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Reovirus Replicase-Directed Synthesis of Double-Stranded Ribonucleic Acid

S. SAKUMA AND Y. WATANABE
The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

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After the incubation of reovirus replicase reaction mixtures (containing labeled ribonucleoside triphosphates), partially double-stranded ribonucleic acid (pdsRNA) products were isolated by cellulose column chromatography followed by precipitation with 2 M NaCl. The pulse-labeled reaction product contained a significantly large amount of pdsRNA that became complete dsRNA as reaction time increased, indicating that pdsRNA was an intermediate of the replicase reaction. The newly synthesized RNA strand (^{3}H-labeled) of the pdsRNA was resistant to ribonuclease digestion, suggesting that single-stranded RNA regions were part of a preexistent unlabeled RNA template. These observations, together with the electrophoretic behavior of the pdsRNA in polyacrylamide gel, are consistent with the hypothesis that dsRNA is synthesized by the elongation of a complementary RNA strand upon a preexistent template of single-stranded RNA (i.e., messenger RNA). The direction of the RNA strand elongation was determined by carrying out the replicase reaction in the presence of ^{3}H-cytidine triphosphate (or ^{3}H-uridine triphosphate) and adenine triphosphate-α-^{32}P followed by a chase with excess unlabeled cytidine triphosphate (or uridine triphosphate). The dsRNA product was digested with T1 ribonuclease and the resulting 3′-terminal fragments were isolated by chromatography on a dihydroxyboryl derivative of cellulose. Examination of the ratio of ^{3}H to ^{32}P in these fragments indicated that RNA synthesis proceeded from the 5′ to 3′ terminus.

The infection of L cells with reovirus induces two ribonucleic acid (RNA) polymerases that support virus multiplication: a replicase and a transcriptase (1, 8, 11). Replicase mediates the synthesis of double-stranded RNA (dsRNA) and is found in template-bound form in a particulate fraction of the infected L-cell cytoplasm (10). With respect to buoyant density in a $CsCl$-Cs_2SO_4 mixture and thermal denaturation, the dsRNA synthesized by reaction with replicase and treated with pancreatic ribonuclease was found to be indistinguishable from reovirus dsRNA (8). As analyzed by polyacrylamide gel electrophoresis (1, 11), the replicase product was separated into three size classes which correspond to the three sizes of segments of reovirus dsRNA. The sum of these observations indicated that replicase catalyzes the synthesis of reovirus dsRNA.

In other experiments, isotopically labeled dsRNA product, after denaturation, was found to hybridize completely with an excess of unlabeled plus-strand RNA (messenger RNA [mRNA] is defined as plus-strand RNA in this paper). These results indicated that the replication of dsRNA proceeded by the unilateral synthesis of minus-strand RNA upon a preexistent RNA template (8).

It is our hypothesis that a minus-strand RNA elongates upon a single, complete plus-strand RNA to yield dsRNA. This report summarizes our analysis of the intermediate structures of dsRNA synthesis and provides evidence to support this model. In addition, data are presented which show the direction of the elongation of the minus-strand RNA during dsRNA synthesis.

MATERIALS AND METHODS

Buffers, isotopes, and chemicals. The following buffers were used: 0.1 M STE buffer, containing 0.1 M NaCl, 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, and 0.001 M ethylenediaminetetraacetate; 0.3 M STE buffer, as above, but containing 0.3 M NaCl; 0.1 M STES buffer, containing 0.5% sodium dodecyl sulfate (SDS) in 0.1 M STE buffer.

Tritiated uridine-5′-triphosphate and cytidine-5′-triphosphate (^{3}H-UTP and ^{3}H-CTP, respectively; specific activity, both 21 Ci/mmole), adenosine-5′-triphosphate-α-^{32}P (ATP-α-^{32}P; specific activity, 2.93 Ci/mmole) and ^{32}P-orthophosphoric acid were purchased from New England Nuclear Corp., Boston, Mass.; guanosine-5′-triphosphate-α-^{32}P (GTP-α-^{32}P; specific activity, 0.66 Ci/mmole) was obtained from Sigma Chemical Co., St. Louis, Mo. T1 ribonuclease, pancreatic ribonuclease, and snake venom phosphodiesterase were obtained from Worthington Biochemi-

cal Corp., Freehold, N.J. *m*-Aminophenylboric acid-hemisulfate and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodimide metho-*p*-toluensulfonate were from Aldrich Chemical Co., Inc., Milwaukee, Wis. *p*-Aminobenzyl-cellulose (Cellex PAB, exchange capacity, 0.35 mEq per g) was obtained from Bio-Rad Laboratories, Richmond, Calif.

Cells and virus. Conditions for culturing L cells in suspension and infecting them with reovirus type 3, a strain producing only complete virions (5), as well as the preparation of reovirus dsRNA labeled with ^{32}P-phosphate, ^{3}H-uridine, or ^{14}C-uridine, have been described previously (10, 12).

Replicase preparation and reaction conditions. Replicase was extracted from reovirus-infected L cells as described previously (8), except that the large particle fraction was treated with deoxycholate but not with chymotrypsin. Composition of the replicase reaction mixture and incubation conditions were the same as those previously described (8), except that 15 μCi (0.7 nmole) of ^{3}H-UTP or ^{3}H-CTP was added to 0.25 ml of the reaction mixture. When the reaction included ATP-α-^{32}P (10 μCi, 3.42 nmoles) or GTP-α-^{32}P (10 μCi, 15.2 nmoles) in the reaction mixture (0.25 ml), unlabeled ATP or GTP was omitted. When the reaction was chased by the addition of unlabeled UTP or CTP, 0.2 μmole of the nucleotide in a volume of 0.01 ml was added to 0.25 ml of the reaction mixture.

