

Unilateral Synthesis of Reovirus Double-Stranded Ribonucleic Acid by a Cell-Free Replicase System

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Received for publication 19 March 1971

A large-particle fraction obtained from reovirus-infected L cells contained both replicase and transcriptase activity. The *in vitro* replicase reaction slowed down soon after initiation, whereas the transcriptase reaction proceeded at an unabated rate. The replicase and transcriptase were both template-bound and could be separated from one another by controlled chymotryptic digestion followed by centrifugation in a CsCl gradient. The transcriptase was recovered as a sharp band ($\rho = 1.43$) and resembled virus core derived from mature virions. In contrast, replicase activity was distributed throughout the gradient, indicating that replicase is associated with structures of various density in CsCl. In subsequent experiments, the replicase product was found to be indistinguishable from the double-stranded ribonucleic acid (RNA) reovirus genome with respect to its buoyant density in cesium-salt gradients and denaturation-annealing characteristics. A "hybridization-competition" experiment in which the replicase product was denatured and annealed in the presence of an excess of plus-RNA indicated that the *in vitro* replicase reaction proceeded by means of a unilateral synthesis of minus-RNA upon a preexisting plus-RNA template, presumably of single-stranded form.

We have previously shown that in reovirus-infected L cells, a ribonucleic acid (RNA) polymerase is induced which synthesizes double-stranded RNA (dsRNA) segments resembling the genomic segments of reovirus (12). In the present paper, this polymerase will be tentatively designated "replicase," to distinguish it from the reovirus-bound "transcriptase." Transcriptase is found in the subviral particles of reovirus (virus core) and synthesizes single-stranded RNA (ssRNA) by transcribing only one strand of reovirus dsRNA (1, 4, 5, 10, 11). For our purposes, the ssRNA synthesized by transcriptase will be referred to as plus-RNA, and the RNA complementary to it, as minus-RNA.

Recently, Schonberg et al. (9) found that the reovirus dsRNA obtained from pulse-labeled, infected cells contained label only in the minus strand. They concluded from this *in vivo* study that the complementary strands of reovirus dsRNA are formed in an asymmetric manner; i.e., the minus strand is synthesized upon a preformed plus-strand template. We decided to further investigate the mechanism by which reovirus dsRNA is synthesized under the more controlled conditions provided by a cell-free replicase system. We therefore wished to determine (i)

whether replicase and transcriptase present in cell extract are two distinct entities and (ii) whether the cell-free replicase reaction involved the simultaneous synthesis of plus- and minus-RNA strands or, as in replication *in vivo*, the asymmetric ("unilateral") synthesis of either a plus- or minus-RNA strand. To investigate the latter point, we conducted a hybridization-competition experiment employing the *in vitro* replicase product.

MATERIALS AND METHODS

Buffers 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 (pH 7.4) and 0.001 M ethylenediamine-tetraacetate (EDTA); 0.3 and 0.01 M STE buffers, as above, but containing 0.3 and 0.01 M NaCl, respectively; 0.1 M STES buffer, containing 0.5% sodium dodecyl sulfate (SDS) in 0.1 M STE buffer; TMS buffer, containing 0.25 M sucrose, 0.001 M MgCl₂, and 0.01 M Tris-hydrochloride, pH 8.2; TM buffer, as above, but without sucrose. Tritiated uridine triphosphate (³H-UTP; specific activity, 17 Ci/mmmole) and ³²P-orthophosphoric acid were purchased from New England Nuclear Corp., Boston, Mass.; α -chymotrypsin and ribonuclease (pancreatic), from Worthington Biochemical Corp., Freehold, N.J.;

and unlabeled nucleoside triphosphates, from Calbiochem Co., Los Angeles, Calif.

Cells, virus, and viral RNA. Conditions for the suspension culture of L cells and their infection with reovirus type 3 [a strain producing only complete virions (8)] have been described previously (13, 15). Reovirus highly labeled with ^{32}P was prepared by incubating the infected cells in a phosphate- and serum-free medium containing ^{32}P -orthophosphate (10 $\mu\text{Ci}/\text{ml}$) throughout the infection process (about 20 hr). The ^{32}P -labeled virus was purified by a procedure described previously (15), and the virion RNA was extracted with phenol. Adenine-rich ssRNA (2) was eliminated from the viral RNA extract by filtration through a column of Sephadex G-100 gel (1.5 by 30 cm) equilibrated with 0.1 M STES buffer. The purified virion dsRNA eluted as a distinct peak after a void volume of buffer.

