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# Transcription In Vitro by Reovirus-Associated Ribonucleic Acid-Dependent Polymerase[1]

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Digestion of purified reovirus type 3 with chymotrypsin degrades 70% of the viral protein and converts the virions to subviral particles (SVP). The SVP contain 3 of the 6 viral structural proteins and all 10 double-stranded ribonucleic acid (RNA) genome segments but not adenine-rich, single-stranded RNA. An RNA polymerase which is structurally associated with SVP transcribes one strand of each genome segment by a conservative mechanism in vitro. The single-stranded products include large ($1.2 \times 10^6$ daltons), medium ($0.7 \times 10^6$ daltons), and small ($0.4 \times 10^6$ daltons) molecules which hybridize exclusively with the corresponding genome segments. The enzyme obtained by heating virions at 60 C synthesizes similar products. Kinetic and pulse-chase studies indicate that the different-sized products are synthesized simultaneously but at rates which are in the order: small > medium > large.

Ribonucleic acid (RNA) polymerase activity is structurally associated with purified poxviruses (11, 20), reoviruses (4, 27), and cytoplasmic polyhedrosis virus (14). These enzymes transcribe the viral genomes in vitro and may be involved in the synthesis of virus-specific early messenger RNA (mRNA) species in infected cells.

The genome of reovirus type 3 consists of 10 distinct double-stranded RNA segments (28). Single-stranded mRNA corresponding in size and base sequence to a limited number of these fragments is formed in virus-infected cells early in the infectious cycle (39). At later times after infection when new enzymes have been synthesized (25, 38), the remaining segments are copied (2, 8, 38, 40). It was of interest to compare the pattern of virus mRNA synthesis in infected cells with the product made in vitro by the virion-associated polymerase. In earlier reports this comparison was not possible because the product was degraded, presumably by contaminating ribonuclease (4, 27). We now report studies on the properties of the particulate polymerase purified free of ribonuclease. Similar studies have been done recently by Levin et al. (*personal communication*) and Skehel and Joklik (31).

## MATERIALS AND METHODS

**Chemicals and enzymes.** Tritiated ribonucleoside triphosphates were obtained from Schwarz Bio-Research Inc., Orangeburg, N.Y. Calbiochem, Los Angeles, Calif., was the source of unlabeled ribonucleoside triphosphates. $^{32}$P-orthophosphate was from E. R. Squibb & Sons, Inc., New York, N.Y. α-Chymotrypsin and pancreatic ribonuclease were purchased as crystalline enzymes from Worthington Biochemical Corp., Freehold, N.J. Bentonite from Fisher Scientific Co., Fair Lawn, N.J., was processed before use by the procedure of Fraenkel-Conrat et al. (7). Spectral quality glycerol and dimethyl sulfoxide (DMSO) were from Matheson, Coleman and Bell, Rutherford, N.J., and Fisher Co., respectively.

**Cells and virus.** Mouse L-929 cells were grown in suspension culture in Eagle's medium containing 5% fetal bovine serum and infected with reovirus type 3 Abney strain [5 plaque-forming units (PFU)/cell (24, 25)]. At 36 to 48 hr after infection, cells were concentrated by centrifugation, resuspended in phosphate-buffered saline at a density of about $3 \times 10^7$/ml, and homogenized with genetron. The resulting emulsion was centrifuged, and the aqueous phase containing the virus was carefully overlaid onto 5 ml of CsCl solution [0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 8.0), $\rho = 1.39$ g/cm$^3$] and centrifuged (SW-27, 52,000 × *g*, 1 hr). The virus which concentrated as a band in the CsCl layer ($\rho = 1.36$ g/cm$^3$) was collected, banded twice more by isopycnic sedimentation in CsCl, and dialyzed against 0.01 M Tris buffer (*p*H 8). Purified virus labeled in the RNA component was prepared by incubating infected cells in phosphate-free medium containing 2% fetal bovine serum and 5 μc/ml of $^{32}$P-orthophosphate.

**RNA polymerase assay.** The standard reaction mixture consisted of 60 mM Tris buffer (*p*H 8.0), 6 mM $MgCl_2$, 2 mM phosphoenol pyruvate, 40 μg (14 units) pyruvate-kinase, 1 mM each of adenosine-5′-triphosphate (ATP), guanosine-5′-triphosphate (GTP), cytosine-5′-triphosphate (CTP), and uridine-5′-triphosphate (UTP), one of which was $^3$H-labeled

[1] This work was reported in part at the meeting of the Federated American Societies of Experimental Biology, April 1970.

at a final specific activity of 3,300 counts per min per nmole, and unless otherwise stated, 47 μg each of α-chymotrypsin and purified reovirus in a total reaction volume of 0.25 ml. All solutions were routinely treated with bentonite to reduce contaminating ribonuclease activity. Protein was measured by the method of Lowry et al. (17), and virus quantities were determined from these values on the basis that virions contain 85% protein (9). The reaction mixture was incubated for 90 to 120 min at 37 C in the absence of the labeled nucleoside triphosphate to permit the conversion of virions to active subviral particles (SVP). The labeled triphosphate was then added, and the incubation was continued for another 30 min. The reaction was stopped by adding 5% trichloroacetic acid at 0 to 4 C, and the precipitates were collected on nitrocellulose filters, washed with 5% trichloroacetic acid containing 0.02 M sodium pyrophosphate, dried, and counted with an efficiency of about 30% in Liquifluor-toluene in a Packard Tri-Carb scintillation counter.

**Analysis of RNA.** The methods used for phenol extraction of RNA (25) and determination of base composition by paper electrophoresis (23) and estimation of molecular weight by polyacrylamide gel electrophoresis have been described (15, 28). For glycerol gradient centrifugation, incubation mixtures were made 1% in sodium dodecylsulfate (SDS), layered onto 12-ml linear density gradients [5 to 30% glycerol, 0.02 M Tris buffer (pH 8.0), 0.1 M NaCl, and 0.005 M ethylenediaminetetraacetic acid (EDTA)], and centrifuged at 4 C in the Spinco SW-41 rotor at 193,000 × *g* for 7 hr or the equivalent conditions 75,000 × *g* for 18 hr. Fractions of 30 drops (about 0.4 ml) were collected from the bottom and assayed for acid-precipitable radioactivity.

