquid scintillation counter.

Phenol extraction of RNA. Infected cells were scraped from culture dishes and collected by centrifugation. The cells were suspended at 4 C in a buffer of 0.01 M Tris, (pH 7.2), 0.01 M NaCl, and 0.001 M MgCl₂, and were ruptured with 14 strokes in a stainless-steel Dounce homogenizer. The nuclei were removed by low-speed centrifugation (800 × g, 5 min), the supernatant fraction (crude cytoplasm) was made 0.05 M in a sodium acetate (pH 5.0), 0.01 M in EDTA, 0.5% in SDS, and the mixture was then extracted with phenol at 45 C as previously described (10). RNA produced by the polymerase complex in vitro was extracted by the same procedure but at 25 C.

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Preparation of crude polymerase. HeLa cell monolayers were infected with HRV-2 virus in the presence of actinomycin D. At the end of 7 hr, the cells were removed by scraping, collected by low-speed centrifugation, and the cell pellet was frozen at -70°C . At this stage, the *in vitro* RNA polymerase can retain its activity for at least a month. Approximately 4×10^8 frozen cells were suspended in 10 ml of cold buffer containing 0.05 M Tris ($\text{pH } 7.2$), 0.002 M MgCl_2 , and 0.1 M NaCl. The cells were allowed to swell at 4°C for 10 min; they were ruptured with 14 strokes in a stainless-steel Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation at $800 \times g$ for 5 min. The supernatant fraction (the cytoplasmic extract) was centrifuged at $30,000 \times g$ for 20 min, and the pellet was suspended in 2 ml of buffer containing 0.05 M Tris ($\text{pH } 8.0$) and 0.01 M NaCl. This fraction contained cytoplasmic membrane and was designated the crude polymerase complex, and the supernatant fraction was designated the cytoplasmic soluble fraction. The nuclei were suspended in the same buffer. A 1:1 mixture of 10% Nonidet P-40 and 5% sodium deoxycholate (DOC) was added in the ratio of 0.2 ml of detergent mixture per 1 ml of suspended nuclei. A detergent wash of the nuclei was prepared by the method of Ehrenfeld et al. (3).

***In vitro* RNA polymerase assay.** A 0.1-ml sample of enzyme was used for assaying polymerase activity. It was incubated at 34°C for 30 min with 0.1 ml of a mixture containing the following constituents: 0.25 μmoles of the 5'-triphosphates of adenine, cytidine, and uridine (ATP, CTP, and UTP, respectively); 10 μmoles of Tris buffer ($\text{pH } 8$); 1 μmole of MgCl_2 ; 0.25 μmoles of phosphoenolpyruvate; 5 μg of phosphoenolpyruvate kinase; 1 μg of actinomycin D; 1.3 μmoles of dithiothreitol; and 2.5 μCi of ^3H -guanosine-triphosphate (GTP, specific activity 1.3 Ci/mmole). The reaction was terminated by adding 0.5 ml of ice-cold 0.1 M $\text{Na}_4\text{P}_2\text{O}_7$ and 0.5 ml of 50% trichloroacetic acid. The precipitate was collected on a filter, and radioactivity was counted in a scintillation counter.

Reagents. Actinomycin D was purchased from Mann Research Laboratory, Inc., New York, N.Y.; ^3H -uridine (36.8 Ci/mmole) and ^3H -GTP (1.3 Ci/mmole) were purchased from New England Nuclear, Boston, Mass. Unlabeled ATP, CTP, and UTP were purchased from Schwarz BioResearch, Inc., Orangeburg, N.Y. Bovine pancreatic ribonuclease, pyruvate kinase (rabbit muscle), and 2-phosphoenolpyruvate were purchased from Calbiochem, Los Angeles, Calif. ^3H -labeled virion RNA was kindly supplied by K. K. Lonberg-Holm of the Central Research Department, E. I. du Pont de Nemours and Co., Wilmington, Del. (8).

RESULTS

Rate and temperature dependence of the *in vivo* synthesis of HRV-2 viral RNA. When HeLa cells are infected with HRV-2 at a multiplicity of 40 PFU/cell, one cycle of virus growth is completed at 12 hr at 34°C (F. H. Yin, unpublished data). The rate of viral RNA synthesis during one-cycle growth was monitored by the incorporation of

^3H -uridine into viral RNA. The synthesis of HRV-2 RNA can be detected 3 hr after infection, and the rate of synthesis reaches a peak 7 hr after infection and then declines. Viral RNA synthesized at 7 hr after infection is usually 15 to 20 times more than the actinomycin D-treated HeLa cell background. Since rhinoviruses grow to a higher titer at 34°C than at 37°C (11), the effect of temperature on the synthesis of HRV-2 RNA was examined. Figure 1 shows that the amount of viral RNA synthesized at 34°C at 8 hr after infection is twice the amount synthesized at 37°C . HeLa host cell RNA is synthesized at a faster rate at 37°C .