Preparation of RNA polymerase-template complexes from infected cells. A modification of a procedure described previously (12) was used. At 7 to 8 hr post-infection, the cells were centrifuged, washed once with cold phosphate-buffered saline, suspended in five volumes of TMS buffer containing the nonionic detergent, Nonidet P-40 [1% (v/v)], and disrupted in a Dounce homogenizer. The extent of cell destruction was monitored by microscopy, and the homogenization continued until 80% of the cells were disrupted. This and all subsequent procedures were carried out at 4 C. Nuclei and large cellular debris were removed after each of three centrifugations at $860 \times g$ for 10 min in a Sorvall refrigerated centrifuge. The supernatant fluid was next centrifuged at $20,000 \times g$ for 30 min, and the resulting sediment was resuspended in TM buffer containing 1% sodium deoxycholate (volume of buffer was equal to 10^{-3} of the volume of the original infected cell culture). This suspension was thoroughly homogenized in a Dounce homogenizer and centrifuged at $3,000 \times g$ for 10 min to remove large particulate material, after which the supernatant was centrifuged in a Beckman ultracentrifuge (SW50 L rotor) at $200,000 \times g$ for 60 min. After centrifugation, the supernatant was discarded and the gelatinous pellet was resuspended in TM buffer. This material shall be referred to as the large-particle (LP) fraction. At this point, chymotrypsin was added to the fraction at a concentration of 200 $\mu\text{g}/\text{ml}$, and the mixture was incubated at 37 C for 30 min with occasional shaking. The resulting turbid solution was centrifuged in a clinical centrifuge at $1,000 \times g$ for 3 min, and the supernatant fluid was either used immediately for polymerase assay or stored at -70 C . The protein content of this preparation was 2.5 to 3 mg/ml, as determined by the method of Lowry et al. (6).

During digestion with chymotrypsin, replicase activity was little affected for the first 60 min, and then it declined gradually. Transcriptase activity, on the other hand, increased during the first 30 min of digestion and then remained unchanged for at least 90 min. This increase varied, ranging from 1.3- to 3.0-fold from one LP fraction to another.

RNA polymerase assay. RNA polymerase activity was determined by measuring the conversion of ^3H -UTP into acid-insoluble form. The standard assay

system contained, in a total volume of 0.25 ml, the LP fraction (200 μg of protein), 5 μCi of ^3H -UTP (0.3 nmole), 0.2 μmole each of the other three ribonucleoside triphosphates, 3 μmoles of MgCl_2 , 20 μmoles of Tris-hydrochloride (pH 8.2), and 2 μg of actinomycin D. The reaction was carried out at 37 C. In the "chased" polymerase assay, 0.2 μmole of unlabeled UTP (0.01 ml) was added at specified times, and the mixture was incubated for an additional 20 min. The reaction was then terminated by chilling the reaction tube in an ice bath and adding 1.75 ml of cold 0.3 M STE buffer supplemented with EDTA to a final concentration of 10 mM. The mixture was divided into two 1-ml portions, and one portion received 10 μg of ribonuclease (0.01 ml) prior to incubation at 37 C for 30 min. Synthesized ^3H -RNA was precipitated by adding carrier yeast RNA, trichloroacetic acid (10%), and sodium pyrophosphate (0.02 M). The precipitate was then collected on a membrane filter, washed with cold 5% trichloroacetic acid containing 0.02 M sodium pyrophosphate, dried, and counted in a liquid scintillation counter, by using toluene-Liquifluor scintillator.