**Annealing of in vitro product.** Single-stranded, $^{3}$H-labeled RNA from incubation mixtures or glycerol gradients was precipitated at −20 C by adding three volumes of ethanol and 200 to 500 μg of yeast transfer RNA (tRNA) in the presence of 0.15 M NaCl. The RNA was collected by centrifugation and dissolved in 0.2 ml of 0.01 M Tris buffer (*p*H 8.0) containing 0.015 M EDTA and $^{32}$P-labeled double-stranded RNA. The volume was brought to 2.0 ml with DMSO (90% final concentration), and the solution was incubated at 37 C for 30 min to denature the RNA. The sample was then chilled, and the RNA was again precipitated at −20 C by adding ethanol and NaCl. For annealing, the RNA was washed free of DMSO with 5 ml of ethanol and dissolved in 0.5 ml of 0.01 M Tris buffer (*p*H 8.0) containing 0.001 M EDTA and 0.3 M NaCl. The RNA was annealed at 72 C for 18 hr (37) and then tested for resistance to digestion by ribonuclease or analyzed on a polyacrylamide gel. For estimating ribonuclease resistance, ribonuclease was added to a final concentration of 5 μg/ml, and the solution was incubated at 37 C for 30 min before precipitation with trichloroacetic acid and scintillation counting. For electrophoresis, the solution was precipitated with ethanol, and the RNA was dissolved in 0.01 M Tris buffer (*p*H 8), containing 0.001 M EDTA and 10% glycerol. A volume of approximately 0.1 ml was added to 10-cm 5% polyacrylamide gels, and 4 ma/gel was applied for 20 hr at 20 C with 0.04 M Tris buffer (*p*H 7.8), containing 0.02 M sodium acetate, 0.002 M EDTA, and 0.1% SDS. The gels were frozen, sliced, dissolved in $H_2O_2$, and counted (28).

**Self-annealing of product.** A reaction mixture containing 112 μg each of chymotrypsin and purified virus and 2 mM ribonucleoside triphosphates was preincubated as described above. RNA synthesis was then permitted to proceed for 3 hr with $^{3}$H-labeled GTP present at a specific activity of 1,500 counts per min per nmole. During this time, 40 nmoles of guanosine-5′-monophosphate (GMP) was incorporated into RNA. The reaction was stopped by chilling, and the SVP were removed by centrifugation (2,000 rev/min for 3 min). Newly formed RNA was precipitated at −20 C by adjusting the supernatant fraction to 2% potassium acetate and 75% ethanol. The precipitated RNA was dissolved in 0.3 ml of 0.01 M Tris (*p*H 8)–0.001 M EDTA, denatured with DMSO, and annealed as described above. Samples were tested for ribonuclease resistance and analyzed by electrophoresis on a 5% polyacrylamide gel after adding $^{32}$P-labeled, double-stranded RNA as a marker.

## RESULTS

**Characteristics of polymerase-containing subviral particles.** Purified virions of reovirus type 3 are about 65 nm in diameter and consist of two structural protein layers (19, 36). During the early stages of an infectious cycle in mouse L cells, lysosomal enzymes strip the outer protein layer from the parental virions, converting them to subviral particles 45 nm in diameter (29). This intracellular conversion can be simulated in vitro since the inner layer of reovirus structural proteins is resistant to proteolytic digestion. When purified virions are digested with equivalent amounts of chymotrypsin (37 C, 2 hr), the outer structural layer comprising 70% of the viral protein is solubilized. The resulting chymotrypsin-resistant SVP contain a highly active RNA polymerase (27). SVP have an increased buoyant density in CsCl ($\rho$ = 1.44 g/cm$^3$) and can be separated from virions ($\rho$ = 1.36 g/cm$^3$) by isopycnic sedimentation in CsCl. It was also noted that SVP aggregate during exhaustive proteolytic digestion. Consequently, they are readily concentrated from digests by low-speed centrifugation (2,000 rev/min for 3 min). The specific polymerase activities are similar for whole proteolytic digests and sedimented SVP; readdition of the inactive, nonsedimentable fraction to sedimented SVP does not influence their incorporation (Table 1). Polymerase activity can also be demonstrated in virions after heat shock (4). Maximal activity was obtained by heating at 60 C for 1 to 2 min in 0.05 M Tris buffer (*p*H 8.0); after 10 and 20 min at 60 C, the activity decreased to 50% and 20% of the maximal level. SVP prepared by chymotrypsin treatment

TABLE 1. *Reovirus-associated RNA polymerase activity under different conditions*[a]

| Sample assayed | $^3$H-GMP-incorporated[b] |
|---|---|
| Virus | 0.10 |
| Virus + chymotrypsin (47 μg) | 2.42 |
| SVP sedimented from chymotrypsin digest | 2.18 |
| Nonsedimentable fraction of digest | 0.10 |
| SVP + nonsedimentable fraction | 2.21 |
| Virus, 60 C for 1 min | 2.46 |
| SVP, 60 C for 1 min | 2.18 |
| SVP + rifampicin (100 μg/ml) | 2.32 |
| SVP, 60 C for 1 min, + rifampicin (100 μg/ml) | 2.28 |
| SVP + α-amanitin (40 μg/ml) | 2.20 |

[a] In each assay, 47 μg of virus or the resulting subviral particles (SVP) and nonsedimentable fraction were incubated as described in the text. To assay the activity of isolated SVP, the particles were sedimented from the chymotrypsin digest (2,000 rev/min for 3 min) and resuspended in 0.25 ml of fresh reaction mixture containing the labeled triphosphate but no chymotrypsin.

[b] GMP, guanosine-5′-monophosphate; values are expressed as nanomoles incorporated per 30 min.

