Mechanism of Reovirus Double-Stranded Ribonucleic Acid Synthesis In Vivo and In Vitro

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The complementary strands of reovirus double-stranded ribonucleic acid (ds RNA) are synthesized sequentially in vivo and in vitro. In both cases, preformed plus strands serve as templates for the synthesis of the complementary minus strands. The in vitro synthesis of dsRNA is catalyzed by a large particulate fraction from reovirus-infected cells. Treatment of this fraction with chymotrypsin or with detergents which solubilize cellular membranes does not alter its capacity to synthesize dsRNA. The enzyme or enzymes responsible for dsRNA synthesis remain sedimentable at $10,000 \times g$ after these enzyme or detergent treatments, indicating their particulate nature. Pretreatment of this fraction with ribonuclease, however, abolishes its ability to catalyze dsRNA synthesis, emphasizing the single-stranded nature of the template and its location in a structure permeable to ribonuclease. In contrast, the newly formed dsRNA is resistant to ribonuclease digestion at low salt concentrations and hence is thought to reside within a ribonuclease-impermeable structure.

Reovirus double-stranded ribonucleic acid (dsRNA) is formed in vivo by the sequential synthesis of the complementary RNA strands. Viral RNA plus strands, so called because they have the same polarity as viral messenger RNA, serve as the templates for the synthesis of their complementary minus strands. The complementary strands remain together as base-paired dsRNA (4).

These facts provide the conceptual framework for the detailed examination of the mechanism of synthesis of the complementary strands of reovirus dsRNA described in this communication. Our data indicate that there is increasing asynchrony of synthesis of the complementary RNA strands as the replicative cycle progresses, until by 8 hr after infection the cell has acquired a full complement of the plus strands which serve as templates for dsRNA synthesis. The plus strands synthesized after this time are not precursors of dsRNA and do not appear in reovirions. Reovirions, radioactively labeled with uridine 8 or more hr after infection, contain dsRNA labeled predominantly in the minus strand.

These data suggested to us that an enzyme-template complex containing plus-strand precursors of dsRNA accumulates during the first part of the infectious cycle. Accordingly, we isolated a fraction from reovirus-infected cells which synthesizes dsRNA in vitro. By using isotopically labeled nucleoside triphosphates to identify newly synthesized RNA molecules, we determined that all 10 segments of the reovirus genome are synthesized under these conditions. Moreover, these dsRNA molecules synthesized in vitro are labeled exclusively in their minus strands, confirming that preformed plus strands serve as the templates for minus-strand synthesis.

MATERIALS AND METHODS

Cell culture and virus. L-929 mouse fibroblasts were grown in suspension; reovirus type 3 was grown and assayed as previously described (3).

Preparation of enzyme fraction responsible for dsRNA synthesis. L-929 cells were infected with reovirus (100 plaque-forming units per cell) in the presence of 0.5 μg of actinomycin D per ml (3); 9 hr after infection, the cells were collected, washed once in phosphate-buffered saline (10), resuspended in a hypotonic buffer, and homogenized as previously described (3, 10). The homogenate was made isotonic by the addition of 0.1 volume of a 10× concentrated buffer (buffer A) containing 1.2 M KCl, 0.2 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.4), 0.05 M MgCl₂, and 0.06 M β-mercaptoethanol, and the nuclei and large cellular debris were removed by centrifugation at $600 \times g$ for 10 min. The supernatant fluid was then centrifuged at $10,000 \times g$ for 15 min; the resultant pellet was resuspended in standard buffer A and was utilized as the enzyme fraction for dsRNA synthesis.

Assay of the enzyme(s). In vitro synthesis of single-stranded (ss) RNA and dsRNA was carried out in a 1-ml reaction mixture containing 100 mM Tris (pH 7.9), 7.5 mM magnesium acetate, 250 mM potassium acetate, 1 mM dithiothreitol, 2 mM adenosine triphosphate, 2 mM guanosine triphosphate, 0.5 mM ^{3}H-uridine triphosphate (80 mCi/mmole), 0.5 mM ^{3}H-cytidine triphosphate (80 mCi/mmole), and 1.6 mg of protein of the enzyme fraction. After incubation at 37 C as indicated, the total RNA was extracted by the method of Tavitian et al. (9), and ssRNA and dsRNA were separated as described by Franklin (2). Radioactivity in dsRNA was measured as ribonuclease-resistant acid-precipitable counts per minute after treatment with 10 μg of ribonuclease per ml for 30 min at 37 C in the presence of 0.3 M NaCl (3–5).

