Incorporation of In Vitro Synthesized Reovirus Double-Stranded Ribonucleic Acid into Virus Corelike Particles

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When reovirus double-stranded ribonucleic acid (dsRNA) was synthesized in vitro by using a large-particulate fraction (LP-fraction) from reovirus-infected L cells, a significant amount of the 3H-labeled dsRNA product was incorporated into reovirus corelike particles bound to the LP fraction. These corelike particles were found to be indistinguishable from virus core derived by chymotryptic digestion of virions when compared on the basis of their (i) resistance to chymotryptic digestion, (ii) buoyant density in CsCl, (iii) particle size as determined by agarose chromatography, (iv) elution characteristics from diethylaminoethyl-Sephadex, and (v) resistance of the incorporated 3H-dsRNA to ribonuclease digestion in 0.01 M NaCl. When the replicase reaction was partially inhibited by NaCl, there was an accumulation of particles that were less dense than the virus core. All of the results indicate that some virus core assembly takes place during the in vitro replicase reaction.

The reovirus particle has a double-layer structure consisting of an outer and inner capsid (2, 5). The inner capsid, a spherical structure of about 45-nm diameter, surrounds the 10 segments of the double-stranded ribonucleic acid (dsRNA) reovirus genome. This inner capsid will be referred to as the “virus core” in this paper.

We have demonstrated previously that a replicase-template complex, bound to a large-particulate fraction (LP-fraction) prepared from the cytoplasm of reovirus-infected L cells, catalyzed the synthesis of reovirus dsRNA (4, 6); furthermore, that minus-strand ribonucleic acid (RNA) was synthesized in vitro upon a preexisting template of plus-strand RNA (equivalent to reovirus messenger RNA) (4). A similar observation was reported by Acs et al. (1).

During subsequent studies in our laboratory, we observed that in vitro synthesized dsRNA was incorporated into particles that were bound to the LP-fraction and resembled reovirus core particles. Further investigation showed that the incorporation of in vitro synthesized dsRNA into the corelike particles was a step in the assembly of the virus core and that it took place within the LP fraction. These findings may be a clue to the way in which the segmented reovirus dsRNA genome is accurately apportioned and incorporated into the virus during its assembly in the infected cell.

This report summarizes the evidence which demonstrates that in vitro synthesized dsRNA is incorporated into the virus corelike particles during the replicase reaction.

MATERIALS AND METHODS

Buffers, chemicals, and isotopes. The following buffers were used: 0.01 M STE buffer, containing 0.01 M NaCl, 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride at pH 7.4, and 0.001 M ethylenediaminetetraacetate (EDTA); 0.3 M STE buffer, as above, but containing 0.3 M NaCl; TM buffer, containing 0.001 M MgCl2 and 0.01 M tris-hydrochloride at pH 8.0. Tritiated uridine triphosphate (3H-UTP; specific activity, 20 Ci/mmole), 32P-orthophosphoric acid, and uridine-5,6-3H (specific activity, 42.4 Ci/mmole) were purchased from New England Nuclear Corp., Boston, Mass.; α-chymotrypsin and pancreatic ribonuclease were from Worthington Biochemical Corp., Freehold, N.J.; and unlabeled nucleoside triphosphates were from Calbiochem, Los Angeles, Calif.

Cells, virus, viral dsRNA, and virus core. Conditions for culturing L cells in suspension and infecting them with reovirus type 3, a strain producing only complete virions (2), have been described previously (7). Preparation and purification of reovirus labeled with 32P-phosphate or 3H-uridine and the procedure for isolating virus dsRNA (8) and virus core particles (3) from the purified virions have also been reported.

Conditions for replicase reaction. The LP-fraction was prepared from reovirus-infected L cells harvested at 7 hr postinfection as described previously but without chymotryptic digestion (4). The LP-fraction obtained from 108 infected cells was suspended finally in
10 ml of TM buffer. Six different preparations of the LP-fraction with various degrees of replicase activity were used.