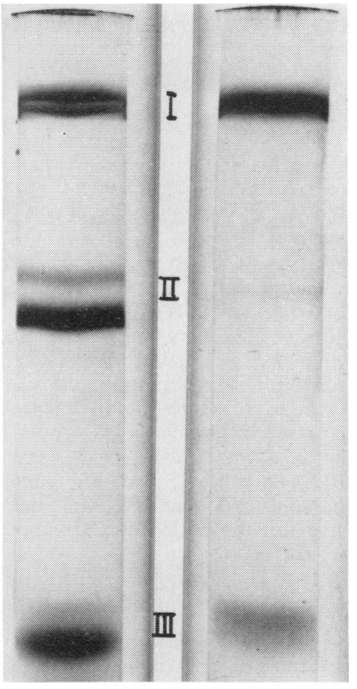


FIG. 1. *Electrophoretic patterns of structural proteins of virions and SVP. Purified reovirus type 3 (left) or SVP (right) prepared by digestion of virus with an equal amount of chymotrypsin (2 hr at 37 C) were solubilized in 0.0 1 M Tris buffer (pH 8) containing 8 M urea, 2% SDS, and 1% β-mercaptoethanol. The samples were dialyzed against 0.001 M phosphate buffer (pH 7.2) containing 8 M urea, 0.1% SDS, and 0.1% mercaptoethanol, and 20 μg of protein were applied to 10-cm 10% polyacrylamide gels. After electrophoresis in 0.1 M phosphate buffer (pH 7.2) containing 0.1% SDS for 22 hr at 4 ma/gel and 20 C, the gels were fixed and stained with 0.025% Coomassie blue in 10% trichloroacetic acid and destained in 7% acetic acid (16).*

are also active after heat treatment (Table 1). Many different purified virus preparations were tested, and their specific enzyme activities varied by less than 10%. SVP prepared by chymotrypsin digestion have been studied most extensively since they contain a relatively stable polymerase of high specific activity but fewer structural proteins than whole virus.

The structural proteins of reoviruses analyzed by polyacrylamide gel electrophoresis include two slightly resolved major polypeptides of molecular weight about 150,000 (I), minor and major components with molecular weights of approximately 80,000 (II), and a third pair of polypeptides with molecular weights of about 40,000 (III) (16, 32; Fig. 1). The polypeptides of molecular weight 80,000 and the predominant component of the pair at 40,000 apparently are part of the outer structural layer of the virus since they are largely solubilized when virions are converted to SVP by brief exposure to urea (16) or chymotrypsin digestion (32; Fig. 1). Mixtures of solubilized virions and SVP containing $^{14}$C- versus $^3$H-labeled amino acids were also analyzed, and the results confirmed that the SVP contain predominantly the two high-molecular-weight polypeptides and one having a molecular weight of about 40,000 (K. Eggen and A. J. Shatkin, *unpublished data.*)

The double-stranded RNA extracted from purified reoviruses consists of three large (L), three medium (M,) and four small (S) segments of approximate molecular weights $2.5 \times 10^6$, $1.4 \times 10^6$, and $0.6 \times 10^6$ to $0.8 \times 10^6$ daltons, respectively (28, 31, 39). Purified reovirus type 3 containing $^{32}$P-labeled RNA was digested with chymotrypsin. The resulting SVP were deproteinized with phenol, and the RNA was analyzed in a 5% polyacrylamide gel. The distribution of radioactivity was identical to that obtained with RNA from virions (*see* Fig. 5), indicating that the SVP contain all 10 double-stranded RNA segments. In contrast to the double-stranded RNA, the single-stranded, adenine-rich RNA, which has been described in preparations of purified reoviruses (1, 3, 26), is not a component of SVP prepared by chymotrypsin treatment. As shown by base composition analysis,

TABLE 2. *Base composition analysis*[a]

| Protein | Cytidylic acid | Adenylic acid | Guanylic acid | Uridylic acid | Ratio of purines to pyrimidines |
|---|---|---|---|---|---|
| Virus RNA.... | 17.2 | 44.8 | 16.8 | 21.2 | 1.6 |
| SVP RNA..... | 23.6 | 26.8 | 23.4 | 26.2 | 1.0 |
| RNA released from SVP.... | 3.4 | 86.7 | 2.7 | 7.2 | 9.0 |

[a] $^{32}$P-labeled purified reovirus type 3 and an equal amount of chymotrypsin were incubated for 2 hr at 37 C in 0.01 M Tris buffer (*p*H 8). The mixture was then chilled and centrifuged for 3 min at 2,000 rev/min to separate the sedimentable SVP from the released RNA. These two fractions and a second sample of radioactive virus were precipitated with 5% perchloric acid and analyzed by paper electrophoresis after alkaline digestion as described previously (23). Values are expressed as moles per cent.

the adenine-rich RNA is in the nonsedimentable portion of the proteolytic digest (Table 2). Krug and Gomatos (12) have reported recently that infectious reovirus can be separated from the adenine-rich RNA.

It was reported previously that the infectivity of some strains of reoviruses can be enhanced by controlled digestion with chymotrypsin (34). However, under the present conditions of proteolytic treatment, the infectivity of reovirus type 3 was markedly reduced. Purified virus diluted in Tris buffer (0.01 M, *p*H 8) was incubated at 37 C for 2 hr. The titer measured by plaque assay on L cells (10) remained at $4 \times 10^8$ PFU/ml in the absence of chymotrypsin but decreased to $7 \times 10^4$ PFU/ml in the presence of 10 $\mu$g of enzyme/ml. A similar inactivation to $7 \times 10^4$ PFU/ml occurred when more concentrated virus ($2 \times 10^{10}$ PFU/ml) equivalent to 400 $\mu$g of protein/ml was treated with 10 $\mu$g of chymotrypsin/ml. The inactivation was apparently not due to aggregation since the titer of SVP was not increased by brief sonic treatment.