Extraction of RNA and Sephadex G-100 chromatography. RNA was extracted from the replicase reaction mixture by 0.5% SDS and phenol and then precipitated with alcohol. The precipitated RNA was dissolved in 0.3 ml of 0.1 M STES buffer and filtered through a column of Sephadex G-100 (0.6 by 30 cm), which had been equilibrated with 0.1 M STES buffer. The RNA synthesized in the reaction mixture was eluted in the void volume of buffer and was precipitated again with alcohol.

Chromatography on cellulose. Single-stranded RNA (ssRNA), synthesized in the reaction by transcriptase (a contaminant of the replicase preparation), was separated from the replicase product by chromatography on cellulose powder (Whatman CF-11), by the method of Franklin (4).

Chromatography on a dihydroxyboryl derivative of cellulose. *N*-(*N*'-[*m*-dihydroxyborylphenyl] succinamyl)aminobenzylcellulose (BPSA-cellulose) was prepared by condensing *p*-aminobenzylcellulose with *N*-(*m*-dihydroxyborylphenyl)-succinamic acid by the method of Weith et al. (14). A column (0.6 by 30 cm) of the BPSA-cellulose was equilibrated with buffer A (0.1 M $MgCl_2$, 1.0 M NaCl, 0.05 M morpholinium chloride; *p*H 8.7). The column was then washed with a succession of 50 ml of buffer A, 50 ml of buffer B (0.1 M $MgCl_2$, 1.0 M NaCl, 0.05 M morpholinium chloride; *p*H 5.5), and finally 100 ml of buffer A.

The replicase product was then chromatographed according to a modification of the method of Rosenberg and Gilham (7). First the RNA (200 μg including yeast RNA) was dissolved in 0.5 ml of 0.05 M morpholinium chloride, *p*H 8.0, heated at 100 C for 3 min, and then cooled rapidly. Next, T1 ribonuclease (10 units, 10 μliters) was added to the denatured RNA, and the mixture was incubated for 10 min at 37 C. This treatment converted approximately 50% of the RNA to a trichloroacetic acid-soluble form. The T1 digest was diluted with 0.5 ml of twice-concentrated buffer A and applied to the column. The material was adsorbed, then eluted with 50 ml of buffer A at a flow rate of 0.2 ml/min, and finally eluted with 30 ml of buffer B. Samples from each 1-ml fraction were mixed with 10 ml of Bray's solution and counted in a liquid scintillation spectrometer to determine radioactivity. Correction was made for spillage of ^{32}P or ^{14}C into ^{3}H.

RESULTS

Isolation of the intermediate of dsRNA synthesis. RNA, synthesized in vitro using replicase and ^{3}H-UTP, was extracted from the reaction mixture by SDS and phenol to remove a previously reported "unstable" portion of the ribonuclease-resistant product (8). The material was then filtered through Sephadex G-100 and chromatographed on a cellulose column to separate the replicase product from contaminating ssRNA. This ssRNA contaminant had been synthesized by reovirus transcriptase which was present in the replicase preparation (8). As shown in Fig. 1a, ssRNA passed out of the cellulose column in 0.1 M STE buffer containing 15% alcohol, whereas dsRNA and partially double-stranded RNA (pdsRNA) were eluted by 0.1 M STE buffer alone. Repeated chromatography of the dsRNA fraction showed that this material was free of ssRNA and that the ^{3}H label contained in the dsRNA fraction was resistant to pancreatic ribonuclease digestion in 0.3 M NaCl (Fig. 1b and Table 1).

The RNA product, free of ssRNA, was then fractionated by precipitation in 2 M NaCl. By this method, it was possible to separate complete dsRNA from dsRNA that contained regions of single-strandedness, i.e., pdsRNA. To study the role of pdsRNA in the synthesis of dsRNA, the following experiments were done. dsRNA was synthesized in three different in vitro reaction mixtures using ^{3}H-UTP as the labeled precursor. The first mixture was incubated for 5 min, the second for 20 min, and the third for 5 min, followed by a 20-min chase with unlabeled UTP. From all three, the RNA products were extracted, and the dsRNA was isolated by cellulose chromatography. These preparations (to be referred to as the 5-min product, the 20-min product, and the 5- to 20-min chased product, respectively) were then treated with 2 M NaCl to precipitate RNA having regions of single-stranded character. The precipitable and nonprecipitable fractions of each sample were then assayed for radioactivity. It was observed that the ^{3}H label in both the precipitable and nonprecipitable RNA fractions was almost as resistant to pancreatic ribonuclease digestion as virion dsRNA. (Table 1). It was also found that the 5-min product consisted of a relatively large