A standard reaction mixture of 0.25 ml total volume contained 20 μmoles of Tris-hydrochloride at pH 8.0, 3.15 μmoles of MgCl₂, 30 μCi (0.7 nmoles) of ³H-UTP, 0.2 μmoles of each of the other three ribonucleoside triphosphates, 2 μg of actinomycin D, and 0.15 ml of the LP-fraction. After incubation at 37 °C for the prescribed period of time, the replicase reaction was terminated by adding 1 to 3 ml of cold 0.3 M STE buffer containing, unless otherwise stated, 0.01 M EDTA. The amount of ³H incorporated into the acid-insoluble fraction was determined by adding carrier yeast nucleic acid when necessary, and then trichloroacetic acid to a final concentration of 5%. The resulting precipitate was collected on a nitrocellulose membrane, dried, and then counted in a liquid scintillation spectrometer with 5 ml of toluene-liquidfluor (New England Nuclear Corp.). Resistance to ribonuclease was determined by incubating samples in 0.3 M STE buffer containing 10 μg of ribonuclease per ml (unless otherwise stated) at 37 °C for 20 min followed by acid precipitation, and the radioactivity assay was as described above.

RESULTS

Binding of in vitro synthesized dsRNA to a readily sedimentable particulate fraction. To determine whether the product dsRNA was released from the cytoplasmic LP-fraction upon completion of synthesis, we carried out the following experiment. The LP-fraction, which had been repelleted from TM buffer by centrifugation at 12,000 × g for 10 min, was incubated under conditions appropriate for the replicase reaction. (³H-UTP was the labeled precursor.) After incubation for various periods of time, the reaction mixture was centrifuged at 12,000 × g for 10 min to pellet the LP-fraction. Then the amount of ³H-labeled dsRNA present in the supernatant and sediment was determined. It was observed that the majority of the newly formed dsRNA was bound to the sediment (Fig. 1).

Homogenization of the sediment in a Dounce homogenizer with either Nonident P-40 (1%) or deoxycholate (0.5%) did not release the replicase product from the sediment (data not shown). These results indicate that the replicase-template complex mediates synthesis of dsRNA while bound to the LP-fraction and that after its synthesis the dsRNA remains associated with detergent-insensitive particulate material.

Release of virus corelike particles from the LP-fraction by chymotryptic digestion. The in vitro dsRNA product associated with the readily sedimentable fraction described above was digested with chymotrypsin and then centrifuged through a preformed CsCl gradient at 131,000 × g for 3 hr. The resulting distribution of ³H-labeled material is shown in Fig. 2. Two major peaks of ribonuclease-resistant ³H-labeled material were revealed: a "lower component" banding in a density region of 1.41 to 1.43 g/cm³ and an "upper component" near the top of the gradient. When the two components were then taken separately and centrifuged again in CsCl for an additional 20 hr, the upper component was seen to be heterogeneously distributed from the middle to the bottom of the gradient, whereas the position of the lower component remained constant. This observation indicates that the lower component sedimented rapidly and reached density equilibrium in 3 hr of centrifugation. It was also found that neither longer periods of chymotryptic digestion (up to 60 min), nor higher concentrations of chymotrypsin (up to 1 mg/0.15 ml of LP-fraction), nor addition of chymotrypsin before, or