Preparation of single-stranded plus-RNA. Single-stranded plus-RNA of three size-classes was obtained by the method described by Banerjee and Shatkin (1). The subviral particles derived from 10 mg of a purified virion preparation by digestion with chymotrypsin were incubated in a 25-ml reaction mixture for 5 hr at 37 C. After the synthesis, the subviral particles were removed by centrifugation at $165,000 \times g$ for 90 min. The single-stranded plus-RNA synthesized was obtained by extraction from the supernatant with phenol and filtration through Sephadex G-25. Approximately 300 μg of plus-RNA was obtained [40 μg of RNA per 1 optical density (OD) unit].

Denaturation and annealing of dsRNA. The procedure for denaturing dsRNA in dimethylsulfoxide (DMSO) and subsequent annealing was essentially the same as that described previously (13, 15). Briefly, the labeled dsRNA dissolved in 0.01 M STE buffer was mixed with 9 volumes of DMSO. [In the competition experiments, single-stranded plus-RNA was added at this time.] The mixture was heated at 70 C for 20 min, and the denatured RNA was precipitated by the addition of carrier yeast RNA (200 μg), a drop of saturated NaCl solution, and 5 volumes of alcohol. The precipitate was collected by centrifugation and washed with 95% alcohol and then acetone. After drying, the precipitate was dissolved in 0.3 ml of cold 0.3 M STE buffer and heated at 70 C for annealing. The extent of annealing was determined by digestion with ribonuclease as described previously (13).

Thermal denaturation of the labeled dsRNA in 0.01 M STE buffer was carried out by heating the samples at various temperatures for 10 min and then quickly chilling in an ice bath. The extent of denaturation was determined by the loss of acid-insoluble label after digestion with 10 μg of ribonuclease in 1 ml of 0.3 M STE buffer at 37 C for 20 min.

RESULTS

Kinetics of replicase reaction. Replicase was assayed by determining the incorporation of

^3H -UTP into a ribonuclease-resistant product. When the determination was made without "chase" (see above), the incorporation of label was greater into ribonuclease-resistant product than into ssRNA for the first 3 to 10 min of the reaction. After about 10 min, it declined to a level below that for ssRNA, whereas ssRNA synthesis maintained an unabated rate of incorporation (Fig. 1). We found that a portion of the label in the ribonuclease-resistant product was unstable and could be "chased out" upon addition of unlabeled UTP. In the experiment shown in Fig. 1a, unlabeled UTP (a 660-fold excess) was added to the reaction mixture 10 min after the polymerase reaction began, and incubation continued for the periods of time shown in the diagram. (This particular concentration of unlabeled UTP blocked >90% of the incorporation of label, but nevertheless stimulated the rate of the transcriptase reaction.) As shown, the amount of label in the ribonuclease-resistant fraction decreased considerably 5 to 10 min after the addition of unlabeled UTP, whereas the label in the ssRNA fraction increased. The presence of an "unstable" portion in the final ribonuclease-resistant product, therefore, was effectively avoided by "chasing," and, as will be shown later, the remaining product demonstrated the characteristics only of dsRNA.

In our investigation of the kinetics of the

replicase reaction, we accordingly added an excess of unlabeled UTP as chaser prior to measuring the incorporation of label into ribonuclease-resistant product and incubated the mixture for 20-min periods before each point of determination during the course of the polymerase reaction (Fig. 1b). We believe the bottom line in Fig. 1b validly represents the actual kinetics of replicase reaction. The kinetics of replicase reaction differ from transcriptase in the rapid slow-down of the replicase reaction. These data are compatible with the view that the replication and transcription of reovirus genome are two distinct processes. (This matter will be dealt with more directly in the following section.)

The nature of the "unstable" portion of ribonuclease-resistant product which became ribonuclease-sensitive after chase is unknown. We have observed that a similar amount of unchased ribonuclease-resistant product became ribonuclease-sensitive when extracted with phenol or heated at 80 C for 5 min in 0.3 M STE buffer. This suggests that a portion of the nascent ssRNA was protected from ribonuclease digestion by its association with protein, by the existence of a short region of base-pairing with the template RNA, or by both.