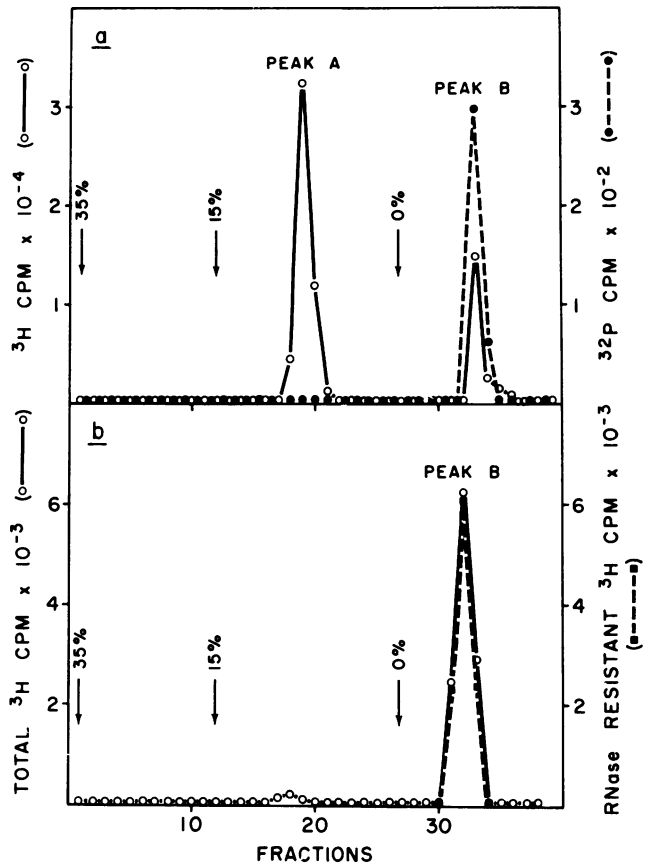

FIG. 1. *Purification of replicase product by chromatography on cellulose. The replicase reaction was carried out in 1.5 ml of reaction mixture and incubated at 37 C for 20 min. RNA was extracted from the reaction mixture, filtered through Sephadex G-100, and chromatographed on cellulose as described in Materials and Methods. (a) Chromatography elution profile of total acid-precipitable RNA with ^{32}P-labeled viral dsRNA used as marker. Arrows indicate the position of buffer change and the percentage of alcohol in the eluting buffer. A portion of peak B material was rechromatographed, and each fraction was counted for total acid-precipitable RNA as well as RNA resistance to ribonuclease treatment (2 μg/ml in 0.3 M STE buffer at 37 C for 30 min). (b) Rechromatography pattern without the ^{32}P marker.*

amount of precipitable radioactive RNA (pdsRNA) and the 20-min product and 5-20-min chased product had significantly less (Table 2). These results suggest that the NaCl-precipitable pdsRNA formed in the 5-min reaction was converted to a nonprecipitable form during the prolonged incubation or chase period, that pdsRNA is the intermediate of dsRNA synthesis, and that the newly synthesized ^{3}H-labeled RNA strand was included in the dsRNA region of the intermediate. The ssRNA region was probably part of the unlabeled preexistent template RNA.

Analysis of pdsRNA by gel electrophoresis. Reovirus dsRNA can be separated into 10 segments by electrophoresis in polyacrylamide gel (9, 12). The migration of these segments is slower than the migration of the single-stranded mRNA which corresponds in length to the largest dsRNA segment (13). It has also been reported that the three kinds of Qβ phage-specific RNA can be separated from each other by acrylamide gel electrophoresis (4). The single-stranded phage RNA migrates fastest, followed by the "replicative form" (complete dsRNA) and finally the "replicative intermediate" which migrates slowest (2). Because of this correlation between the structure of RNA and its rate of migration in polyacrylamide, it seemed reasonable that electrophoretic analysis of the NaCl-precipitable dsRNA intermediate would provide information about its molecular structure and aid in delineating the mode of dsRNA replication. Therefore, NaCl-

TABLE 1. *Digestion of replicase product by pancreatic ribonuclease*

dsRNA prepn[a]	% Ribonuclease resistant[b]		
	Expt 1	Expt 2	Expt 3
1st chromatography on cellulose column	85.3	88.9	94.5
2nd chromatography on cellulose column	ND[c]	ND	87.6
Fractionation in 2 M NaCl[d]			
Supernatant	85.3	86.0	87.7
Precipitate	77.0	83.4	83.7
^{32}P-labeled virion dsRNA[e]	90.2	ND	93.4

[a] Double-stranded ribonucleic acid (dsRNA) was synthesized during a 5-min reaction using ^{3}H-UTP as label in a total volume of 2 ml and purified as described in Materials and Methods.

[b] Ribonuclease resistance was determined by incubating RNA in 1 ml of 0.3 M STE buffer with 10 μg of pancreatic ribonuclease at 37 C for 30 min. RNA was precipitated by 5% trichloroacetic acid and counted. Values are mean of duplicated assay.

[c] Not determined.

[d] Samples of the dsRNA fraction were mixed with 10 mg of high-molecular-weight L-cell RNA, which had been purified by precipitation in 2 M NaCl, and the mixture was adjusted to 2 M NaCl in a total of 1 ml. After 20 hr at 4 C, the resulting precipitate was centrifuged for 10 min at 10,000 $\times$ *g*, then resuspended in 0.3 ml of 2 M NaCl, and centrifuged again. The supernatants were pooled, and the level of radioactivity in both supernatant and precipitate was measured by trichloroacetic acid (5%) precipitation followed by filtration on nitrocellulose membranes.

[e] ^{32}P-labeled virion dsRNA was added before the 2 M NaCl fractionation. Over 95% of the ^{32}P was recovered in the nonprecipitable fraction.

precipitated pdsRNA from the 5-min product was subjected to electrophoresis in polyacrylamide gel using ^{32}P-labeled virion dsRNA as a marker. The ^{3}H label was widely distributed along the gel. Some part of it migrated within the region of marker dsRNA; none of it migrated slower than the largest dsRNA segment, and a considerable amount migrated faster than the smallest dsRNA segment (Fig. 2a). Electrophoretic migration of the intermediate of reovirus dsRNA synthesis, therefore, was faster than complete dsRNA but slower than ssRNA, demonstrating that the molecular structure of the intermediate is different from that of the replicative intermediate of ssRNA phage (4).

The fraction of dsRNA product which was not precipitated in 2 M NaCl was then electrophoresed in polyacrylamide (Fig. 2b and c). The migration of these products was limited, for the most part, to the region of the marker dsRNA. In addition, there is a significant correspondence between the peaks of marker dsRNA, the 20-min product (*not shown*), the 5-20-min chased product (Fig. 2c), and to a lesser degree the 5-min product (Fig. 2b). These results plus the data in Table 2 suggest that early in the synthesis of dsRNA, the intermediate has ssRNA regions which later become double-stranded.

