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Properties of RNA Transcriptase in Reovirus Subviral Particles*

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Abstract. Subviral particles containing reovirus RNA transcriptase have been isolated from extracts of virus-infected mouse fibroblast cells. The purified particles which lacked the outer protein capsomeres of the mature virion had a buoyant density of 1.43–1.44 g/ml in CsCl and contained all of the double-stranded RNA genome of the intact virus. The particles were free of nuclease activity. RNA synthesis required all four ribonucleoside triphosphates and was dependent on magnesium or manganese; optimal activity required potassium or ammonium ions. In the presence of a ribonucleoside triphosphate regenerating system, reaction rates were linear for 20 hr. RNA yields of 40-fold in excess of input template could be obtained. Completed RNA chains were released from the subviral particles. In the course of RNA synthesis, the double-stranded RNA template was fully conserved. The RNA products formed *in vitro* displayed profiles in sucrose gradients similar to those found for *in vivo* reovirus mRNA. The RNA products were single-stranded and did not self-anneal. Over 90 per cent of the transcriptase products could be annealed with template double-stranded RNA. The annealed products migrated in acrylamide gels as double-stranded RNA, indicating efficient *in vitro* transcription.

Introduction. Reovirus RNA transcriptase is an integral component of the mature virus and is carried into the host cell during infection.[1, 2] The intimate association of the reovirus double-stranded RNA genome with the virion transcriptase provides an efficient mechanism for the *in vivo* transcription of early mRNA without the need for host cell polymerases or virus-induced enzymes.[3]

In the present studies subviral particles containing reovirus RNA transcriptase have been prepared from reovirus-infected L-cells. The particulate enzyme is highly active and relatively stable, and it synthesizes single-stranded RNA products *in vitro* similar to those produced *in vivo*.[4–6] The enzyme appears to be the same as that previously described.[1, 2, 6–8] In the course of these studies, two other enzyme activities have been found to be associated with such particles (*a*) a phosphatase which converts nucleoside triphosphates to nucleoside diphosphates and P_i;[9] and (*b*) a pyrophosphate exchange between ribonucleoside triphosphates and pyrophosphate.[10] This report describes a simple purification of reovirus subviral particles, the properties of the particulate RNA transcriptase, and the nature of the RNA products.

Materials and Methods. Virus and cell strains: The Dearing strain of reovirus type 3 (American Type Culture Collection, Washington, D.C.) and mouse fibroblast L-929 (L-cells) cells were used in all cultures. Chymotrypsin and pancreatic RNase A were products of the Sigma Chemical Co. (St. Louis, Mo.); ^{3}H- and ^{14}C-labeled nucleosides, nucleotides, and amino acids were obtained from New England Nuclear Corp., Boston.

Growth conditions: L-cells were grown in spinner cultures in minimum essential medium (Eagle) containing 10% (v/v) fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.). Lysates of reovirus-infected cells were prepared[11] and plaque assays were performed as previously described.[11,12]

Isolation and purification of reovirus RNA transcriptase: Sixteen hours after infection, cultures were harvested by centrifugation and resuspended in 20 ml of RSB buffer[13] (10 mM Tris-HCl, pH 7.4, 1.5 mM $MgCl_2$, and 10 mM NaCl) per liter of culture (10^9 cells), centrifuged, washed, and resuspended as before. Cells were disrupted with a Dounce homogenizer and the nuclei and cellular debris sedimented at 1200 × *g* for 5 min. The disruption and sedimentation steps were repeated and the pooled supernates were designated fraction 1 (Table 1). Fraction 1 was treated for 5 min at 0°C with sodium dodecylsulfate at a final concentration of 0.5%, diluted with 2 vol of RSB, and centrifuged 20 min at 30,000 × *g*. The pellet was resuspended in 10 ml of RSB per liter of original culture and dialyzed for 19 hr versus 2 × 100 vol of RSB. The dialyzed suspension (fraction 2) was incubated with chymotrypsin (50 μg/ml) for 20 min at 37°C and centrifuged at 30,000 × *g* for 20 min. The pellet was resuspended in 10 ml of RSB per liter of original culture (fraction 3). An equal volume of 8 M urea in RSB was added to fraction 3 and after 5 min at 0°C, the suspension was diluted with 2 vol of RSB, layered over a solution of RSB containing 20% glycerol (v/v) and 0.1% Brij-35 (Technicon Corporation, Tarrytown, N.Y.) and centrifuged for 30 min at 105,000 × *g*. The pellet, composed of subviral particles, was resuspended in 5 ml of RSB per liter of original culture and designated fraction 4. Unless otherwise noted, operations were performed at 0–4°C.