**Properties of polymerase.** In the presence of all four ribonucleoside triphosphates, an ATP-generating system, and $Mg^{2+}$ at *p*H 8 to 9, the SVP-associated polymerase is active for many hours. (The ATP-generating system is not required for incubations of 30 min or less.) Under these conditions, there is a net synthesis of reovirus-specific, single-stranded RNA. The in vitro activity of deoxyribonucleic acid (DNA)-dependent RNA polymerase is markedly influenced by the addition of salt or by increasing the concentration of triphosphates relative to $Mg^{2+}$; salt stimulates by promoting the release of completed chains (18, 33) and increased amounts of triphosphates inhibit by chelating $Mg^{2+}$ and reducing its effective concentration (5). In the case of the reovirus-associated polymerase, addition of NaCl, KCl, or $NH_4Cl$ to the SVP incubation mixture inhibited incorporation; 0.04 M salt reduced incorporation by 2 to 10% and 0.2 M by 29 to 44%. The concentration ratio of triphosphates to $Mg^{2+}$ is also important for the SVP-associated enzyme activity. In the presence of 2 mM triphosphates, the optimum concentration of $Mg^{2+}$ was 12 mM (Fig. 2). When the level of triphosphates was reduced twofold, the optimum $Mg^{2+}$ concentration also decreased by 50%. However the RNA product obtained was not changed by the addition of salt or alteration of the ratio of triphosphates to $Mg^{2+}$; patterns similar to those in Fig. 3 were observed in each instance.

The SVP-associated polymerase is not inhibited by actinomycin (4, 27), an antibiotic which blocks DNA-dependent RNA transcription by binding to the template (21). Rifampicin, a specific inhibitor of bacterial DNA-dependent RNA polymerase which binds to the enzyme (30), also has no effect on the reovirus polymerase. A concentration of 100 $\mu$g of rifampicin/ml added to SVP or heated SVP (60 C for 1 min) did not alter the extent of incorporation (Table 1). The pattern of RNA synthesized in the presence of rifampicin was identical to that made in the absence of the drug (Fig. 3). $\alpha$-Amanitin, a potent inhibitor of mammalian DNA-dependent RNA polymerase (35), also was without effect

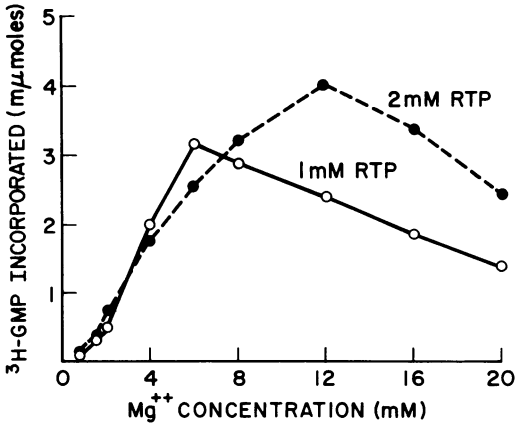


FIG. 2. *Effect of $Mg^{2+}$ and ribonucleoside triphosphates (RTP) on in vitro RNA synthesis. Purified virus (47 $\mu$g) and an equal amount of chymotrypsin were incubated for 1 hr at 37 C in 0.25 ml of complete reaction mixture containing the indicated concentrations of $Mg^{2+}$ and RTP. Acid-precipitable radioactivity was then determined as described in the text.*

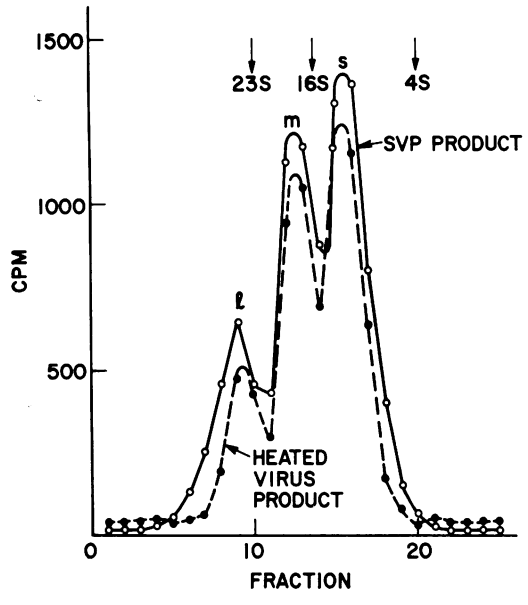


FIG. 3. *Sedimentation of RNA products in glycerol gradients. Virus was digested with chymotrypsin or heated (60 C for 1 min) and incubated under standard assay conditions. The acid-precipitable $^3$H-labeled products were analyzed in 5 to 30% glycerol gradients as described in the text. Arrows indicate the positions of E. coli ribosomal and soluble RNA centrifuged under the same conditions.*

on the particle-associated polymerase at a concentration as high as 40 μg/ml (Table 1).

SVP containing $^{32}$P-labeled RNA were used to examine whether the particle-associated polymerase transcribes double-stranded RNA by a semiconservative or conservative mechanism. $^{32}$P-labeled virions were digested with an equivalent amount of chymotrypsin for 2 hr at 37 C, and the resulting SVP were sedimented and washed with 0.05 M Tris buffer (*p*H .8). A portion ($10^5$ counts/min, approximately 20 μg) was then incubated for 60 min in a reaction mixture containing $^3$H-GTP. During this time, 4.1 nmoles of GMP were incorporated into RNA representing a twofold (or on a one-strand basis, fourfold) net synthesis and release of single-stranded product. At the end of the incorporation period, the incubation mixture was centrifuged (2,000 rev/min for 3 min) and more than 99% of the $^{32}$P-labeled template RNA was sedimented as SVP. No released $^{32}$P was detected in the nonsedimentable fraction. The pelleted SVP were then extracted with phenol and the ribonuclease sensitivity of the resulting RNA was compared to RNA extracted from purified virus. Both RNA molecules were double-stranded, as shown by their acid precipitability after ribonuclease digestion (5 μg/ml, 37 C, 30 min, 0.3 M NaCl). These findings indicate that synthesis is conservative as reported by Skehel and Joklik (31).