Net elongation of RNA strand during chase period. The incorporation of ^{3}H-UTP into dsRNA was readily stopped by "chasing" with excess unlabeled UTP (Table 2, reference 8). The following experiments were done to determine whether elongation of the newly synthesized RNA strand into dsRNA was taking place under the conditions of the chase.

In the first experiment, a reaction mixture containing ^{3}H-UTP and ATP-α-^{32}P (experiment 1 in Table 3) or ^{3}H-CTP and ATP-α-^{32}P (experiment 2 in Table 3) and other components necessary for the replication reaction was divided into three portions. Reaction 1 was stopped with SDS and phenol after 5 min of incubation and reaction 2 after 20 min. Reaction 3, after 5 min, received unlabeled UTP (experiment 1) or CTP (experiment 2), and then incubation was continued for 20 min and finally stopped by the addition of SDS and phenol. The replicase product was quantitatively extracted from these reaction mixtures and purified by chromatography on a cellulose column. The level of radioactivity in the dsRNA fraction was then measured (Table 3). After the addition of excess unlabeled UTP or CTP, there was only a small increase in ^{3}H-UTP or ^{3}H-CTP incorporation, whereas radioactivity from ATP-α-^{32}P was incorporated into dsRNA at a rate similar to that of the reaction in which no unlabeled UTP or CTP was added. The increase in the amount of ^{32}P incorporated during the chase period was approximately 2.5-fold. Assuming that there was no reinitiation of minus-strand synthesis (8) and that AMP was randomly distributed, these data suggest that the new RNA strand was elongated approximately 2.5-fold under the conditions of the chase period.

In the second experiment, the replicase reaction was carried out in the presence of ^{3}H-UTP. ^{3}H-labeled 5-min product and 5-20-min chased product were prepared and purified as before. These replicase products then were denatured to ssRNA by 95% dimethylsulfoxide, and the molecular weight of the newly synthesized (^{3}H-labeled) RNA strand was determined by sedimentation in a sucrose gradient containing formaldehyde (10; Fig. 3). The sedimentation coefficients of ^{32}P-RNA (denatured virion dsRNA added as marker) were similar to those of the three size classes of

TABLE 2. *Fractionation of replicase product in 2 M NaCl*[a]

Incubation conditions	Supernatant (counts/min)/precipitate (counts/min) (percent non-precipitable)		
	Expt 1	Expt 2	Expt 3
5 Min	822/692 (54.3)	5,008/1,473 (77.3)	2,760/1,556 (59.2)
20 Min	2,448/552 (81.6)	10,932/674 (94.2)	5,075/776 (86.9)
5 Min and chased 20 min	1,262/97 (92.9)	6,253/334 (94.9)	3,134/310 (91.0)
^{32}P-virion dsRNA[b] (control)	980/12 (98.8)		5,200/153 (97.2)

[a] Experiments 1, 2, and 3 were carried out with three different replicase preparations in 1.5 ml of reaction mixture. After incubation in the presence of ^{3}H-UTP for the indicated time intervals, the replicase product was extracted and purified as described in Materials and Methods. The RNA was fractionated in 2 M NaCl, as described in Table 1, and samples of precipitable and nonprecipitable RNA were counted.

[b] ^{32}P-double-stranded ribonucleic acid (dsRNA) was mixed with the 5-min product, and distribution of ^{32}P between precipitable and nonprecipitable fractions were determined.

reovirus mRNA (10), indicating that the denaturation of dsRNA into ssRNA was essentially complete and that no renaturation had occurred during sedimentation analysis. Therefore, the distribution of ^{3}H in the gradient represented the molecular-weight distribution of the newly synthesized RNA strand. As shown in Fig. 3a and b, the ^{3}H-labeled RNA in the 5-20-min chased product sedimented within the region of denatured marker ^{32}P-virion RNA to a greater extent than the 5-min product. The observed increase in average molecular weight during the chase period was estimated to agree with the 2.5-fold elongation suggested by the result shown in Table 3. Therefore, it was concluded that the incorporation of labeled nucleotides represented net elongation of the RNA strand and that this elongation continued during the chase period.

Direction of RNA chain elongation: experimental design. As described for the experiments in Table 3, the replicase product was isolated from a reaction mixture that had been incubated with ^{3}H-CTP (or ^{3}H-UTP) and ATP-α-^{32}P as the labeled precursors and then chased by the appropriate unlabeled pyrimidine nucleotides (CTP or UTP, respectively). Under the chase conditions employed, the incorporation of ^{32}P-adenosine monophosphate (AMP) should continue and ^{3}H-labeled CTP or UTP incorporation should cease (Table 3). The dsRNA product was heat-denatured and then digested with T1 ribonuclease. The resulting RNA fragments were chromatographed on a column of BPSA-cellulose to isolate the fragments of the 3'-terminal end of the RNA from the internal fragments and 5'-terminal fragments of the RNA (to be referred to as non-3'-terminal fragments). Finally, the ratios of ^{3}H/^{32}P in both the 3'-terminal and non-3'-terminal fragments were determined. This test system assumes that if the 3'-terminal fragments of the dsRNA product, prepared and isolated in the manner described above, were found to have a significantly lower ^{3}H/^{32}P ratio than the non-3'-terminal fragments, this would be evidence that the replicase-directed RNA synthesis proceeds from the 5' to 3' terminus.