Products in *in vivo* RNA synthesis. To examine the products of *in vivo* HRV-2 RNA synthesis, the RNA synthesized in infected cells was labeled with ^3H -uridine for 2 hr, starting 5 hr after infection. The products were analyzed by centrifugation through a sucrose gradient and by electro-

FIG. 1. Effect of temperature on HRV-2 RNA synthesis *in vivo*. Duplicate sets of HeLa cells were infected in the presence of actinomycin D with HRV-2 at a multiplicity of 40 PFU/cell. ^3H -uridine was added to each monolayer to a concentration of 5 $\mu\text{Ci}/\text{ml}$ at the time of infection. One series of plates was incubated at 34°C and the other at 37°C . At various times after infection, the total label incorporated into viral RNA was determined as follows. The cell pellet was suspended in 1 ml of 0.01 M sodium acetate ($\text{pH } 5.0$) containing 1% SDS, to lyse the cells. After cell lysis, 1 ml of cold 50% trichloroacetic acid was added to precipitate the RNA. The precipitate was collected on a filter and washed three times with cold 50% trichloroacetic acid, and the radioactivity was counted in a liquid scintillation counter with Bray's liquid scintillator solution. Uninfected cells represent cellular RNA synthesized in the absence of actinomycin D. (●) RNA synthesized at 34°C ; (●---●) RNA synthesized at 37°C .

phoresis on polyacrylamide gels. Figure 2 shows the classes of HRV-2-specific RNA when analyzed by electrophoresis on polyacrylamide gel. Peak III is the single-stranded virion RNA since it comigrates with the virion RNA and is completely digested by pancreatic ribonuclease at 40 $\mu\text{g}/\text{ml}$ in 2.25 SSC, (0.34 M NaCl plus 0.34 M sodium citrate). Peak II is the double-stranded replicative form since it is the only peak that remains after ribonuclease digestion (Fig. 2). Peak I is tentatively designated the double-stranded replicative intermediate since (i) it barely enters the gel, analogous to polio replicative intermediate (9); (ii) it is precipitable by 1 M NaCl; and (iii) it disappears after ribonuclease digestion.

When the ^3H -uridine-labeled *in vivo* RNA is analyzed by sedimentation through a sucrose

FIG. 2. Composite profile of gel electrophoresis of HRV-2 RNA. HeLa cells were infected with HRV-2 and viral RNA was labeled by the addition of ^3H -uridine (20 $\mu\text{Ci}/\text{ml}$) for 2 hr at 5 hr after infection. The infected cells were collected, lysed, and the lysate was extracted with phenol at 45 C as described in Materials and Methods. The RNA was analyzed by electrophoresis on 2.3% polyacrylamide gels (0.8 by 10 cm). Electrophoresis was for 16 hr at 4 ma/gel. Migration of RNA is from left to right. (—○—) HRV-2 RNA from infected cells; (—○—) HRV-2 RNA from infected cells treated for 30 min at 37 C with pancreatic ribonuclease (40 $\mu\text{g}/\text{ml}$) in 0.34 M sodium chloride—0.034 M sodium citrate (2.25 $\times$ SSC). (—●—) HRV-2 virion RNA.

gradient, two peaks of RNA with relative sedimentation values of 18 and 28S are obtained (Fig. 3). The 18S is completely ribonuclease-resistant when the ribonuclease digestion is done after centrifugation. If the digestion is performed prior to centrifugation, however, the ribonuclease-resistant peak sediments with a sedimentation value of about 14S. The 28S peak and a small shoulder at 32S consist mainly of single-stranded