![FIG. 1. Association of the ³H-labeled, ribonuclease-resistant product with a readily sedimentable fraction. Reaction mixtures, 0.5 ml each, were incubated at 37 °C for the indicated periods of time. The reactions were stopped by chilling followed by addition of 1.5 ml of cold 0.3 M STE buffer (containing 0.01 M EDTA). Each mixture was centrifuged at 12,000 × g for 10 min at 4 °C, and then the supernatant was removed carefully. The sediment was resuspended in 2 ml of 0.3 M STE buffer and recentrifuged as above, and the supernatants were combined. The pellet was resuspended in 2 ml of 0.3 M STE buffer. Both the supernatant and sediment fraction were assayed for level of ³H-incorporated into trichloroacetic acid-insoluble material after digestion with ribonuclease (10 μg/ml of 0.3 M STE buffer at 37 °C for 20 min). Symbols: ○, ribonuclease-resistant ³H-RNA contained in sediment fraction; □, ribonuclease-resistant ³H-RNA contained in supernatant.](http://jvi.asm.org/)
FIG. 2. Fractionation of the chymotrypsin-digested, ribonuclease-resistant, 3H-labeled replicase product in a CsCl gradient. A reaction mixture of 6.7 ml was incubated for 60 min and then centrifuged at 25,000×g for 40 min at 4°C. The pellet, which contained most of the 3H-labeled replicase product, was resuspended in 10.0 ml of TM buffer containing 1 mg of chymotrypsin and incubated at 37°C for 30 min. The digested replicase product was adjusted to a density of 1.25 g/cm³ by adding solid CsCl, stored for 18 hr at 4°C, and then centrifuged through a preformed CsCl gradient (1.47–1.28 g/cm³ in TM buffer) at 131,000×g for 3 hr at 4°C in a Beckman SW41 rotor. 32P-labeled virus cores, which had been isolated from 32P-labeled virions by chymotryptic digestion and purified by isopycnic centrifugation in CsCl, were co-sedimented as marker. After centrifugation, the gradient was fractionated from the bottom and a 25-ml aliquot of each fraction was assayed for the amount of 3H or 32P present. Symbols: △, total acid-insoluble 3H; ○, ribonuclease-resistant acid-insoluble 3H; ○, marker 32P-labeled virus core particles; □, density as determined by refractometry.

Nature of the lower component. The density of the lower component corresponds closely to that of marker 32P-labeled core (1.43 g/cm³) prepared by chymotryptic digestion of 32P-labeled virus and purified by banding in a CsCl gradient before co-sedimentation with the lower component (Fig. 2). Based on the peak width of marker virus core, it appeared that the lower component was somewhat heterogeneous. From similar analyses with different preparations of LP-fraction, it was determined that the lower component consisted of two distinct fractions, a major portion having a density of 1.43 g/cm³ and a minor portion at 1.40 to 1.41 g/cm³.

Besides similarity in buoyant density and resistance to chymotryptic digestion, the lower component shared the following characteristics with virus core derived by chymotryptic digestion of virions.

(i) According to analysis by polyacrylamide gel electrophoresis, the phenol-extracted, 3H-labeled RNA from the lower component consisted of segments which correspond for the most part to the three size classes of reovirus dsRNA segments (Fig. 3a).

(ii) Both 3H-labeled RNA in the lower component and dsRNA in the virus core are resistant to ribonuclease digestion in either 0.3 M or 0.01 M NaCl (Table 1). Furthermore, after phenol extraction both 3H-RNA and reovirus dsRNA were resistant to ribonuclease in 0.3 M NaCl. These results indicated that the 3H-RNA in the lower component was dsRNA that had been synthesized in vitro during the replicase reaction and that this dsRNA was protected from ribonuclease digestion in 0.01 M NaCl in a manner similar to that which protects dsRNA in the virus core.

(iii) In an analysis by filtration through agarose beads (Bio-Gel A-150 m) the lower component and the virus core eluted at the same position, whereas the complete virion eluted separately in the column’s void volume (Fig. 4). Comparison of the peak width and symmetry of the elution pattern for the lower component and the virus core suggested that the lower component was relatively homogeneous with regard to particle size.

(iv) In chromatography on diethylaminoethyl (DEAE)-Sephadex the lower component appeared somewhat heterogeneous, but the majority of it eluted sharply at the same concentration of NaCl as the virus core (Fig. 5). This demonstrates that surface charge of the lower component is similar to that of virus core.

All of these similarities between the lower component and virus core lead to the conclusion that the lower component apparently consisted of virus corelike particles which contained dsRNA synthesized during the in vitro replicase reaction.