**Analysis of product: size.** In reovirus-infected tissue culture cells, the segments of double-stranded genome RNA are transcribed to form single-stranded viral mRNA molecules of comparable length (2, 8, 40). The in vitro product of the SVP-associated polymerase is also single-stranded and copied from the genome template (27). However, the product was previously found to be smaller than the shortest double-stranded RNA segments, apparently due to degradation by contaminating ribonuclease rather than premature termination of newly synthesized chains. When traces of nuclease are removed from the polymerase incubation mixture by bentonite treatment or preincubation with chymotrypsin, the products are also similar in length to the L, M, and S genome segments. All components of the incorporation mixture except $^3$H-GTP were incubated for 2 hr at 37 C with 47 μg of chymotrypsin, a concentration equal to the amount of virus present. The missing triphosphate which had been bentonite-treated was then added, and the mixture was incubated an additional 30 min. The mixture was then chilled, brought to 1% SDS, and analyzed by sedimentation in a glycerol gradient. The single-stranded product consisted of three peaks (l, m, and s) which sedimented at 25, 18, and 12*S* as compared to 23 and 16*S* ribosomal RNA and 4*S* soluble RNA from *E. coli* (kindly provided by S. Pestka; Fig. 3). The product obtained after a 30-min incubation with heated virions or heated SVP (*not shown*) separated into the same three peaks.

The sedimentation values are very similar to those reported previously for the denatured segments of viral RNA and to viral mRNA from infected cells (1, 8, 39). They correspond to molecular weights for single-stranded RNA of about $1.2 \times 10^6$, $0.7 \times 10^6$, and $0.4 \times 10^6$ daltons. The quantity of radioactivity in each peak after an incubation time of 30 min (or 3 hr) was not equal but in the ratio for l/m/s of about 1.0/1.7/1.8. Since the chain lengths of the l, m, and s single-stranded RNA species are about 3,600, 2,000, and 1,000 nucleotides, respectively, it can be calculated that, for each molecule of l synthesized, there were approximately three molecules of m and six molecules of s formed.

**Hybridization with genome RNA.** The composition of the single-stranded $^3$H-labeled in vitro product was examined further by annealing with $^{32}$P-labeled viral RNA. The proportion of product which was ribonuclease-resistant after annealing increased from 4% in the absence of viral RNA to a maximum of 89% (Fig. 4). A concen-

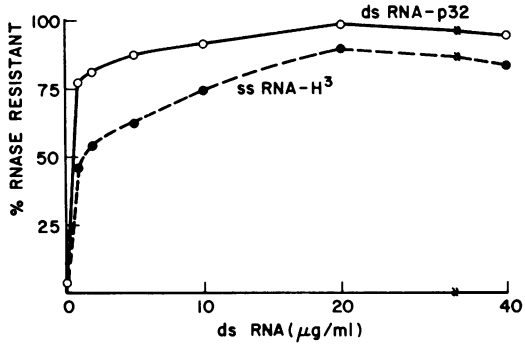


FIG. 4. *Annealing of reovirus RNA and polymerase product. $^{32}$P-labeled, double-stranded (ds) RNA (900 counts per min per μg) was extracted from purified virus and denatured and annealed with $^{3}$H-labeled, single-stranded RNA product (10,500 counts/min). The samples were then assayed for ribonuclease resistance as described in the text.*

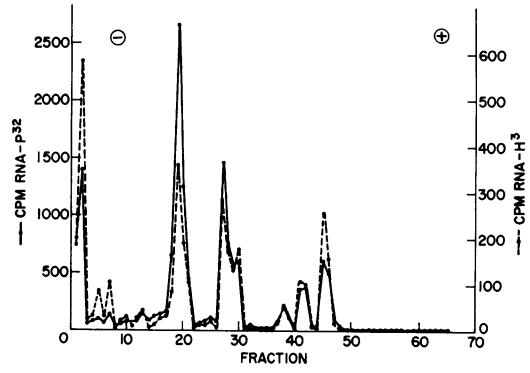


FIG. 5. *Electrophoretic pattern of in vitro product hybridized with viral RNA. $^{3}$H-labeled, single-stranded (ss) RNA was synthesized with 47 μg of heated virus (60 C for 2 min). After 60 min of incorporation under standard conditions (no chymotrypsin), the product was isolated by alcohol precipitation. $^{32}$P-labeled, double-stranded (ds) RNA extracted with phenol from purified virus was hybridized with the $^{3}$H-labeled product and analyzed in a 5% polyacrylamide gel.*

tration of 20 μg/ml of double-stranded genome RNA was required for maximal hybridization of the single-stranded product and this concentration was used for subsequent annealing experiments. To test whether all or a limited number of genome segments are transcribed in vitro, mixtures of single-stranded product and double-stranded viral RNA were analyzed by polyacrylamide gel electrophoresis. Before annealing, the $^{3}$H-labeled, single-stranded RNA remained near the origin of the 5% gel, whereas the $^{32}$P-labeled, double-stranded RNA segments migrated according to their respective molecular weights. After hybridization, the pattern of $^{3}$H-labeled product and viral $^{32}$P-RNA were coincident, indicating that all double-stranded segments are transcribed in vitro. The hybridized product of heated virus (60 C for 2 min) is shown in Fig. 5. An identical pattern was obtained with the 60-min product from chymotrypsin-treated virus. A small amount of material apparently aggregates or remains single-stranded during the annealing process and consequently remains near the gel origin after electrophoresis.

The sharp separation of the l, m, and s peaks in glycerol density gradients suggests that there is little or no degradation of the in vitro products (Fig. 3). To test whether the s segments are free of degraded l and m RNA, the three classes of RNA products were separated on glycerol gradients and hybridized to genome RNA. From a gradient identical to that in Fig. 3, the three peak fractions of l RNA were combined, precipitated with ethanol, mixed with $^{32}$P-labeled viral RNA, denatured with DMSO, annealed, and analyzed on a 5% gel. RNA from the m and s classes were similarly tested. As shown in Fig. 6, each class of