CsCl density gradients: A portion of fraction 4 labeled with ^{3}H-uridine in its double-stranded RNA was layered on 5 ml of a CsCl solution with an average density of 1.4 g/ml in 10 mM Tris (pH 7.4)–1 mM EDTA and centrifuged at 92,000 × *g* in an SW-39 Spinco rotor at 5°C for 18 hr. Fractions were collected dropwise by piercing the bottom of the tube; refractive indices were measured using an Abbe Refractometer (Bausch and Lomb Co., Rochester, N.Y.) and converted to buoyant densities. Radioactivity was determined by precipitating aliquots with 5% trichloroacetic acid in the presence of 30 μg of bovine serum albumin carrier. The precipitates were collected on Millipore filters (Millipore Co., Bedford, Mass.), washed with 5% trichloroacetic acid, dried, and counted in Liquiflor scintillation fluid (New England Nuclear Corp.) in a liquid scintillation spectrometer (Nuclear-Chicago, Des Plaines, Ill.).

Electron microscopy: Enzyme fractions were plated on Formvar-carbon-coated grids, stained with 2% phosphotungstic acid, pH 7.4,[14] and examined in a Siemens Elmskop 1A electron microscope.

Assay of reovirus RNA transcriptase: Previously described assays were modified for these studies.[2,6] Reaction mixtures in a final volume of 0.1 ml contained 100 mM Tris-HCl (pH 7.9); 2 mM dithiothreitol (DTT); 7.5 mM $MgCl_2$; 300 mM potassium acetate (or 1.5 mM $MnCl_2$ and 500 mM ammonium acetate); 2 mM each of ATP, CTP, and GTP; 1 mM ^{3}H-UTP with a specific radioactivity of 30,000 cpm/nmole, and 0.2–1 enzyme unit. Unless otherwise noted, reaction mixtures were incubated at 37°C for 30 min and incubation was terminated by the addition of 1 ml of cold 7% trichloroacetic acid. Acid-precipitable material was collected and counted as above. One unit of enzyme activity is defined as the incorporation of 1 nmole of ^{3}H-UMP into RNA in 30 min. Specific activity is expressed as units per milligram of protein.

Preparation and purification of RNA transcriptase products: A 5-ml reaction mixture containing Mg^{2+} and K^+ was incubated for 2 hr, treated with EDTA and bentonite at final concentrations of 10 mM and 0.5%, respectively, and then centrifuged at 30,000 × *g* for 20 min to sediment the particles and bentonite. The ^{3}H-RNA products in

the supernate were concentrated by ethanol precipitation, dissolved in 20 mM sodium acetate–1 mM EDTA buffer (SE), and passed through Sephadex G-25 (0.9 × 25 cm) in the same buffer.

Sucrose gradients of purified RNA products: A portion of the ^{3}H-RNA preparation was layered on 4.6 ml of a 5–20% sucrose gradient containing 0.1 M potassium acetate (pH 5.5) and 1 mM EDTA, and centrifuged at 98,000 × *g* in an SW-39 Spinco rotor for 4.5 hr at 12°C in the Spinco model L ultracentrifuge (Beckman-Spinco Co., Mountainside, N.J.). Fractions were collected from the bottom of the tube and acid-precipitable radioactivity was determined as described above.