Assay for the distribution of radioactive label in the minus strand of the synthesized dsRNA. The in vivo- or in vitro-labeled dsRNA was denatured at 95 C (1) and then reannealed in the presence of an eightfold excess (based on absorbance at 260 nm) of unlabeled plus strands previously prepared in vitro (3), yielding a new dsRNA duplex whose minus strand was derived from the synthesized labeled product. The reannealed product was treated with ribonuclease (10 μg/ml) at 37 C for 30 min in the presence of 0.3 M NaCl, and trichloroacetic acid-insoluble radioactivity was determined. All of the counts in the reannealed dsRNA represent the radioactivity in the minus strands of the original duplex.

Acrylamide gel electrophoresis. Electrophoresis in a 5% acrylamide-0.25% methylene-bisacrylamide gel and analysis of the distribution of radioactive label in the gel were carried out as previously described (3).

RESULTS AND DISCUSSION

Sequential synthesis of the complementary strands of dsRNA in vivo. Pulse-labeling experiments, employing ^{3}H-uridine as a precursor of newly synthesized RNA, established that the complementary strands which comprise the reovirus dsRNA are asymmetrically labeled during the logarithmic phase of viral replication. At the times examined (8 or more hr after infection) and under the conditions of labeling (30-min pulse), only the minus strand of dsRNA contained ^{3}H-uridine (4). To establish the period in the replicative cycle during which the plus strands which are precursors of dsRNA are formed, infected L cells were labeled for 1 hr with ^{3}H-uridine at hourly intervals during the replicative cycle. The cells were harvested at the end of the labeling period, and the dsRNA was extracted and purified (Fig. 1). The labeled dsRNA was denatured and annealed with excess unlabeled plus strands, and the amount of radioactivity which was recovered in the reannealed double strands was compared with the radioactive label present in the original duplex molecules. As shown in Fig. 1, early in the replicative cycle, the complementary strands of the dsRNA were equally labeled. However, as the
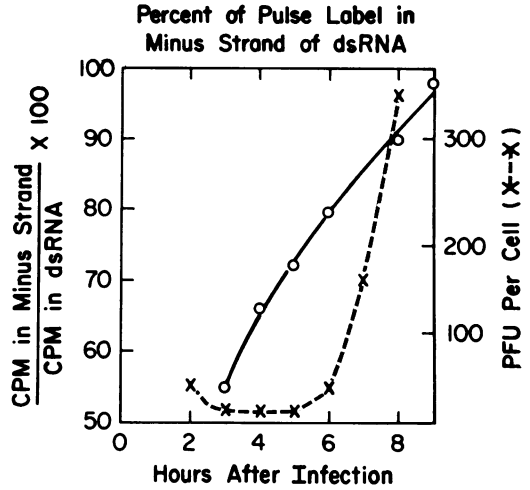

Percent of Pulse Label in
Minus Strand of dsRNA

FIG. 1. Kinetics of synthesis of the minus strand of reovirus dsRNA as measured by pulse-labeling in vivo. L-929 cells (1.8 × 10⁹) were infected with reovirus [100 plaque-forming units per cell]. At the end of the adsorption period, the culture was divided into eight samples; each was incubated with 10 μCi of ^{3}H-uridine per ml (400 μCi/μmole) for successive 1-hr intervals from 2 to 8 hr after infection. At this concentration, the label is incorporated linearly for at least 3 hr. Labeling period was terminated by centrifuging the cells. Total RNA was extracted, and the ^{3}H-labeled dsRNA was purified. The per cent of acid-precipitable label in dsRNA minus strands (○) at each time interval was determined, and the formation of infectious virus (plaque-forming units per cell, ×) at each time point was measured.

infection progressed, the labeling of the complementary strands during the period of the 1-hr pulse became increasingly asymmetric, until, by 8 to 9 hr after infection, only the minus strands were labeled.

Our own data and those of others (6, 12) indicate that plus strands comprise the bulk of the RNA synthesized during the logarithmic phase of viral growth. Thus, the failure of labeled plus strands to appear in dsRNA during the period of the ^{3}H-uridine pulse is not a consequence of deficient synthesis of plus strands; rather, it reflects the hiatus between the time of synthesis of the plus strands which are dsRNA precursors and their incorporation into double strands.

The data in Fig. 1 indicate that plus strands which are destined to be incorporated into dsRNA are synthesized both during the latent and logarithmic phases of viral infection. Although these data do not allow us to calculate the time required for plus strands to be incorporated into double strands, they do demonstrate that, at the earliest time points, the synthesis of plus

strands and their incorporation into dsRNA are nearly synchronous. The increasing asymmetry of labeling as the infection progresses reflects a lag between the time of synthesis of dsRNA precursor plus strands and their incorporation into double strands.