Chromatographic isolation of the 3'-terminal fragments of dsRNA replicase products. The methodology for separation of the 3'-terminal fragments from the non-3'-terminal fragments by BPSA-cellulose chromatography is based on the specific interaction of the dihydroxyboryl group of the cellulose derivative with the unsubstituted 2'3'-dihydroxyl groups of the 3'-terminal fragments (7, 14). When the product of T1 ribonuclease digestion of denatured dsRNA is applied to the column, the 3'-terminal fragments are retained while the non-3'-terminal fragments having 3'-phosphorylated guanosine monophosphate (GMP) residues at the 3'-terminus (3) are eluted without retention (7).

Figure 4a characterizes the elution profiles of various nucleic acid components under our conditions of BPSA-cellulose chromatography. All the ribonucleoside-5'-monophosphates were retained to various degrees on the column and eluted, as represented by regions II and III. To characterize the elution of T1-digested reovirus dsRNA, ^{3}H-labeled virion dsRNA was first denatured and then digested by T1 ribonuclease as described above. Under these conditions, 50% of the dsRNA became trichloroacetic acid soluble. Based on the specificity of T1 ribonuclease (3), most of the ^{3}H-labeled RNA fragments should have 3'-phosphorylated GMP residues on the 3'-terminus. The T1 digest was mixed with a snake venom phosphodiesterase digest of ^{14}C-labeled virion dsRNA, which served as a marker for uridine-5'-monophosphate (6), and the mixture was chromotographed on the BPSA-cellulose

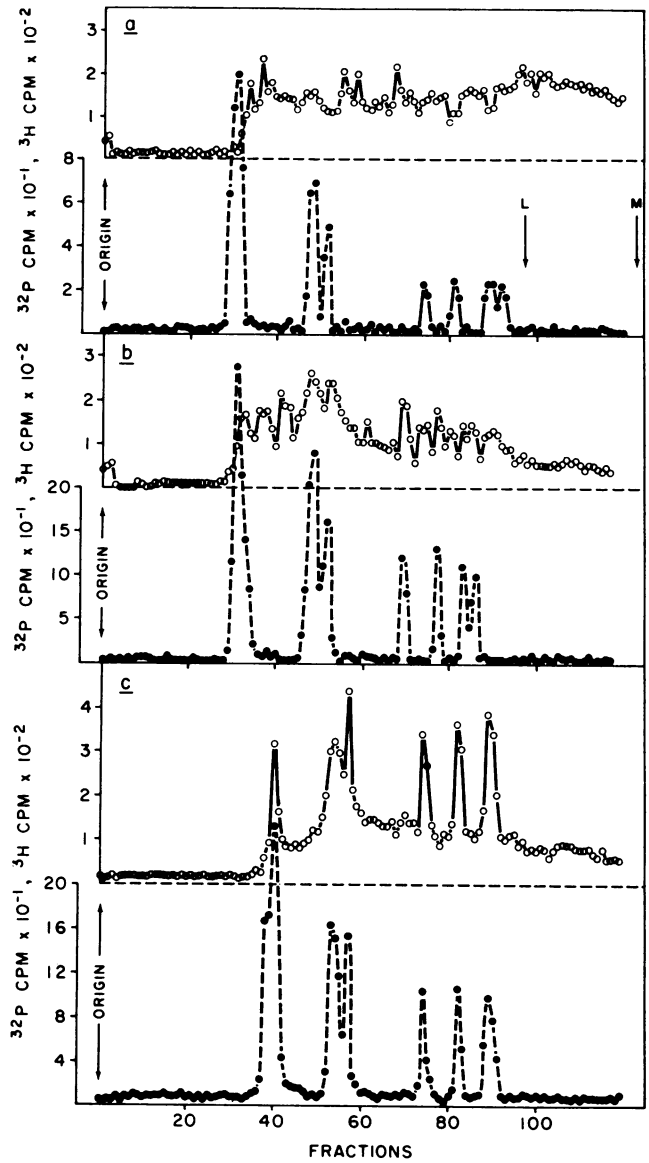

FIG. 2. *Electrophoretic analysis of the replicase product in polyacrylamide gel. NaCl-precipitable ^{3}H-pdsRNA product from the 5-min product (a) and ^{3}H-dsRNA products not precipitable in NaCl from the 5-min product (b) and the 5- to 20-min chased product (c) were mixed with ^{32}P-labeled virion dsRNA and subjected to electrophoresis in polyacrylamide gel by the method described previously (12). Reaction conditions are described in Table 2. Approximate positions of large (L) and medium (M) classes of reovirus mRNA are indicated by arrows (a). Migration proceeded from left to right.*

column. As shown in Fig. 4b, ^{14}C-uridine-5'-monophosphate was, as predicted by the results of 4a, retarded by the column and eluted in region II. The ^{14}C-labeled material that eluted in region III has not been identified. The majority of ^{3}H-uridine-labeled fragments were not retained by the column, due to phosphorylation at the 3' end, and therefore eluted in region I. The ^{3}H-labeled RNA fragments from the 3'-termini, having unsubstituted dihydroxy groups at the 3' end, were expected to elute in regions II and III, but, because they represented only a small portion of the T1 digest, their elution was not detectable. Therefore, it was necessary to do another analysis to

TABLE 3. *Pulse-chase experiments of replicase reaction with double labeling*

Reaction conditions	Radioactivity in dsRNA[a] fraction		
	^3H (counts/min)	^{32}P (counts/min)	$^3H/^{32}P$ ratios
Expt 1, labeled precursors: ^3H-UTP and ATP-α-^{32}P[b]			
5-Min incubation	100,500	65,900	1.53
20-Min incubation	256,900	187,500	1.44
5 Min, then chased by UTP for 20 min	100,700	163,900	0.61
Expt 2, labeled precursors: ^3H-CTP and ATP-$\alpha^{32}P$[c]			
5-Min incubation	25,000	8,950	2.79
20-Min incubation	48,677	19,297	2.81
5-Min, then chased by CTP for 20 min	28,320	22,900	1.24

[a] Double-stranded ribonucleic acid.