Partial dissociation of replicase from transcriptase. To verify that replicase and transcriptase are two distinct entities, the LP fraction was

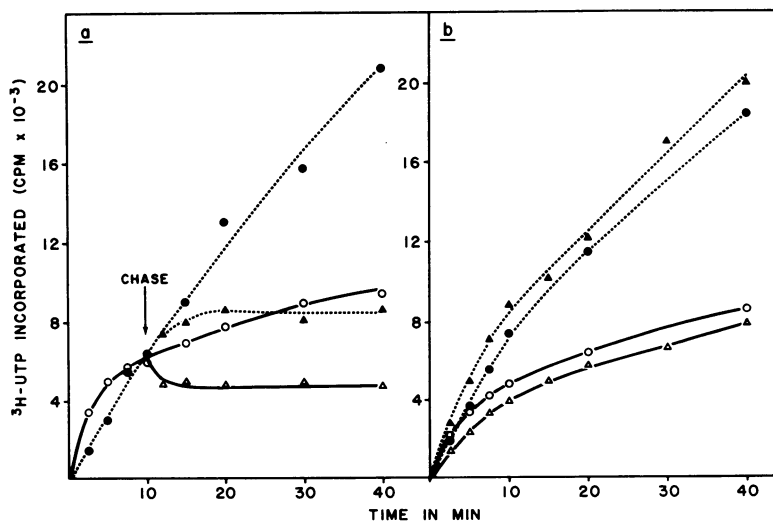


FIG. 1. Kinetics of replicase and transcriptase reactions. RNA polymerase assay was carried out in our standard assay system. (a) Unlabeled UTP (0.2 μmole per 0.25 ml of reaction mixture) was added at 10 min after the polymerase reactions began, and at the times indicated samples were analyzed for ^3H incorporated into ribonuclease-sensitive and resistant RNA. A control experiment was carried out in the same manner without the addition of unlabeled UTP. (b) Unlabeled UTP (0.2 μmole per tube) was added at each point indicated and incubated for an additional 20 min. ^3H incorporation was determined. Symbols: (○) ^3H in dsRNA without chase; (△) ^3H in dsRNA after chase. (●) ^3H in ssRNA without chase; (▲) ^3H in ssRNA after chase.

centrifuged through a preformed gradient of CsCl (Fig. 2). A sharp, readily visible band, was observed at a buoyant density of 1.43 g/cm³. This band coincided with a virus core marker (not shown) which was derived from purified virions by chymotryptic digestion. Electron microscopy could not distinguish between the particles present in this band and virus core derived from mature virions (8).

A majority of the transcriptase activity was recovered in the subviral particle band ($\rho = 1.43$

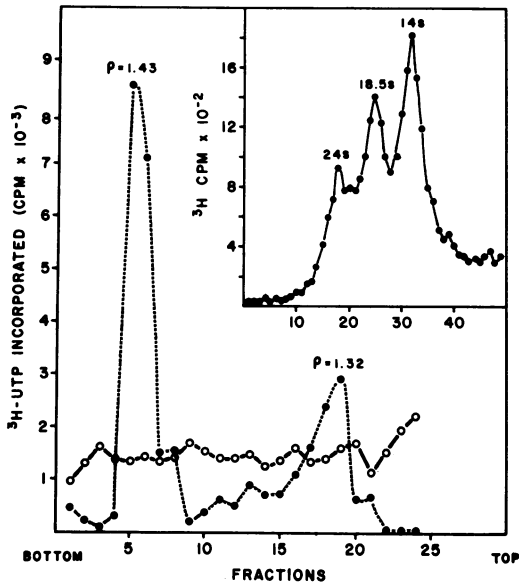


FIG. 2. Distribution of replicase and transcriptase activity in a CsCl gradient. A 1-ml amount of the LP fraction (digested with 200 μ g of chymotrypsin for 90 min at 37 C) was centrifuged in a preformed gradient of CsCl ($\rho = 1.29$ to 1.46 g/cm³) in TM buffer for 2 hr at 165,000 \times g (SW50 L Beckman ultracentrifuge rotor). The gradient was then fractionated into 24 tubes. Each fraction was immediately diluted to 5 ml with cold TM buffer and centrifuged at 165,000 \times g for 1 hr to pellet the polymerases. The pellet was resuspended in 0.45 ml of TM buffer, and 0.15 ml was removed for polymerase assay in the standard assay system. Incubation was for 30 min and chase with unlabeled UTP was for 20 min. Symbols: (O) replicase activity; (●) transcriptase activity. Recoveries of the activities were 80 to 100% for transcriptase and 60 to 80% for replicase in four repeated experiments. The inset is a sedimentation profile of ssRNA synthesized with the subviral particle fraction of density 1.43 g/cm³. This fraction was further dialyzed against TM buffer overnight and incubated in a reaction mixture reported by Borsa (3) for 30 min at 37 C. The product RNA was extracted by the SDS-phenol method and centrifuged in a sucrose gradient (5 to 20% in 0.1 M STES buffer) with a sedimentation marker at 200,000 \times g for 150 min at 15 C in a SW50 L rotor.