Isolation of RNA template: Purified subviral particles were extracted with phenol. RNA in the aqueous phase was precipitated with ethanol, collected after 2 hr at −20°C, and dissolved in SE buffer. The double-stranded RNA was purified by chromatography on Whatman CF11 cellulose powder (W. and R. Ralston and Co., Ltd., England).[15,16]

Acrylamide gel electrophoresis of template RNA: Purified double-stranded RNA was separated electrophoretically for 10 hr in 5% polyacrylamide gels (0.6 × 8 cm) into nine distinct bands as previously described.[3,17] The distribution of radioactive RNA components was determined by slicing the gels into 0.7-mm pieces, extracting the slices with 1 ml of Soluene-100 (Packard Instrument Co., Downers Grove, Ill.) for 18 hr at 50°C, and counting in Bray's[18] scintillation solution.

Annealing of template RNA with RNA transcriptase products: The annealing procedure was a modification of that previously described.[4] Portions of double-stranded RNA diluted in H_2O were heated at 97°C for 7 min followed by rapid cooling in ice. Aliquots were mixed with given amounts of ^{3}H-single-stranded RNA products and the solution was brought to 300 mM NaCl and 5 mM K-PO_4, pH 7. The solution was heated at 95°C for 1 hr and then slowly cooled to 45°C over a 7-hr span.

Assay for annealing efficiency: Efficiency of annealing was measured by the extent to which single-stranded ^{3}H-RNA was converted to an RNase-resistant form in the presence of 300 mM NaCl, a procedure which permits the hydrolysis of single-stranded but not double-stranded RNA.[4] The assay mixture in a final volume of 0.5 ml contained 10 mM Tris-HCl (pH 7.5), aliquots of the annealed RNA product, 5 μg of RNase A, and 30 or 300 mM NaCl. A control tube contained 30 mM NaCl but no RNase. After 20 min at 37°C, acid-precipitable radioactivity was determined as above.

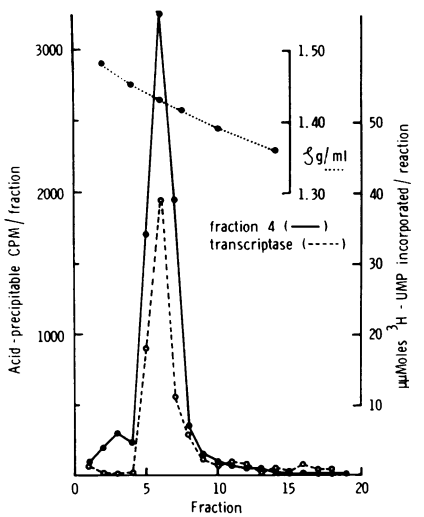
Protein was determined by the method of Lowry *et al.*[19] RNA was determined by the orcinol procedure of Mejbaum.[20]

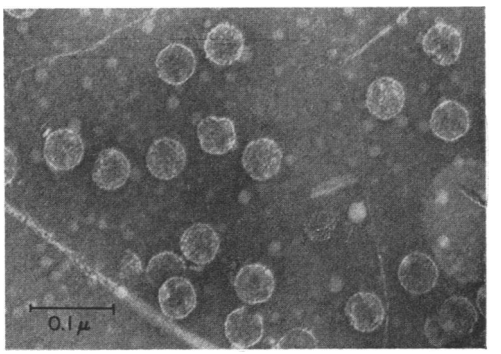
Results. Reovirus RNA transcriptase purification: One of the primary objectives of these studies was to purify subviral particles containing reovirus RNA transcriptase from crude extracts of reovirus-infected cells in order to examine the transcription mechanism of the particulate enzyme. A typical purification resulting in a 640-fold increase in enzyme specific activity is summarized in Table 1. In several preparations chymotrypsin treatment of fraction 2 increased enzyme activity from two- to five-fold. The specific activity of fraction 4 varied among preparations from 180 to 420 units/mg protein. Electron-microscopic examination of fraction 2 revealed that sodium dodecylsulfate