FIG. 3. Sucrose gradient analysis of HRV-2 RNA extracted from infected cells. The RNA of infected cells was labeled with ^3H -uridine (20 $\mu\text{Ci}/\text{ml}$) starting 5 hr after infection. At 7 hr after infection, a cytoplasmic extract was prepared as described in Materials and Methods and was extracted with phenol at room temperature. The RNA was divided into three equal portions. Two of the portions were layered onto two, 37-ml, 15 to 30% (w/w) linear sucrose gradients (0.01 M Tris [pH 7.2], 0.01 M EDTA, 0.1 M NaCl, 0.2% SDS) and centrifuged at 20,000 rev/min for 16 hr at 25 C in a Spinco SW27 rotor. Another portion was treated with pancreatic ribonuclease (40 $\mu\text{g}/\text{ml}$) in 2.25 $\times$ SSC for 30 min at 37 C. SDS was added to 0.5%, and the RNA was layered on a sucrose gradient identical to that for the nontreated RNA. Fractions (1 ml) were collected from the bottom of the gradient. To each fraction of one of the untreated gradients 4 ml of 2.25 $\times$ SSC containing 40 μg of pancreatic ribonuclease was added. The fractions were incubated for 30 min at 37 C. RNA in all fractions was precipitated with 20% trichloroacetic acid and collected and counted as in Fig. 1. (—○—) RNA from infected cell, no ribonuclease treatment; (—○—) ribonuclease treatment after centrifugation; (—●—) ribonuclease treatment before centrifugation.

RNA and double-stranded replicative intermediate RNA.

Properties of the RNA-polymerase complex. The isolation of the crude polymerase complex was described in Materials and Methods. Figure 4 shows the incorporation in vitro of ^3H -GTP into trichloroacetic acid-insoluble material by various cell fractions. The fraction which contains the cytoplasmic membranes of infected cells is the only fraction that has significant activity (Fig. 4). The incorporation of ^3H -GTP by the polymerase complex is linear for 30 min at 34 C, and the rate of incorporation of ^3H -GTP is the same at pH 7 and 8. The amount of the incorporation of ^3H -GTP is a function of the concentration of the crude polymerase complex.

The requirements for ^3H -GTP incorporation by the membrane-associated RNA polymerase complex are shown in Table 1. All four nucleoside triphosphates and magnesium are required. The magnesium ion concentration for optimal reaction is 5 mM. Manganese ion at 4 mM in the pres-

ence of 5 mM magnesium inhibits ^3H -GTP incorporation (Table 1).

Sedimentation characteristics of detergent-treated RNA polymerase complex. The polymerase complex retains greater than 90% of the original activity after solubilization of the membranes with 0.5% DOC. All the activity is retained in the supernatant fraction after centrifugation at $30,000 \times g$ for 20 min. This activity sediments in a sucrose gradient as a broad peak with a sedimentation value of approximately 120S (Fig. 5).

RNA products of the in vitro polymerase reaction. An in vitro reaction was performed with ^3H -GTP, and the products were first extracted with phenol as described above then treated with 0.3 M KOH for 16 hr at 37 C. This results in a 75% loss of trichloroacetic acid-insoluble ^3H radioactivity indicating that most of ^3H -GTP was incorporated into RNA.

Figure 6 shows the in vitro products of the crude membrane-bound polymerase complex after sedimentation of the RNA products through a sucrose gradient. There is a heterogeneous peak from 16 to 28S, with a broad shoulder greater than 28S. Treatment of the RNA with ribonuclease before sedimentation produces a large peak which sediments at about 14S (Fig. 6). In contrast to the RNA products of the crude polymerase complex, the RNA products of the complex after solubilization with 0.5% DOC sedimented as shown in Fig. 7. There is only one peak sedimenting at about 16S and no peak at 28S. Treatment of the RNA products with ribonuclease before centrifugation produces a peak which sediments at about 14S (Fig. 7), indicating that the solubilized polymerase complex is unable to synthesize replicative intermediate and single-stranded RNA.

FIG. 4. Time course of RNA synthesis by polymerase complex in vitro. Each point represents duplicate assays as described in Materials and Methods. Infected cells: (—●—) cytoplasmic membrane fraction (crude enzyme); (---○---) detergent wash of nuclei; (---●---) cytoplasmic soluble fraction. Uninfected cells: (—●—) cytoplasmic membrane fraction.

TABLE 1. Requirements for ^3H -GTP incorporation by the RNA polymerase complex^a

Reaction mixture	^3H -GTP incorporated (counts/min)
Complete ^b	2,100
—ATP, —CTP, and —UTP	150
—Mg ²⁺	150
+0.002 M Mg ²⁺	1,600
+0.005 M Mg ²⁺	2,050
+0.010 M Mg ²⁺	1,950
+0.020 M Mg ²⁺	1,500
+0.005 M Mg ²⁺ , 0.004 M Mn ²⁺	150

^a Abbreviations: GTP, guanosine triphosphate; ATP, adenosine triphosphate; CTP, cytidine triphosphate; UTP, uridine triphosphate.