g/cm³) and minor activity was detected in a more diffuse band distributed around the density of 1.32 g/cm³. This lighter peak was reduced when the LP fraction was digested with chymotrypsin for a longer period of time before centrifugation, suggesting that the presence of transcriptase in this band was due to incomplete digestion of the LP material. When the heavier subviral particle fraction was incubated in a reaction mixture suitable for transcriptase reaction (3), three size-classes of ssRNA resembling the messenger RNA (mRNA) formed in infected cells were synthesized (Fig. 2, inset). This result is consistent with the conclusion of others that transcriptase is associated with the subviral particles of reovirus (1, 4, 5, 10, 11). In contrast, replicase activity was not associated with the subviral particles but was deposited variously over the gradient. Exogenous virus-specific RNA (dsRNA, denatured dsRNA, or plus-ssRNA) was not required as a template for the replicase present in these fractions. In addition, the dsRNA synthesized by the different fractions of the gradient was similar to that synthesized by the unfractionated replicase preparation, i.e., the synthesis of three major classes of dsRNA, as shown in a previous paper (12). These results indicate that the replicase was bound to a template and that the complex was associated with structures of various density in CsCl. We conclude from these data that replicase and transcriptase exist as two distinct forms of an enzyme-template complex.

Physical properties of the replicase product.

The replicase product was extracted with SDS and phenol from the reaction mixture which had been incubated for 30 min and chased for 30 min. Ethanol-precipitated RNA was then dissolved in 1 ml of 0.3 M STE buffer containing 10 μ g of ribonuclease, incubated at 37 C for 20 min, and finally deproteinized by phenol and filtered through a column of Sephadex G-100 (1.5 by 30 cm; reference 12). The ribonuclease-resistant replicase product eluted as a distinct peak after a void volume of buffer.

Buoyant density. The replicase product obtained in this manner was found to have a buoyant density of 1.60 g/cm³ in Cs₂SO₄ (not shown) and 1.85 g/cm³ in an 8:1 CsCl-Cs₂SO₄ mixture (7), as determined by equilibrium density gradient centrifugation (Fig. 3). The ³H-labeled replicase product banded coincidentally with the ³²P-labeled virion dsRNA.

Thermal denaturation and annealing. The replicase product and virion dsRNA both underwent sharp thermal denaturation (T_m of 92 C) and were rendered completely ribonuclease-sensitive at 95 C (Fig. 4). Denaturation also resulted from heating at 70 C in 90% DMSO

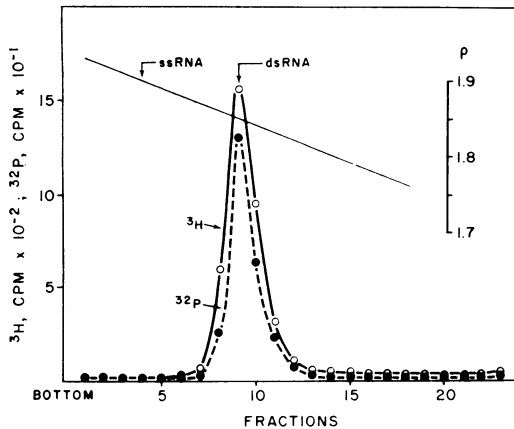


FIG. 3. Equilibrium density gradient centrifugation of the ^3H -labeled replicase product and ^{32}P -labeled virion dsRNA in a $\text{CsCl}-\text{Cs}_2\text{SO}_4$ (8:1) mixture. Centrifugation was carried out at $84,000 \times g$ for 96 hr at 22 C, by using an SW50.1 rotor. The position of ssRNA (arrow) was determined in a separate experiment by using the plus-RNA synthesized by reovirus transcriptase.