TABLE 1. *Purification of reovirus RNA transcriptase.*

Fraction	Volume (ml)	Total protein (mg)	Total units	Specific activity
1	40	244.2	106.2	0.4
2	10	2.1	73.2	35.0
3	5	1.2	171.9	143.0
4	5	0.6	153.9	256.0

The purification procedure is described in *Materials and Methods.*





(*Above*) FIG. 2.—Electron micrograph of subviral particles in fraction 4. Electron microscopy was performed as described in *Materials and Methods:* magnification is 180,000; particle diameter is 50 mμ.

(*Left*) FIG. 1.—CsCl buoyant density gradient of subviral particles in fraction 4. One-tenth ml of ^{3}H-labeled fraction 4 (Table 1) containing 15,650 cpm in its template double-stranded RNA was layered on CsCl and centrifuged as described in *Materials and Methods.* Radioactivity was assayed as previously described. Aliquots from each fraction of a separate but similar gradient were assayed for RNA transcriptase; endogenous ^{3}H-double-stranded RNA radioactivity (——), RNA transcriptase (- - -).

treatment partially removed the outer capsomeres of the virus. However, maximal transcriptase activity was expressed only after chymotrypsin treatment. A preparation of fraction 4 labeled with ^{3}H-uridine in its double-stranded RNA template was centrifuged to equilibrium in CsCl; a single radioactive peak with a buoyant density of 1.43 g/ml was observed (Fig. 1). In a similar gradient an assay for RNA transcriptase activity in each fraction showed a peak at the same buoyant density (Fig. 1). Electron micrographs (Fig. 2) of the peak fraction revealed that the virus was stripped of its outer capsomeres and resembled particles previously described.[2,21,22] Particles of fraction 4 contained 27% RNA compared to 14% RNA reported for the intact virion.[23]

Properties of the reovirus transcriptase: (*a*) The presence of all four ribonucleoside triphosphates—ATP, CTP, UTP, and GTP—was required for transcriptase activity in fractions 2–4. In addition, neither ribonucleoside diphosphates nor deoxyribonucleoside triphosphates served as substrates. (*b*) At rate-limiting enzyme concentrations RNA synthesis proceeded linearly for about 1 hr (Fig. 3). However, the addition of a ribonucleoside triphosphate regenerating system sustained linear kinetics of RNA synthesis for as long as 10 hr (Fig. 3) and in some cases up to 20 hr. (*c*) Synthesis was directly proportional to the amount of enzyme added up to a concentration of 1.5 enzyme unit/0.1 ml of incubation volume. (*d*) The pH optimum of the enzyme was 7.9. (*e*) The transcriptase activity exhibited an absolute dependence upon the presence of divalent cations such as Mg^{2+} or Mn^{2+}. Monovalent cations were also required for maximal activity. At optimal Mg^{2+} concentrations (7.5 mM), maximal activity was obtained with 0.3 M K^+. When Mg^{2+} was replaced by

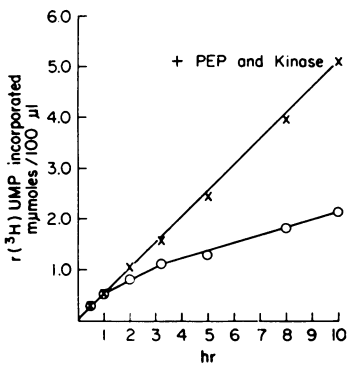


FIG. 3.—Kinetics of RNA transcription in the presence and absence of a ribonucleoside triphosphate regenerating system. Two reaction mixtures (0.2 ml), each containing 0.6 enzyme unit of fraction 4, were incubated for 10 hr at 37°C. One incubation was supplemented with 1.6 μmoles phosphoenol pyruvate and 5 μg of pyruvate kinase. At the indicated intervals, aliquots were assayed for acid-precipitable radioactivity (*Materials and Methods*).