The highly asymmetric labeling of the complementary strands, which was observed at 8 hr and at later times in the growth cycle which continues for more than 12 hr, suggested to us that dsRNA precursor plus strands accumulate early in the infectious cycle and that the plus strands synthesized after 8 hr do not function as template for dsRNA synthesis. To test this hypothesis, virus-infected cells were labeled with ^{3}H-uridine as described in the legend of Fig. 1 from 8 to 11 hr after infection; the double-stranded RNA was extracted, and the distribution of label in the complementary strands was examined. Ninety per cent of the label present in the original dsRNA was recovered in minus strands, indicating that during this 3-hr period, which comprises a significant proportion of the logarithmic phase of viral growth, the plus strands which are formed are not incorporated into double strands. This result explains why Watanabe et al. (12) were unable to demonstrate a chase of pulse-labeled plus strands into dsRNA. The plus strands which were labeled in their experiments were synthesized 9 hr after infection. Our data show that virtually all dsRNA precursor plus strands have been formed by this point in the replicative cycle and predict that pulse-chase experiments performed at earlier time points will demonstrate a precursor-product relationship between plus strands synthesized early in the infectious cycle and dsRNA.

To determine whether the dsRNA found in reovirions is synthesized by the same mechanism as the bulk of the newly synthesized dsRNA, infected L cells were labeled with ^{3}H-uridine from 8 to 9 hr after infection. The radioactively labeled virus was isolated from these cells, and the dsRNA was extracted from the virion. This RNA was denatured and reannealed with excess unlabeled plus strands. All of the label present in the original dsRNA was conserved in a double-stranded form after reannealing with excess unlabeled plus strands (Table 1). Hence, the dsRNA isolated from intact virions exhibits a labeling pattern identical to that of the bulk of the dsRNA formed in a reovirus-infected cell.

Synthesis of dsRNA in vitro. The in vivo experiments described above suggested to us that a protein-plus strand complex accumulates during the infectious cycle and that the asymmetric distribution of ^{3}H-uridine in the complementary strands of dsRNA reflects a step in the maturation of the virus, namely, the synthesis of the minus

TABLE 1. *In vivo pulse-labeling of the minus strand of the reovirus dsRNA genome[a]*

Isolated viral dsRNA	Ribonuclease-resistant acid-precipitable counts/min
Untreated.....................	3,805
Denatured.....................	155
Reannealed with excess unlabeled plus strands..........	3,730

[a] L-929 cells were infected, and, at 8 hr after infection, 10 μCi of ^{3}H-uridine per ml was added to the culture; 1 hr later, the cells were harvested, and the virus was isolated and purified as previously described (8). Viral dsRNA was extracted and purified, and radioactive label in the minus strand of the dsRNA was determined. Recovery of all of the label in the annealed product demonstrated that 98% of the radioactivity in the viral dsRNA was in the minus strand.

strand to form the duplex molecule. Assuming this, we isolated a fraction from reovirus-infected cells which in vitro synthesizes dsRNA by using preformed RNA plus strands as template.

The enzyme(s) contained within this fraction catalyzed the synthesis of both single- and double-stranded RNA, but the kinetics of synthesis of these two molecular species are markedly different (Fig. 2). Ninety per cent of the dsRNA synthesis occurred during the first 10 min of incubation at 37 C. The rate of dsRNA synthesis decreased thereafter and by 30 min had ceased. In contrast, ssRNA synthesis proceeded in linear fashion and at a faster rate than dsRNA.

To determine whether the dsRNA formed in vitro was similar in size to the 10 segments of dsRNA contained within the reovirion, the in vitro-synthesized dsRNA was analyzed by electrophoresis on polyacrylamide gels (Fig. 3); as expected, it exhibited an electrophoretic profile identical to that obtained with dsRNA isolated from intact reovirions. Although our method of isolating a fraction capable of synthesizing dsRNA was different from that reported previously by Watanabe et al. (11), the results we report here are in complete agreement with their findings. However, neither the experiments of Watanabe et al. nor our own experiments described above characterize the distribution of label in the complementary strands of dsRNA. To determine this distribution, the dsRNA products of an in vitro synthetic reaction were purified and reannealed with an excess of unlabeled viral strands. Virtually all of the label originally present in dsRNA was conserved in the reannealed duplex molecules (Table 2). Hence, the dsRNA which is synthesized in vitro, like the dsRNA
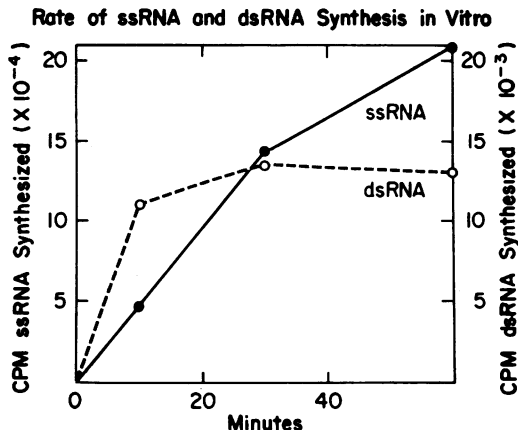
Rate of ssRNA and dsRNA Synthesis in Vitro