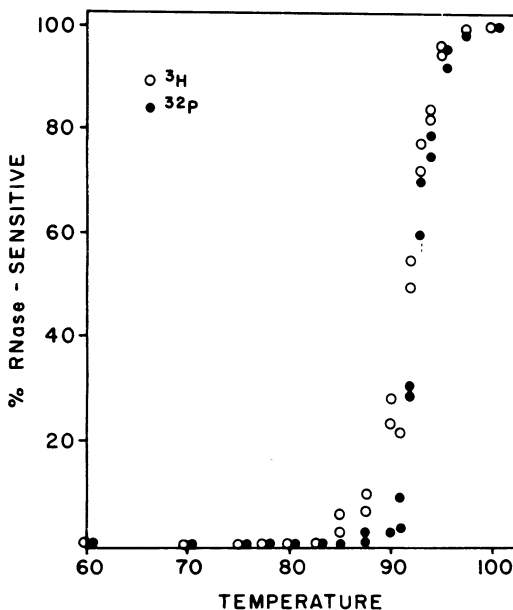


FIG. 4. Thermal denaturation of the ^3H -labeled replicase product and ^{32}P -labeled virion dsRNA. The analysis was conducted in 0.7 ml of 0.01 M STE buffer containing ^3H -dsRNA (3,000 count/min, 0.3 μg) and ^{32}P -dsRNA (1,800 count/min, 0.01 μg).

(Fig. 5). When the ^3H -labeled replicase product and the ^{32}P -labeled virion dsRNA were combined in a 27:1 mass ratio and denatured and annealed, the annealing of both ^3H and ^{32}P radioactivities

proceeded gradually and was near completion after 22 hr. The annealing kinetics of both the replicase product and virion dsRNA were similar (Fig. 5). In contrast, the self-annealing of only the ^{32}P -labeled virion RNA (i.e., in the absence of replicase product) proceeded at a significantly slower rate (Fig. 5), because of the decreased concentration (13). The fact that the mixed annealing of the ^3H - and ^{32}P -labeled RNA followed similar kinetics suggested that the complementary strands of each species behaved identically with respect to annealing and that some strand interchangeability between the two occurred.

Mixed annealing in the presence of an excess of plus-RNA. The denaturation and subsequent annealing of a mixture of the ^3H -labeled replicase product and ^{32}P -labeled virion dsRNA were carried out in the presence of an increasing amount of unlabeled plus-RNA which had been synthesized *in vitro* by the reovirus-core transcriptase. If the ^3H label was uniformly distributed between the plus and minus strands of the replicase product, the labeled plus-RNA would be diluted upon denaturation by the unlabeled plus-RNA that was added. Thus, most of the original labeled plus-RNA might be expected to be lost from the annealed product. On the other hand, the labeled minus-RNA originally present in the replicase product would be retained completely in the annealed product. The theoretical value for the retention of label in the annealed product would therefore be 50%. This was the

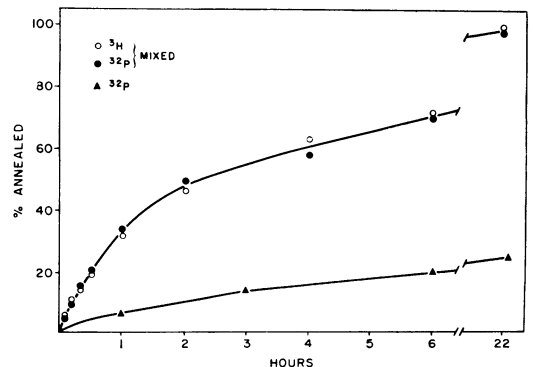


FIG. 5. Annealing kinetics of the denatured ^3H -labeled replicase product and ^{32}P -labeled virion dsRNA. The denaturation of RNA in DMSO and subsequent annealing were carried out as described in the text. The mixed annealing was performed in 0.3 ml of 0.3 M STE buffer containing ^3H -dsRNA (2,700 count/min, 0.27 μg) and ^{32}P -dsRNA (2,240 count/min, 0.01 μg). Annealing of ^{32}P -dsRNA alone was carried out in a similar manner although without the addition of ^3H -dsRNA.

case with the ^{32}P -labeled dsRNA extracted from virions (Fig. 6); about 40% of the ^{32}P was lost from the annealed product. In contrast, the ^3H contained in the replicase product was totally retained after annealing.