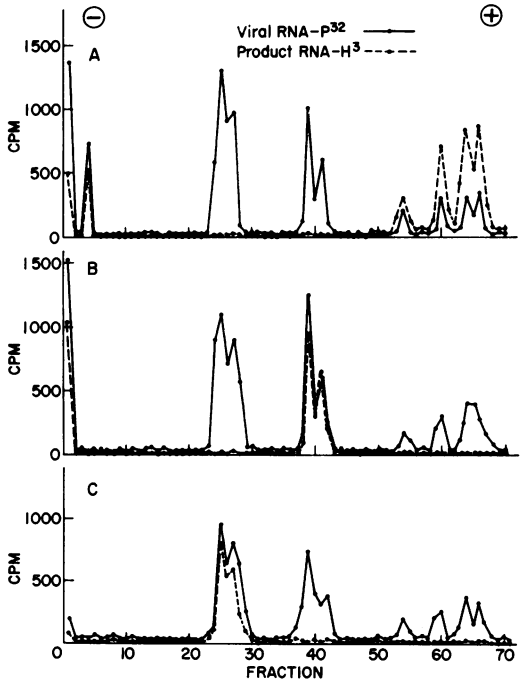


FIG. 6. *Hybridization of separated classes of single-stranded product with viral RNA. Reaction products from three assay tubes were combined after incorporation for 60 min under standard conditions. After sedimentation in a 5 to 30% glycerol gradient, peak fractions of s (A), m (B), and l (C) RNA were precipitated with ethanol, annealed with $^{32}$P-labeled RNA from purified virus and analyzed in 5% polyacrylamide gels.*

single-stranded $^3$H-RNA hybridized only with genome segments in the homologous size class. As found previously with single-stranded RNA from infected cells, there was no cross-hybridization (2, 40). This high specificity indicates that the three classes of RNA in the in vitro product have unique base sequences and that the smaller segments are not contaminated by degraded fragments of the larger ones.

**Self-annealing of product.** The annealing procedure was also used to determine whether one or both strands of the template RNA are transcribed in vitro. $^3$H-labeled product synthesized during 3 hr of incubation was concentrated to 9 $\mu$g/ml (optical density at 260 nm = 0.22) and annealed. At this concentration, duplex formation would be expected to increase the ribonuclease resistance of in vitro product to 90% if both strands were equally represented (Fig. 4). However, only 6% of the self-annealed RNA remained acid-precipitable after ribonuclease digestion. An identical value was obtained for the RNA treated with ribonuclease before annealing. Furthermore, no double-stranded RNA could be detected by gel electrophoresis after self-annealing. As reported previously for viral mRNA synthesized in infected cells (2, 25, 37), the in vitro product apparently is copied from only one strand (or alternate regions of both strands) of the double-stranded RNA template.

**Kinetics of synthesis.** In an attempt to measure the rates of synthesis of the three classes of RNA, the in vitro products were analyzed after increasing intervals of incorporation. Assay mixtures were incubated under standard conditions. At the indicated times, incorporation was stopped by addition of SDS to a final concentration of 1%, and the mixtures were applied directly to glycerol gradients. Only completed chains which had been released from SVP were detected since treatment with 1% SDS does not disrupt the particles. Template-bound nascent strands, whether complete or incomplete, sedimented with the SVP to the bottom of the gradient. Under these conditions, there is a sequential release of completed chains; the s chains of about 1,000 nucleotides in length were present at 1 min, m chains of 2,000 nucleotides at 5 min, and l chains of 3,600 nucleotides at 10 min (Fig. 7A). These results suggest that, during the early periods of in vitro transcription, the rates of synthesis of s, m, and l molecules are different and in the order s > m > l.

Accurate determinations of the rates of chain growth are difficult to make since both the specific activity of the RNA precursor and the quantity of virus incubated markedly influence the values obtained. When the specific activity of GTP was increased fivefold and 87 $\mu$g of virus was incubated, completed s segments were detected within 30 sec, the shortest period studied. Small amounts of m appeared at 1 min, and l RNA was observed at 2 min. When the virus concentration was increased further to 112 $\mu$g, completed chains of all three classes of RNA were found after 1 min (Fig. 7B). Again the quantity of completed segments were in the order s > m > l. On the basis of these results, a minimum estimate for the maximum rate of l chain synthesis is 60 nucleotides/sec (3,600 nucleotides/60 sec). As shown below, the

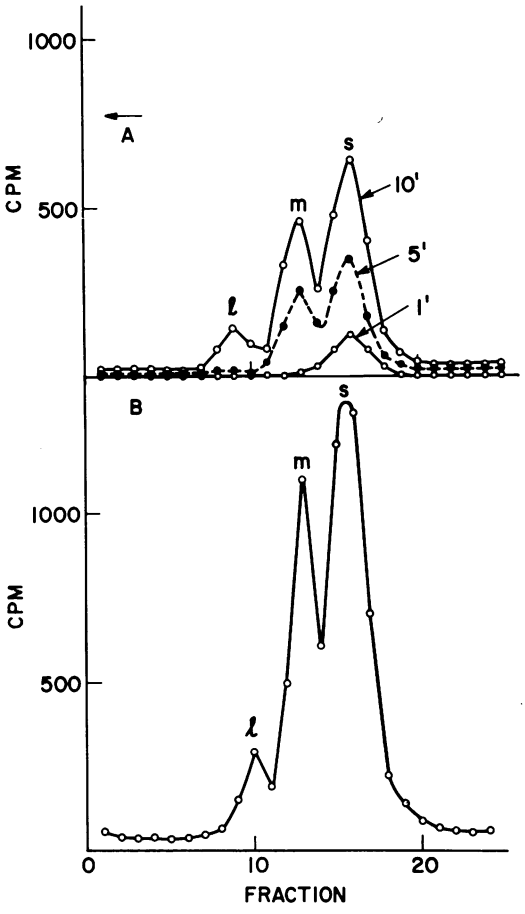


FIG. 7. *Sedimentation patterns of released RNA products. (A) Polymerase reactions were carried out under standard conditions. At the indicated times, incorporation was terminated by adding SDS (1% final concentration). The mixtures were centrifuged in 5 to 30% glycerol gradients and fractions were assayed for acid-precipitable radioactivity as described in the text. (B) Virus and $^3$H-GTP were increased to 112 $\mu$g and a specific activity of 15,000 counts per min per nmole, respectively. The polymerase reaction was terminated after 1 min, and the released products were analyzed as in A above.*

rates of synthesis of m and s are greater, particularly during these early stages of transcription.