FIG. 2. *In vitro synthesis of reovirus ssRNA and dsRNA in cell-free extracts of virus-infected L-929 cells. Each time point represents a 1-ml reaction mixture.*
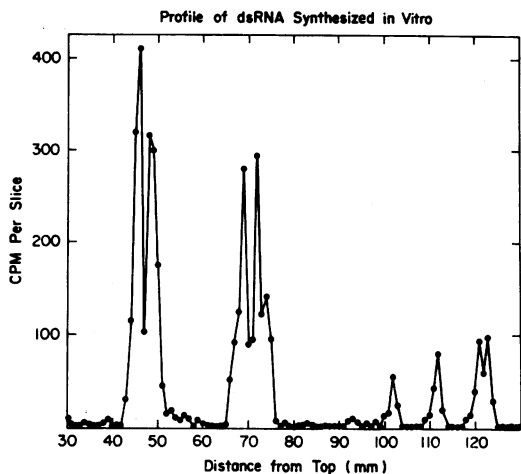
Profile of dsRNA Synthesized in Vitro

FIG. 3. *Acrylamide gel electrophoresis of reovirus dsRNA synthesized in vitro. RNA synthesis was carried out in a 1-ml incubation mixture of cell-free extracts, and the dsRNA was isolated. Electrophoresis in a 5% acrylamide-0.25% methylene-bisacrylamide gel and analysis of the distribution of radioactive label were carried out as previously described (3). The electrophoretic pattern is identical to that of viral dsRNA, indicating that the minus strands of all 10 dsRNA segments were synthesized proportionately.*

formed late in the infectious cycle in vivo, is labeled exclusively in its minus strand.

Partial characterization of dsRNA "synthetase." Previous studies have identified the reovirus inclusion bodies as the intracellular sites of synthesis of dsRNA (7). Although these inclusions are themselves free from cytoplasmic membranes, they cosediment with the large particulate fraction

TABLE 2. *In vitro synthesis of the dsRNA minus strand[a]*

Treatment of dsRNA synthesized in vitro	Acid-precipitable counts/min		
	Expt 1	Expt 2	Expt 3
Untreated...............	6,202	4,550	4,125
Ribonuclease (0.3 M NaCl)................	6,213	4,350	3,950
Ribonuclease (0.03 M NaCl)................	165	200	110
Denatured, reannealed with excess plus strands, ribonuclease (0.3 M NaCl)..........	6,041	4,310	3,760

[a] L cells (2 × 10^8) were infected with reovirus (60 plaque-forming units per cell); at 9 hr after infection the cells were collected, and the particulate extracts for dsRNA synthesis were prepared. RNA synthesis was carried out in 1-ml reaction mixtures; the dsRNA was isolated, and the incorporation of label into the minus strand was determined. The per cent of label found in the minus strand was 97, 99, and 95 in experiments 1, 2, and 3, respectively.

which contains mitochondria, lysosomes, and some endoplasmic reticulum. Treatment of this fraction with Triton X-100 or sodium deoxycholate had little or no effect on the synthesis of single- or double-stranded RNA, further confirming that the enzyme(s) engaged in dsRNA synthesis are located in particulate cytoplasmic structures devoid of membranes (Table 3). Treatment of the large granule fraction with chymotrypsin did not alter the capacity of this fraction to synthesize dsRNA, indicating that the dsRNA "synthetase" is either protected from or insensitive to chymotrypsin digestion. As expected, chymotrypsin markedly enhanced ssRNA synthesis by activating the RNA transcriptase in the reovirions contained within this fraction.