[b] The replicase reaction was carried out in 8.25 ml of reaction mixture containing ^3H-UTP (495 μCi, 29.7 nmoles) and ATP-α-^{32}P (333 μCi, 116 nmoles). Other conditions were the same as those for the standard assay system described previously (8). dsRNA fraction (containing partially double-stranded ribonucleic acid) was obtained as described in the Materials and Methods.

[c] The reaction was carried out in 8.0 ml of reaction mixture containing ^3H-CTP (500 μCi, 23.4 nmoles) and ATP-α-^{32}P (333 μCi, 86.7 nmoles). Other conditions are the same as above.

determine the eluting position of the 3'-terminal fragments. This analysis was done with in vitro synthesized dsRNA in anticipation of our final analysis of in vitro synthesized dsRNA. As shown in Fig. 2, in vitro synthesized dsRNA contains a significant amount of pdsRNA that would yield a heterogeneous mixture of 3'-terminal fragments upon digestion with T1 ribonuclease, so this preliminary analysis was also necessary to determine the elution pattern of a heterogeneous mixture of 3'-terminal fragments. dsRNA was synthesized in a 20-min in vitro reaction using ^3H-UTP and GTP-α-^{32}P as the labeled precursors. Since the replicase reaction was not chased with unlabeled UTP, a random distribution of ^3H and ^{32}P should be present in the replicase product. The replicase product was isolated and digested with T1 ribonuclease, and the resulting RNA fragments were applied to the BPSA-cellulose column. The column was eluted with 50 ml of buffer A (*p*H 8.7) followed by 30 ml of buffer B (*p*H 5.5) which would remove any 3'-terminal fragments still linked to the column through the dihydroxy groups (7). Analysis of the chromatograph was based on the following properties of the T1 ribonuclease digestion products: (i) all the radioactive non-3'-terminal fragments have at least one ^{32}P-GMP residue at the 3'-terminus and elute in peak I, (ii) at most, only 25% of the 3'-terminal fragments are labeled with a ^{32}P-GMP residue at the 3'-terminus, so that the ratio of $^3H/^{32}P$ among 3'-terminal fragments is higher than among non-3'-terminal fragments (the 3'-terminal fragments with CMP, uridine monophosphate [UMP] or AMP at the 3'-terminus are not labeled with ^{32}P, whereas the 3'-terminal fragments with a single GMP residue at the 3' terminus are labeled with ^{32}P). Results of the experiment are shown in Fig. 4c. Only 35 of the total 80 fractions collected are shown. Fractions 36 to 50, which were eluted in buffer A had such low levels of radioactivity that calculation of $^3H/^{32}P$ ratios are not meaningful for this analysis. A measurable peak of radioactivity was eluted in buffer B (fractions 51 to 80) but the $^3H/^{32}P$ ratios were significantly lower than the ratios in region I. According to our interpretation, these fractions did not contain a detectable amount of 3'-terminal fragments. From the elution pattern it is clearly evident that the $^3H/^{32}P$ ratio is higher in region II than in region I, indicating that at least some of the 3'-terminal fragments of the replicase product were retarded by the BPSA-cellulose column and were eluted in region II. If the majority of 3'-terminal fragments were eluted in region II, the $^3H/^{32}P$ ratios in region II should have been higher than those observed. But this result is reasonable when the contour of the elution curve is considered. The shoulder that extends into region II probably contains trailing non-3'-terminal fragments whose low $^3H/^{32}P$ ratio masks the predicted high ratio of the 3'-terminal fragments that are also eluting in region II. Nevertheless, the difference in the ratios is sufficient to distinguish the 3'-terminal fragments from the non-3'-terminal fragments.