The completion and release of RNA segments is a sequential process and a comparison of the chain lengths and time of completion suggests that the rates of synthesis are s > m > l. A more direct test of the relative rates of formation is possible by making use of the observation that incomplete nascent RNA strands remain template bound. As shown in Fig. 8, only a small fraction of the newly formed RNA is released from SVP after 1 min of incubation. The proportion of product which is released increases as more chains are completed and by 30 min 97% of the product is released. (In contrast to SVP, the RNA product from heated SVP or virus is all released after only 5 min of incubation.) To examine the relative rates of synthesis of s, m, and l molecules, incorporation under standard conditions was allowed to proceed for 1 min. (The specific activity of the $^3$H-GTP was increased to 15,000 counts per min per nmole to detect the synthesis of small amounts of RNA.) The SVP were then separated from the released RNA. Under these conditions, the released product consists exclusively of s chains as shown by sedimentation analysis (Fig. 9). In addition, the released RNA hybridized only with S segments of genome RNA. A sample of SVP was extracted with phenol, and the template-bound chains were analyzed in a glycerol gradient. The nascent incomplete strands sedimented in a broad peak. The maximum at about 9S indicates that, on the average, the nascent strands are shorter than S molecules. A second aliquot of SVP was washed with 0.05 M Tris buffer and reincubated for 30 min in the presence of $^{32}$P-CTP (specific activity = 3,000 counts per min per nmole) but no $^3$H-labeled precursor. During this 30-min "chase" period, the $^3$H-labeled nascent chains which were formed in the 1-min "pulse" incubation were completed and released. If the rates of synthesis of the three RNA classes were similar, the amounts of $^3$H-labeled l, m, and s would have been in the ratio 3/3/4 in the released product, i.e., identical to the ratio of double-stranded template segments. However, the total quantity of $^3$H in the completed l, m, and s chains was not in the ratio 3/3/4, but 1/4/8, indicating that the rates of synthesis are in the order s > m > 1. The results also show that, although only s molecules are completed during the first minute of incorporation, m and l chains are being formed simultaneously. In other similar experiments, simultaneous synthesis of all three classes of RNA was also observed during a 30-sec

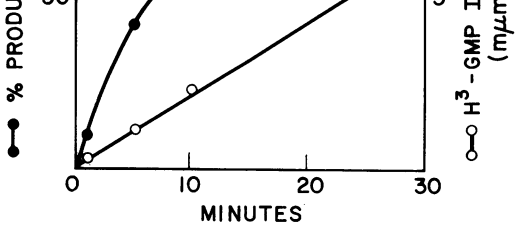


FIG. 8. *Kinetics of release versus synthesis of RNA. Duplicate reaction mixtures were incubated under standard conditions, except that 112 μg of virus were present. At the indicated times, one was assayed for total acid-precipitable radioactivity. The second was centrifuged to sediment the SVP, and the released products in the nonsedimentable fraction were precipitated with 5% trichloracetic acid and counted.*

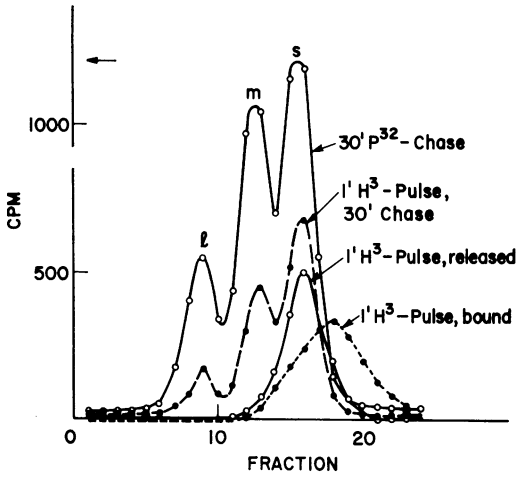


FIG. 9. *Sedimentation patterns of "pulse-chase" RNA. Standard reaction mixtures containing $^3$H-GTP of increased specific activity (15,000 counts per min per nmole) were incubated in duplicate for 1 min and then centrifuged to separate the released RNA and SVP. The released product was layered onto a 5 to 30% glycerol gradient (1-min $^3$H-pulse, released). One of the resulting SVP pellets was extracted with phenol- and the ethanol-precipitated RNA applied to a second gradient (1-min $^3$H-pulse, bound). The second SVP pellet was washed twice with 0.05 M Tris buffer (pH 8) and resuspended in 0.25 ml of a fresh reaction mixture lacking chymotrypsin but containing $^{32}$P-CTP (specific activity = 3,000 counts per min per nmole) in place of $^3$H-GTP. The mixture was incubated at 37 C for 30 min, terminated by adding SDS, and the $^3$H and $^{32}$P-labeled products analyzed in a glycerol gradient (1-min $^3$H-pulse, 30-min chase; 30-min $^{32}$P-chase).*

pulse. Thus the sequential release of complete chains probably results from the higher rates of synthesis of the shorter chains rather than from sequential transcription. RNA chains which were partially formed during a 1-min pulse and subsequently completed and released in a second "chase" were annealed with reovirus genome RNA and analyzed by electrophoresis in a 5% polyacrylamide gel. The profile was similar to that shown in Fig. 5, indicating that in a population of particles all 10 segments are probably simultaneously transcribed during the first minute of synthesis.