Nature of the upper component. The upper component, obtained from the top region of a CsCl gradient as described in Fig. 2, was contaminated by 3H-labeled ribonuclease-sensitive single-stranded RNA (ssRNA) which had been synthesized by the transcriptase present in the LP-fraction (4). To remove this contaminating ssRNA, the upper component was treated with ribonuclease (2 μg/ml in 0.3 M STE buffer at 37°C for 20 min) and filtered through Sephadex G-100 (1.5
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FIG. 3. Acrylamide gel electrophoretic patterns of $^3$H-labeled RNA extracted from the lower component (a) and upper component (b). RNA was extracted by sodium dodecyl sulfate and phenol from the lower component (fractions 7–13) and the upper component (fractions 22–27, see text for details) of the experiment shown in Fig. 2. Appropriate amounts of $^{32}$P-labeled virion dsRNA were added to 20,000 counts/min of lower component and 27,000 counts/min of upper component. Both mixtures were electrophoresed in polyacrylamide gel (8). Symbols: ●, $^3$H-RNA; — marker $^{32}$P-labeled virions dsRNA.

by 20 cm) which had been equilibrated in 0.3 M STE buffer. The upper component, now free of ssRNA, was collected in the void volume of the column.

The $^3$H-labeled RNA from the upper component was extracted by phenol and analyzed by polyacrylamide gel electrophoresis. As seen in Fig. 3b, the upper component $^3$H-RNA migrates to the same positions along the gel as the $^3$H-dsRNA from the lower component. With regard to resistance to ribonuclease digestion in 0.01 M NaCl, when the upper component was compared to the virus core and free dsRNA, it was observed that the upper component dsRNA was partially resistant, whereas virus core dsRNA was fully resistant and free dsRNA was almost totally sensitive (Table 1). This result suggested that the $^3$H-dsRNA in the upper component was asso-
associated with material which only partially protected it from digestion by ribonuclease.

Effect of NaCl on replicase reaction: accumulation of lower component less dense than virus core.

**TABLE 1. Resistance to ribonuclease digestion of $^3$H-labeled double-stranded ribonucleic acid (dsRNA) contained in upper and lower components**

<table>
<thead>
<tr>
<th>Materials</th>
<th>0.3 M NaCl$^a$ (counts/min)</th>
<th>0.01 M NaCl$^a$ (counts/min)</th>
<th>% ribonuclease resistance in 0.01 M NaCl$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^3$H lower component</td>
<td>4,907</td>
<td>4,637</td>
<td>94.5</td>
</tr>
<tr>
<td>$^3$H upper component</td>
<td>3,507</td>
<td>1,436</td>
<td>41.5</td>
</tr>
<tr>
<td>$^{32}$P-virus core (control)$^e$</td>
<td>1,762</td>
<td>1,546</td>
<td>87.7</td>
</tr>
<tr>
<td>$^{32}$P-dsRNA (control)$^e$</td>
<td>2,335</td>
<td>89</td>
<td>3.8</td>
</tr>
</tbody>
</table>

$^a$ Acid-insoluble radioactivity was determined after treatment with ribonuclease in 0.3 or 0.01 M STE buffer. Materials tested were incubated for 20 min at 37 C in 1.0 ml of the indicated buffer which contained 2 μg of pancreatic ribonuclease. The products were precipitated by adding carrier yeast RNA and trichloroacetic acid, and the precipitates were collected on nitrocellulose membrane and counted. Values are the mean of duplicated assays.

$^b$ NaCl at 0.01 M/NaCl at 0.3 M × 100.

$^c$ Control samples are totally resistant to ribonuclease treatment in 0.3 M NaCl.

It has been observed that the in vitro synthesis of dsRNA is inhibited by NaCl and that the degree of inhibition varies directly with the concentration of NaCl (Fig. 6). By employing this correlation, replicase reaction mixtures were incubated in various concentrations of NaCl and then digested with chymotrypsin. The $^3$H-labeled products were analyzed by sedimentation in CsCl gradients as shown in Fig. 7. Although not as quantitative as the experiment shown in Fig. 6, a similar pattern of inhibition of dsRNA synthesis by NaCl was observed. More significantly, the data shown in Fig. 7 clearly show a shift in density of lower component, which varies with salt concentration. With 0.01 or 0.03 M NaCl in the reaction mixture, the lower component banded coincidentally with marker virus core at 1.43 g/cm$^3$, but in the presence of 0.1 or 0.3 M NaCl the density of this material shifted to 1.38 to 1.40 g/cm$^3$. These data indicated that the addition of NaCl to the replicase reaction mixture caused an accumulation of virus corelike particles with a RNA-protein ratio that was less than that of the virus core.