The resistance of the dsRNA "synthetase" to chymotrypsin digestion was reminiscent of the insensitivity of the particle-bound RNA transcriptase to this proteolytic enzyme, and we were intrigued by the possibility that dsRNA synthesis occurs within nascent viral particles. Because the intact core is impermeable to ribonuclease, we reasoned that, if the dsRNA precursor plus strands reside within the core, then they should be shielded from ribonuclease digestion. We therefore compared the amount of dsRNA synthesized in the presence and absence of ribonuclease. dsRNA synthesis was markedly inhibited by the addition of ribonuclease at the start of the incubation (Table 4). However, addition of ribo-

TABLE 3. *Effect of deoxycholate, Triton, and chymotrypsin on dsRNA synthesis in vitro[a]*

Treatment	(a) ssRNA synthesized (counts/min)	(b) dsRNA synthesized (counts/min)	(c) Reannealed products[b] (counts/min)	Per cent of label in minus strand (c/b × 100)
Untreated...	180,000	20,510	19,120	93
DOC........	171,060	19,430	18,520	95
Triton.......	269,980	19,250	18,260	95
Chymotryp-sin........	1,312,000	20,477	19,010	93

[a] One portion of cell-free extract was treated for 1 min in the cold with deoxycholate (DOC) at a concentration of 0.5% and centrifuged at 12,000 × g for 15 min, washed once with standard buffer A, recentrifuged, and resuspended in the same buffer. A second portion was treated in the cold for 1 min with 0.8% Triton X-100 and centrifuged, washed, and resuspended. The reaction mixtures for RNA synthesis are described in Materials and Methods. Chymotrypsin (100 μg/ml) was added to one incubation containing untreated extract. Acid-precipitable radioactivity in ssRNA, dsRNA, and the minus strand of dsRNA was determined. All of the in vitro label found in dsRNA was in the minus strand.

[b] Denatured dsRNA was reannealed in the presence of excess unlabeled plus strands.

nuclease 15 min after the initiation of RNA synthesis abolished ssRNA synthesis but had no effect on the amount of dsRNA produced. These data confirm that dsRNA synthesis is completed after 15 min of incubation in vitro; they also indicate that the dsRNA precursor plus strands are accessible to ribonuclease and are not sequestered within ribonuclease-impermeable structures. However, the ribonuclease digestion was performed in an incubation mixture which contained 0.25 M KCl and 7.5 mM Mg^{2+}. Under these conditions, dsRNA is resistant to ribonuclease digestion. Hence, from these data we could not be certain whether the newly formed dsRNA was also in a site accessible to ribonuclease or whether it had been sequestered within a structure which rendered it insensitive to ribonuclease. To distinguish between these two possibilities, ribonuclease was added after 15 min to an incubation mixture which had been diluted 10-fold with water to bring the final salt concentration to 0.025 M. At this lowered salt concentration, labeled dsRNA which was added to the incubation mixture was degraded by ribonuclease. However, the dsRNA which was synthesized in vitro remained resistant to ribonuclease digestion.

These data have led us to propose the following

TABLE 4. *Effect of ribonuclease on ssRNA and dsRNA synthesis in vitro[a]*

Ribonuclease addition (min)	Acid-precipitable counts/min	
	ssRNA	dsRNA
Without ribonuclease	61,922	7,931
15-30	2,873	6,904
0-30	1,462	1,012

[a] Ribonuclease (10 μg/ml) was added to the incubation mixtures at the indicated time intervals. Total RNA was extracted, and acid-precipitable radioactivity in dsRNA was determined. Values for ssRNA are total acid-precipitable counts per minute minus dsRNA counts per minute.

model for the structure which catalyzes dsRNA synthesis. RNA plus strands, which are precursors of dsRNA, are bound within a provirion that is permeable to ribonuclease. Synthesis of complementary minus strands on plus-strand templates may be accompanied by the condensation of viral proteins to form a structure which is impermeable to ribonuclease.

It cannot be determined from the data presented here whether the enzyme(s) responsible for ssRNA synthesis on dsRNA templates also catalyze the synthesis of minus strands on plus-strand templates to produce dsRNA. However, the possibility is intriguing, and there is presumptive evidence which supports such a hypothesis. (i) Based on the results described here as well as other evidence (*manuscript in preparation*), dsRNA is synthesized in a nucleoprotein particle which contains "dsRNA polymerase" and is a direct precursor of the mature virion. (ii) The enzyme which catalyzes the synthesis of ssRNA is associated with the same structure. (iii) Nascent provirion particles containing dsRNA and ssRNA transcriptase are incorporated intact into virions. We presume that the "dsRNA polymerase" is also incorporated into virions unless it has been excluded from the particle immediately after dsRNA synthesis, which in our view seems unlikely. Because the core is composed of only three polypeptide species and because one (or more) of these represents ssRNA transcriptase, we suggest that the "dsRNA polymerase" and the ssRNA transcriptase may be alternate activities of the same protein(s).

ACKNOWLEDGMENTS

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