Annealing in the presence of an excess of plus-RNA proceeded conspicuously faster (Fig. 7) than that done in the absence of plus-RNA (Fig. 5) because of the dependence of annealing on concentration. Plateau levels of 100% for ^3H and 60% for ^{32}P were achieved. A major conclusion from the preceding experiments is that the dsRNA synthesized *in vitro* by replicase is composed of an unlabeled plus-RNA strand and a nascent ^3H -labeled minus-RNA strand. Presumably, therefore, the *in vitro* replicase reaction proceeds unilaterally by means of the synthesis of the minus-RNA strand upon a preexisting RNA template.

The annealing data also show that the ^{32}P -labeled plus-RNA in the virion dsRNA was not completely replaced by the unlabeled plus-RNA even in the presence of a 400-fold excess of unlabeled plus-RNA (Fig. 6). The reason for the approximately 10% discrepancy between the theoretical value of 50% reduction and the observed value of 40% is unknown. This phe-

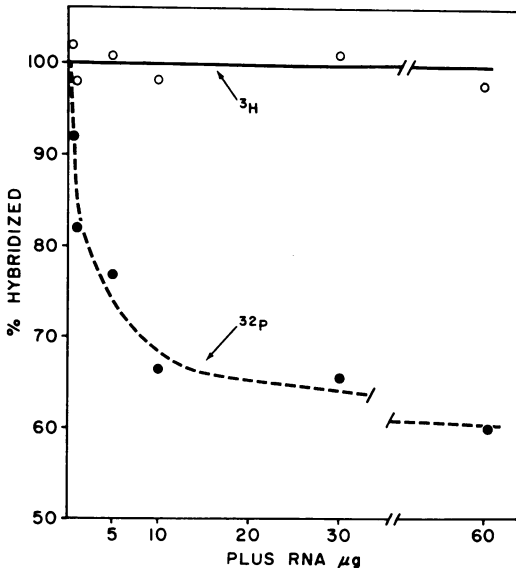


FIG. 6. Mixed annealing of the ^3H -labeled replicase product and ^{32}P -labeled virion dsRNA in the presence of unlabeled plus-RNA. To the mixture of ^3H -dsRNA (3,076 count/min, 0.3 μg) and ^{32}P -dsRNA (1,600 count/min, 0.005 μg) was added the indicated amount of unlabeled plus-RNA, and the mixture was denatured in DMSO. The denatured RNA was annealed in 0.3 ml of 0.3 M STE buffer for 20 hr.

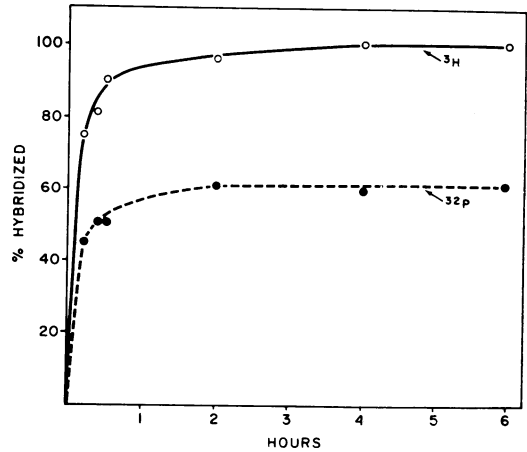


FIG. 7. Kinetics of mixed annealing of the ^3H -labeled replicase product and ^{32}P -labeled virion dsRNA in the presence of an excess of plus-RNA. Each annealing mixture (0.3 ml) contained ^3H -dsRNA and ^{32}P -dsRNA in the amounts given in the legend to Fig. 5 and, in addition, 25 μg of unlabeled plus-RNA.

nomenon, however, may be related to the fact that a small portion of the DMSO-denatured, virion dsRNA anneals at a faster rate than the major portion (13). If so, the discrepancy does not essentially affect the above conclusion.