**Failure to detect complete virion formation.** Since the outer layer of the reovirus capsid was digested by chymotrypsin (3), it was possible that the virus corelike particles, whose detection required chymotryptic digestion of the replicase reaction mixture, were the product of the digestion of the outer capsid. To rule out this

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**Fig. 4.** Agarose chromatography of the lower component, virion, and virus core. (a) The $^3$H-labeled virus core particles were prepared by chymotryptic digestion of purified virions labeled with $^3$H-uridine and purified by isopycnic centrifugation in CsCl (3). The $^3$H-labeled virus core, which banded at a density of 1.43 g/cm$^3$, was mixed with purified $^{32}$P-labeled virions and filtered through a column (1.7 by 50 cm) of Bio-Gel A-150 m (100 to 200 mesh, equilibrated in 0.3 M STE buffer) at a flow rate of 3 ml per hr. Fractions of 3 ml were collected. The radioactivity of $^3$H and $^{32}$P was determined for each fraction. (b) The $^3$H-labeled lower component (1.42–1.43 g/cm$^3$), obtained from an experiment similar to the one shown in Fig. 2, was mixed with the $^{32}$P-labeled virions and chromatographed as in (a). Symbols: ●, $^3$H; ○, $^{32}$P (for both a and b).
possibility, it was necessary to determine whether complete virions were also assembled during the replicase reaction. First, $^{32}$P-labeled purified reovirus and 200 $\mu$g of chymotrypsin were added to 0.25 ml of the replicase reaction mixture. After 30 min of incubation, the mixture was filtered through Sephadex G-100 to remove acid-soluble material and then centrifuged through a preformed CsCl gradient. The $^{32}$P-labeled virions were only slightly digested under these conditions. Furthermore, there was no $^{32}$P label in the density region of 1.41 to 1.43 g/cm$^3$ where the $^3$H-labeled lower component banded, and there was no $^3$H-label in the region of $^{32}$P-labeled virions (Fig. 8a). The same distribution was observed when 50 $\mu$g of chymotrypsin was added to the replicase reaction except that there was a smaller amount of lower component (not shown). These results demonstrated that the conditions of chymotryptic digestion employed in this experiment were not strong enough to disrupt the outer capsid and convert the intact virions to virus cores. Therefore, the $^3$H-labeled lower component was apparently not a breakdown product of complete virions.

To examine this possibility in another way, we added $^{32}$P-labeled virions without chymotrypsin to the replicase reaction mixture. After incubation, the mixture was homogenized in an equal volume of Freon under conditions necessary for dissociating virions from virus-infected L cells (8). The aqueous phase obtained after centrifugation of the freonized mixture was filtered through Sephadex G-100 and analyzed by sedimentation in a CsCl gradient as in the preceding experiment. As shown in Fig. 8b, approximately 80% of input $^{32}$P-labeled virions banded sharply at density 1.36 to 1.37 g/cm$^3$, and no significant $^3$H-label was found at this density. Since $^{32}$P-labeled virions were not degraded under the conditions of the replicase reaction, any newly synthesized virions, likewise, would not have been degraded. Therefore, we concluded that com-
FIG. 7. Change in the buoyant density of the lower component formed in the presence of NaCl. Reaction mixtures (0.75 ml each) with final NaCl concentrations of none (a), 0.03 M (b), 0.1 M (c), and 0.3 M (d) were incubated in the presence of 200 μg of chymotrypsin. After 1 hr of incubation, the reaction mixtures were chilled in an ice bath, mixed with 32P-labeled virus core, and centrifuged through a CsCl gradient as described in Fig. 2. A sample from each fraction of the gradient was mixed with 1.0 ml of 0.3 M STE buffer containing 10 μg of ribonuclease and incubated at 37°C for 20 min. Then the level of 3H in acid-insoluble material was determined. Symbols: ●, ribonuclease-resistant, acid-insoluble 3H; ○, marker 32P-labeled virus core particles.