Analysis of dsRNA product synthesized in the

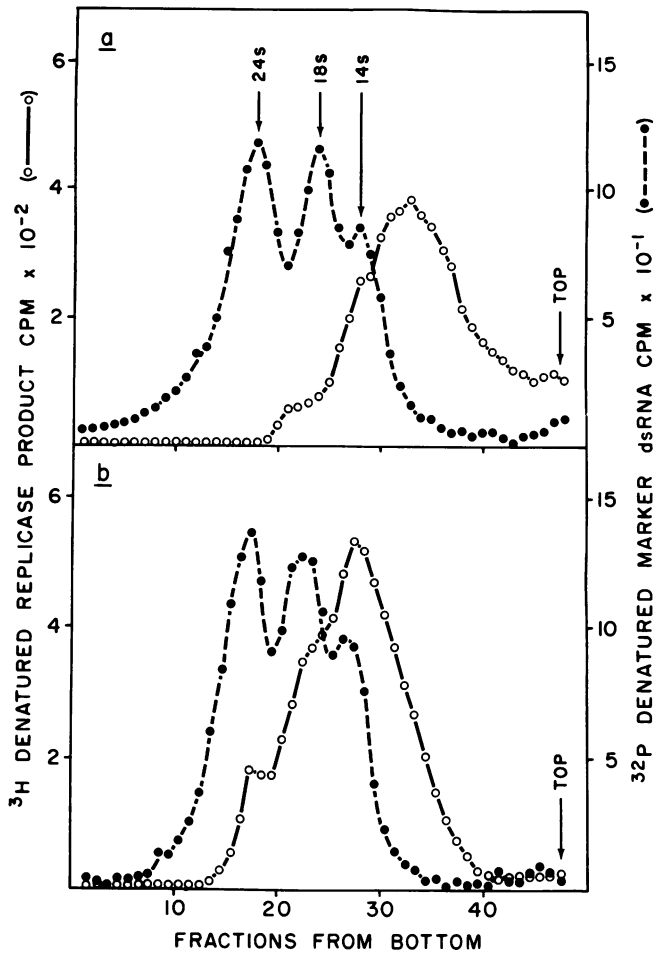

FIG. 3. *Sedimentation of denatured replicase product in a sucrose gradient containing formaldehyde. A mixture of dsRNA and pdsRNA from experiment 1 of Table 2 was combined with ^{32}P-labeled virion dsRNA, denatured in 95% dimethylsulfoxide at 37 C for 30 min, and then precipitated with alcohol. The precipitated RNA was dissolved in 0.2 ml of 0.1 M STES buffer containing 0.1% formaldehyde and sedimented through a sucrose gradient (5 to 20% sucrose in 0.1 M STES buffer containing 0.1% formaldehyde) at 100,000 × g for 140 min at 20 C. Sedimentation coefficients of the ^{32}P-labeled marker RNA were determined in a separate experiment in which reovirus mRNA was cosedimented as described previously (10). (a) ^{3}H-labeled denatured 5-min product, (b) ^{3}H-labeled denatured 5- to 20-min chased product.*

presence of ^{3}H-CTP (or ^{3}H-UTP) and ATP-α-^{32}P. As described in the experimental design, the dsRNA product, isolated from a reaction mixture that had been incubated with ^{3}H-CTP and ATP-α-^{32}P and then chased by unlabeled CTP, was digested with T1 ribonuclease and then chromatographed (Fig. 5a). As controls, dsRNA products from reaction mixtures incubated for 20 min or for 5 min in the presence of ^{3}H-CTP and ATP-α-^{32}P (without chase) were analyzed in the same manner (Fig. 5b and c). Only the chased product showed a significant decrease in ^{3}H/^{32}P ratio in the region of peak II, where 3′-terminal fragments have been shown to elute. Although region III was also richer in ^{32}P than ^{3}H, this result is less significant due to the lower level of radioactivity in this region (Fig. 5a).

When labeling was done with ^{3}H-UTP and ATP-α-^{32}P followed by a chase with unlabeled UTP, a decrease in the ^{3}H/^{32}P ratio was also obtained. The ^{3}H/^{32}P ratio in the regions of peak I and II are summarized in Table 4. Clearly, the chased dsRNA product had a decreased ^{3}H/^{32}P ratio in the region containing 3′-terminal fragments as compared to the ratio in the region of non-3′-terminal fragments. From these results, it

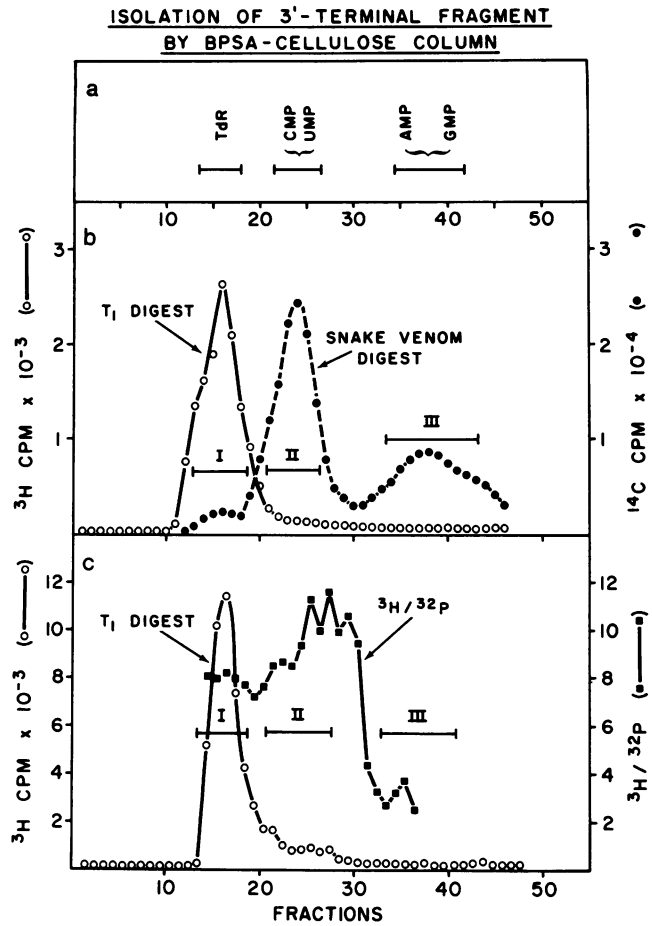

FIG. 4. *Chromatography of various nucleotides and RNA fragments of a BPSA-cellulose column. (a) A composite diagram of four chromatographic results, showing eluting positions of the four rubonucleoside-5'-monophosphates and deoxythymidine (TdR). (b) Elution pattern of mixed chromatography of the T1 ribonuclease digest of the reovirus dsRNA which had been labeled with ^{3}H-uridine and the snake venom phosphodiesterase digest of the reovirus dsRNA which had been labeled with ^{14}C-uridine. Digestion of dsRNA with T1 ribonuclease was done as described in Materials and Methods. For digestion with snake venom enzyme, 1 μg of dsRNA was mixed with 250 units of enzyme in 0.5 ml of morpholinium buffer (pH 8.0) containing 5 mM $MgCl_2$ and incubated at 37 C for 1 hr. (c) Elution pattern of the T1 ribonuclease digest of dsRNA which was synthesized by replicase during a 20-min in vitro reaction using ^{3}H-UTP and GTP-α-^{32}P as labeled precursors. The amount of ^{3}H and the ratio of ^{3}H to ^{32}P in each fraction are plotted. Elution pattern of ^{32}P is not shown.*

is concluded that the direction of minus-strand RNA synthesis, mediated by replicase, is from the 5′ to 3′ terminus.