Nascent strands formed during a 1-min pulse are not released in the chase period if synthesis is prevented by deleting one of the four ribonucleoside triphosphates from the second incubation mixture. Completion of the chains is apparently a prerequisite for their release from SVP. To ensure that synthesis had occurred during the 30-min chase period in the experiment shown in Fig. 9, $^{32}$P-labeled CTP was included in the second incubation mixture. After the 30-min chase when essentially all the $^{32}$P-labeled products are completed chains, the ratio of l/m/s was 1/1.7/1.8. These values are the same as those observed for the products of a 30-min incorporation (Fig. 3), but differ markedly from the values of 1/4/8 for the quantities of incomplete, nascent l, m, and s chains present at 1 min after synthesis. Although the overall rate of RNA synthesis is linear throughout the first 30 min of incorporation, the individual rates of synthesis of l, m, and s change during this interval. The proportion of the total newly synthesized RNA accounted for by s molecules decreases from 61% after 1 min to 40% at 30 min. Concomitantly, m increases from 31 to 38% and l from 8 to 22%. After synthesis has proceeded for 30 min or more, a steady state is reached with respect to the relative rates of synthesis of the three classes of RNA. Incubation for 1 min with $^{3}$H-GTP after incorporation has proceeded with unlabeled precursors for 30 min or longer results in the synthesis and release of completed l, m, and s molecules in the ratio 1/1.7/1.8 (Fig. 10). It was also found that under these conditions more than 98% of the $^{3}$H-labeled RNA is released from the SVP. This is in marked contrast to the RNA made during the first minute of incubation which is largely bound to SVP. The change in both the ratio and extent of binding of s, m, and l products with increasing time of incubation suggests that the SVP are structurally altered during synthesis.

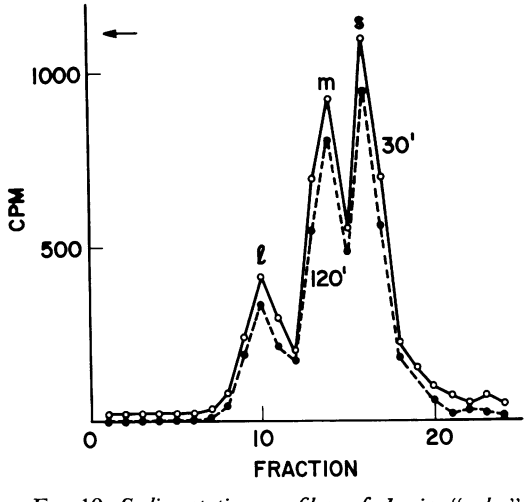


FIG. 10. *Sedimentation profiles of 1-min "pulse" RNA after 30 and 120 min of incubation. Duplicate reaction mixtures containing 47 μg of virus and chymotrypsin were incubated for 2 hr at 37 C without GTP. Unlabeled GTP was then added and the incubation continued. After 30 min, $^{3}$H-GTP (final specific activity = 15,000 counts per min per nmole) was added to one of the assay tubes. One minute later, this reaction was terminated with 1% SDS and the products were analyzed on a glycerol gradient. The second tube was similarly pulsed and processed after 120 min.*

## DISCUSSION

The RNA polymerase which is structurally associated with purified reoviruses transcribes one strand of the double-stranded genome RNA by a conservative mechanism in vitro. As shown by sedimentation and hybridization studies, the enzyme in heat-treated or chymotrypsin-digested particles copies all 10 segments of the genome. Correct initiation and termination apparently occur since there is net synthesis of single-stranded products corresponding in length to the L, M, and S genome fragments.

The enzyme remains active at 37 C for many hours and is resistant to inactivation by chymotrypsin and inactivated slowly at 60 C. The stability of the enzyme may be accounted for, at least in part, by its firm association with template. Although the polymerase in virus or SVP remains active and tightly bound to the genome RNA after heating for 2 min at 60 C, the structural integrity of the particles is altered. The structure and composition of heat-treated particles remain to be determined, but it is known that they no longer sediment in CsCl gradients with unheated virions or SVP (*unpublished data*). In view of the tight binding between enzyme and template, the mechanism by which the polymerase returns to the initiation site after chain termination is unclear. Reinitiation would be facilitated if the double-stranded RNA segments were packed within the virions in an open circle

configuration, i.e., with the initiating and terminating ends of the segments close together.

In reovirus-infected cells the initial viral mRNA is probably copied from the parental genome by the particle-associated polymerase. During the early part of the infectious cycle, only a limited number of genome segments are transcribed (39). In contrast, the polymerase in vitro copies all the double-stranded segments (31). Early viral mRNA formation in cells may be restricted by a heat-labile, chymotrypsin-sensitive viral structural protein or by a preexisting cellular protein. It will be of interest to examine the protein composition and pattern of in vitro RNA synthesis by "natural" SVP purified from cells at different times after infection.

The active SVP prepared by chymotrypsin digestion contain three major proteins, two of about 150,000 and one of 40,000 molecular weight (32), and a trace amount of one of the proteins of molecular weight about 80,000 (Fig. 1). The polymerase may correspond to one or more of these polypeptides or to a minor component which is not readily detected in the polyacrylamide gels. The particles may also contain proteins analogous to the recently described initiation (6) and termination (22) factors for RNA synthesis in *E. coli*. These possibilities can be explored if the SVP can be solubilized without irreversible denaturation of its component proteins.

The results of kinetic analyses and hybridization experiments indicate that all genome segments are transcribed simultaneously in vitro. Concomitant transcription may result from the presence of 10 (or more) enzyme molecules in each particle, i.e., 1 per RNA segment. Alternatively, each particle in a random population may contain a single polymerase molecule with various segments being copied in the different particles during a given time interval. The latter possibility is supported by the observation that net synthesis occurs only after 30 min of incubation although all segments are transcribed during the first minute of incorporation. The observed order for the rates of synthesis of the single-stranded products, s > m > l, may reflect the presence of a greater number of particles making s chains rather than their higher rate of synthesis. The preferential synthesis of s chains diminishes during the in vitro incubation, and the quantity of l, m, and s strands changes from a ratio of 1/4/8 at the early times of incubation (approximately 1 min) to steady-state values of 1/1.7/1.8 after 30 min. Possibly the s segments have a favored initiation site which is altered by structural rearrangement of the particles as synthesis proceeds; transcription of L and M genome fragments may also require prior formation of s molecules. Some of these possibilities are currently under investigation with $\gamma$-$^{32}$P-labeled ribonucleoside triphosphates, and the in vitro products have been found to contain GTP at the 5′-termini (Cold Spring Harbor Symp. Quant. Biol., *in press*). The results should provide a better understanding of the process of transcription.

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