^b The complete system is described in Materials and Methods.

FIG. 5. Sedimentation of RNA polymerase complex. A 2-ml amount of the crude polymerase complex was made to 0.5% in sodium deoxycholate and layered onto the following gradient: a 37-ml linear, 7–47% (w/w) sucrose gradient in 0.01 M Tris (pH 7.2)–0.01 M NaCl. Sedimentation was performed in a Spinco SW27 rotor for 3 hr at 37,000 rev/min at 4 C. Fractions (1 ml) were collected from the bottom. A 0.5-ml sample of each fraction was assayed for enzyme activity. The concentration of each component in the enzyme assay was the same as that described in Materials and Methods, but the total assay volume was 1.0 ml. Sedimentation is from right to left.

DISCUSSION

Infection of HeLa cells with HRV-2 causes the synthesis of cell-associated viral RNA: single-stranded viral RNA, double-stranded replicative form, and the putative replicative intermediate. The single-stranded RNA from the infected cell has the same migration rate on electrophoresis as the single-stranded RNA isolated from the virion; they are therefore assumed to be identical in size. We have no evidence that any cell-associated single-stranded RNA is larger than that of the virion (2). In vitro products of the polymerase complex when sedimented through a sucrose gradient appear to be more heterogeneous in size than the in vivo products extracted from the infected cell. The relative percentage of the ribonuclease-resistant RNA is higher in the in vitro products. Furthermore, the yield of virus and the total synthesis of viral RNA in vivo, as measured by the uptake of ^3H -uridine, are lower at 37 C than at 34 C; the incorporation of ^3H -GTP into

FIG. 6. Sucrose gradient analysis of RNA synthesized by crude polymerase complex. To 2 ml of crude polymerase, complex components of the assay were added to give a final volume of 4.0 ml. The mixture was incubated at 34 C for 30 min then extracted with phenol at room temperature. One-half of the ^3H RNA was layered directly onto a sucrose gradient and analyzed as in Fig. 3. The other half was treated with pancreatic ribonuclease (40 $\mu\text{g}/\text{ml}$ in 2.25 SSC; 30 min; 37 C) layered onto a sucrose gradient and analyzed as in Fig. 3. (—●—) No ribonuclease treatment; (---●---) ribonuclease treatment.

RNA in vitro by the polymerase complex is the same at both temperatures. If the in vivo effect of temperature is directly on RNA synthesis, then we conclude that the in vitro system does not accurately reflect the in vivo synthesis. We have no evidence of RNA chain initiation nor of release of single-stranded RNA from the template. Either point, or some other point, could be sensitive to temperature.

The sedimentation rate in a sucrose gradient of the double-stranded RNA is slower if the sedimentation is performed after ribonuclease treatment rather than sedimentation before ribonuclease treatment. A similar result is obtained when the double-stranded RNA is labeled in vitro with ^3H -GTP and treated with ribonuclease before and after sedimentation. We can speculate that the size or the configuration, or both, of the double-stranded RNA is changed after ribonuclease treatment, causing it to sediment at a slower rate.

The polymerase complex has a sedimentation

FIG. 7. Sucrose gradient analysis of RNA synthesized by crude polymerase complex after treatment with sodium deoxycholate. The crude polymerase complex (2 ml) was made 0.5% in deoxycholate. RNA synthesis, phenol extraction, sucrose gradient analysis, and ribonuclease treatment were performed as in Fig. 3 and 6. (—●—) No ribonuclease treatment; (---●---) ribonuclease treatment.

value of approximately 120S after treatment of the membrane fraction with DOC. The polymerase activity is spread over a broad peak, suggesting that the complex may be attached to membrane pieces of various sizes; it may contain variable numbers of polymerase molecules and variable numbers of nascent chains. We have no experiments in which the endogenous RNA template is not required. Clearly, the type of RNA labeled with ^3H -GTP by the polymerase complex after detergent treatment is different from the RNA labeled by the complex before detergent treat-

ment. The detergent-treated complex synthesizes only double-stranded RNA. Similar results have been observed in other detergent-treated polymerase systems (1). Since there are nucleases present in detergent-treated as well as non-detergent-treated cytoplasmic extracts of infected HeLa cells (Yin, *unpublished data*), we can speculate that the integrity of the membrane may be necessary for the synthesis of single-stranded RNA by somehow protecting it from the exposure of nucleases.

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