DISCUSSION

It has been shown that with respect to thermal denaturation and buoyant density in CsCl-Cs_2SO_4 mixture the replicase product, upon removal of ssRNA regions by digestion with ribonuclease, was indistinguishable from virion dsRNA (8). This observation implies that the dsRNA regions of the pdsRNA are completely double stranded. Previous results also showed that only minus-strand RNA (complementary to reovirus mRNA) is newly synthesized during the process of in vitro dsRNA synthesis (8).

The results presented here demonstrate that pdsRNA was formed during the reovirus dsRNA synthesis catalyzed by the virus-induced replicase and that pdsRNA was an intermediate form of dsRNA synthesis. Newly synthesized minus RNA has been shown to elongate from the 5′ to 3′ terminus and to become almost totally incorporated into the dsRNA region of the interme-

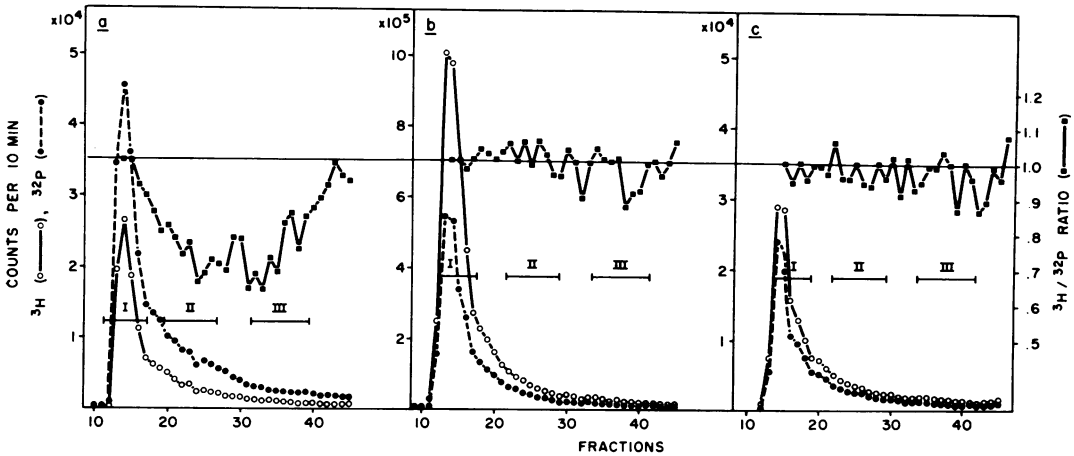

FIG. 5. *Chromatography of the T1 ribonuclease digest of the dsRNA synthesized in the presence of ^3H-CTP and ATP-α-^{32}P. The replicase product was synthesized and purified as described in Table 3. These products were heat-denatured, digested with T1 ribonuclease, and then chromatographed; (a) 5- to 20-min chased dsRNA product, (b) 20-min product, and (c) 5-min product. The scales of radioactivity of ^3H and ^{32}P are shown on the left side of each diagram. The positions of peaks I, II, and III were determined from the results of Fig. 4. The ratios of ^3H to ^{32}P are standardized by assigning a ratio of 1 to region I. Actual values of counts per minute of $^3H/^{32}P$ ratios are presented in Table 4.*

TABLE 4. *$^3H/^{32}P$ ratios of 3'-terminal and non-3'-terminal RNA fragments*[a]

Labeled precursors	Incubation conditions (min)	$^3H/^{32}P$ ratios		
		Peak I (a)	Peak II (b)	b/a
Expt 1, ^3H-UTP and ^{32}P-ATP	5	1.24	1.19	0.96
	20	1.34	1.28	0.96
	5 and chased	0.71	0.46	0.65
Expt 2, ^3H-UTP and ^{32}P-ATP	5	1.13	0.94	0.83
	20	0.99	0.90	0.91
	5 and chased	0.42	0.27	0.64
Expt 3, ^3H-CTP and ^{32}P-ATP	5	1.20	1.31	1.09
	20	1.67	1.70	1.02
	5 and chased	0.57	0.38	0.67

[a] In experiments 1 and 2, double-stranded ribonucleic acid was synthesized using ^3H-UTP and ATP-α-^{32}P, and in experiment 3 using ^3H-CTP and ATP-α-^{32}P. The experimental conditions are described in Table 3. The RNA was denatured, digested with T1 ribonuclease, and then chromatographed. Chromatography pattern for experiment 3 is shown in Fig. 5. The $^3H/^{32}P$ ratio represents (total counts per minute of ^3H) per (total counts per minute of ^{32}P) in regions of peak I and II. The peak II position was determined as shown in Fig. 5.

diate form, indicating that the single-stranded regions are a preexistent RNA template.

It is possible to hypothesize two mechanisms of in vitro dsRNA synthesis. One, referred to here as model I, involves the elongation of minus-strand RNA upon single, complete, plus-strand RNA to yield dsRNA. A second possible mechanism, model II, involves the incorporation of newly synthesized minus-strand RNA by replacement of the minus strand of the complete, preexistent dsRNA molecule. According to our results, the intermediate of in vitro dsRNA synthesis, pdsRNA, migrates faster in polyacrylamide gel than complete dsRNA and a considerable amount migrates as fast as reovirus mRNA (Fig. 2a). If the intermediate had the configuration predicted

by model II, at least some of it would migrate slower and none would migrate faster than complete dsRNA (2). Therefore, we conclude that model I is the better representation of in vitro dsRNA synthesis and perhaps a good approximation of dsRNA synthesis as it occurs in infected cells.

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