FIG. 8. Search for evidence of formation of complete virions during the replicase reaction. (a) Purified 32P-labeled virions and 200 μg of chymotrypsin were added to a standard, 0.25-ml replicase reaction mixture. After 30 min of incubation, the mixture was filtered through Sephadex G-100 which had been equilibrated with 0.3 M STE buffer. The first peak of radioactive material was eluted in the void volume of the column. This fraction, which contained most of input 32P, was centrifuged through a CsCl gradient as described in Fig. 2. A sample of each fraction from the gradient was applied to glass-fiber filters, and the levels of 3H and 32P were determined. (b) Purified 32P-labeled virions without chymotrypsin were added to 0.25 ml of a replicase reaction mixture. After 30 min of incubation, 0.3 ml of Freon was added, the mixture was vigorously homogenized in a Dounce homogenizer and then centrifuged at 1,000 × g for 10 min. The aqueous phase was filtered through Sephadex G-100 and sedimented as before in a CsCl gradient. Symbols: ●, 3H; ○, 32P.
plete virions were not assembled during the in vitro synthesis of dsRNA.

DISCUSSION

This study has demonstrated that during the in vitro replicase reaction, which employed a replicase-template complex bound to a particulate fraction of virus-infected cells, in vitro synthesized reovirus dsRNA was incorporated into chymotrypsin-resistant particles. These particles, the lower component, have physical characteristics which resemble the reovirus core particles derived by the chymotryptic digestion of virions.

It was observed that the lower component and the virus core resemble each other with regard to buoyant density, surface charge (based on elution from DEAE-Sephadex), and particle size (based on elution from agarose beads). Furthermore, in vitro synthesized dsRNA associated with the lower component was protected from ribonuclease digestion in 0.01 M NaCl to the same extent as dsRNA from virus core.

When virion dsRNA and in vitro synthesized dsRNA from the lower component were analyzed by polyacrylamide gel electrophoresis, slight differences became apparent. As illustrated in Fig. 3a, there was a considerable amount of intermediate size in vitro synthesized dsRNA present in the lower component and a difference in the ratio of ${}^3$H incorporated into the three size classes relative to genomic RNA. If it is assumed that the level of ${}^3$H in each class of dsRNA represents the absolute amount of each class present, then the reovirus corelike particles in the lower component may have significantly less dsRNA per particle than the virus core. Previous work has shown that the virus core lacking the largest piece of dsRNA (ca. 15% of total RNA) has a density in CsCl that is 0.015 g/cm$^3$ less than the virus core with all segments of dsRNA (3). Therefore, the heterogeneity of the lower component, reflected by the range in buoyant density (1.43–1.40 g/cm$^3$), may be due to the incorporation of incomplete sets of reovirus dsRNA into the corelike particles.

It is also possible that density heterogeneity is a reflection of the variation in protein content of the particles. Unfortunately, contamination of the LP-fraction with preexistent virus core particles (4) makes meaningful protein analysis or electron microscopy of the lower component impossible.

If the incorporation of in vitro synthesized dsRNA into corelike particles represent synthesis of dsRNA within a protein matrix of the virus core, formed before or during the in vitro reaction, it would be reasonable to expect that partial inhibition of the in vitro replicase reaction would result in the accumulation of virus core particles that would contain less dsRNA and therefore be less dense than virus core in CsCl. The result of an experiment in which the replicase reaction was partially inhibited by NaCl (Fig. 5) is consistent with this model.

Analysis of the data on the upper component is more difficult to interpret. It seems to be a heterogeneous mixture of complexes that consist of in vitro synthesized dsRNA and proteinaceous material. Electrophoretic analysis shows that ${}^3$H-labeled dsRNA of the upper and lower components are similar and suggests that failure to form corelike particles is not related to incomplete synthesis of dsRNA. Rather, it may be that the replicase-template complex is associated with an incomplete core protein matrix and therefore would be sensitive to chymotryptic digestion or exposure to CsCl, or both.

The observations of this study show that core protein and replicase-template complex are closely associated at the virus-synthesizing sites of infected cells and that dsRNA synthesis and virus core formation occur simultaneously. These findings suggest that, rather than separate replication of dsRNA segments and subsequent linkage of these segments during virus assembly, the segments of the reovirus genome are synthesized as a complete set within a matrix of virus-specific proteins.

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LITERATURE CITED