DISCUSSION

Existence of replicase as a separate entity from transcriptase. Evidence has been provided that the replication and transcription of the reovirus genome are independent processes catalyzed by two distinct enzyme-template complexes which are distinguishable by their buoyant densities in CsCl (Fig. 2). The reason for the heterogeneous distribution of replicase in CsCl has not been studied, however.

It is possible that replicase and transcriptase are two individual proteins. Alternatively, replicase might be converted to transcriptase as the enzyme-template complex is incorporated into the subviral particles. The latter possibility is compatible with experimental evidence that transcriptase is detectable only in the subviral particles of infected cells and the virus core of mature virions and that these particles apparently lack replicase activity. The fact that dsRNA replication in infected cells disappears soon after protein synthesis is inhibited by an addition of cycloheximide (14) is also compatible with this hypothesis, since continued synthesis of replicase would be required.

Examination of the *in vitro* replicase reaction and product. The dsRNA synthesized *in vitro* by replicase contained three major size-classes of

dsRNA segments (12) and appeared to be indistinguishable from virion dsRNA with respect to double-strand characteristics (Fig. 3-5). That the replicase product could be denatured and annealed in the same manner as the virion dsRNA (Fig. 4, 5) provided a rationale for the hybridization-competition experiment. This experiment allowed the determination of whether the *in vitro* dsRNA synthesis involved the simultaneous synthesis of both plus- and minus-RNA strands. The results (Fig. 6, 7) indicated that the dsRNA segments synthesized *in vitro* were composed of a nascent ³H-labeled minus-RNA strand and an unlabeled plus-RNA strand which was preexistent in the enzyme preparation.

The replicase preparation contained transcriptase activity and incubation for the replicase reaction stimulated synthesis of ssRNA in addition to the replicase product. This ssRNA appeared to be indistinguishable from the plus-RNA synthesized by reovirus-core transcriptase in that it also contained three size-classes of ssRNA resembling the messenger RNA formed in reovirus-infected cells (Fig. 2). The ssRNA did not self-anneal or hybridize with plus-RNA (*unpublished data*). These observations suggest that no free minus-RNA was present in the products synthesized by the cell-free replicase and transcriptase preparation. We believe, therefore, that the *in vitro* replicase reaction proceeds by the synthesis of minus-RNA upon a plus-RNA template, presumably of single-strand form, rather than by replacement of the minus-strand of dsRNA. The latter possibility has not been completely ruled out. Our interpretation of the data reported here is in agreement with the model of reovirus dsRNA replication recently proposed by Schonberg et al. (9). The possibility that the replicase product is triple-stranded is unlikely in view of the similarity of the product to virion dsRNA in all physical properties determined.

Assuming the validity of the above interpretation, the fact that the replicase product did not contain any detectable amounts of labeled plus-RNA suggests that the plus-RNA synthesized by the contaminating transcriptase did not subsequently serve as a template for further replicase activity. This failure to reinitiate the synthesis of minus-RNA by using the nascent plus-RNA as template is consistent with the demonstration that the *in vitro* replicase reaction slows down rapidly (Fig. 1).

It is interesting that the nascent plus-RNA did not readily serve as template in the cell-free system. One hypothesis to explain this would be that for the nascent plus-RNA to become func-

tional as a template for replicase action, a concomitant protein synthesis would be required. The protein(s) could be either a replicase or those proteins required for the formation of an active replicase-template complex. We have previously shown that, in reovirus-infected cells, the inhibition of protein synthesis by cycloheximide leads to a rapid cessation of viral dsRNA synthesis, and the plus-RNA (mRNA) synthesized and accumulated in the presence of cycloheximide is not later incorporated into dsRNA after removal of the drug (14).

ACKNOWLEDGMENT

This investigation was supported by Public Health Service research grant AI-02454 from the National Institute of Allergy and Infectious Disease.

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