Mn^{2+} (1.5 mM), optimal enzyme activity required 0.5 M NH_4^+; in both systems Na^+ was a poor substitute for NH_4^+ or K^+. (*f*) Transcriptase activity was not inhibited by actinomycin D,[24] nogalamycin,[25] rifampicin,[26] or inorganic phosphate. However, 1 mM and 5 mM inorganic pyrophosphate inhibited transcription by 68 and 97% respectively. (*g*) A net RNA synthesis by the transcriptase of 22-fold in excess of the input double-stranded RNA has been obtained. A 0.5-ml reaction mixture supplemented with 5 μmoles of phosphoenol pyruvate and 25 μg of pyruvate kinase was incubated with 2.5 enzyme units of fraction 4 for 14 hr at 37°C. The input template RNA contained a total of 12 nmoles of RNA nucleotides; this increased to 260 nmoles of RNA nucleotides after 14 hr. Since only one strand of each double-stranded RNA template segment was being copied (see below), the net synthesis represented a 43-fold increment over the template RNA. (*h*) The addition to the incubation mixture of a variety of RNA polymers did not affect the enzyme activity of fraction 4. The polymers tested included reovirus single-stranded RNA product and double-stranded RNA template and the synthetic ribopolymers, poly C, poly A, poly G:C, poly A:U, and poly I:C. (*i*) RNA transcriptase preparations were examined for nuclease activity by incubating several ^{3}H-labeled RNA polymers including RNA transcriptase products, ^{3}H-poly C, and 28S L-cell ^{3}H-rRNA. Less than 1% of each polymer was rendered acid-soluble after 1 hr at 37°C, suggesting that the transcriptase was free of nuclease. However, to preclude the presence of traces of endonuclease, the 28S ^{3}H-rRNA was analyzed in 5–20% sucrose gradients before and after exposure to the enzyme. In both gradients, all of the radioactivity migrated as a single peak with identical sedimentation coefficients.

Characterization of the RNA transcriptase products: (1) *Sedimentation in sucrose gradients:* Tritium-labeled RNA transcriptase products, prepared and isolated as described in *Materials and Methods*, were analyzed in sucrose gradients (Fig. 4). Three major RNA peaks with sedimentation coefficients of 24, 19, and 14S were observed which corresponded to the three size classes of single-stranded RNA formed in reovirus-infected cells.[5,6] Similar RNA profiles were obtained when Mg^{2+} and K^+ were replaced by Mn^{2+} and NH_4^+, respectively, in the reaction mixture.

After centrifugation over 95% of the particulate enzyme was recovered in the pellet whereas 96% of the RNA product remained in the gradient, indicating that completed strands were released from particle.

(2) *Single-strandedness and nonself-complementarity of the transcriptase products:* When purified ^{3}H-labeled RNA products were incubated with RNase A (5 μg/ml)

in the presence of 30 or 300 mM NaCl (see *Materials and Methods*), 98% of the RNA was rendered acid soluble, reflecting the single-stranded character of the transcriptase products. Moreover, when incubated under annealing conditions, the transcriptase products did not self-anneal.

(3) *Complementarity of transcriptase single-stranded RNA products with denatured double-stranded RNA template:* ^{3}H-labeled single-stranded RNA products were annealed with heat-denatured double-stranded RNA as described in *Materials and Methods*. Over 90% of the single-stranded RNA could be incorporated into a double-stranded product as determined by its resistance to RNase in the presence of 300 mM NaCl. The ^{3}H-labeled annealed products were further characterized by electrophoresis in a polyacrylamide gel with carrier double-stranded ^{14}C-RNA as described in *Materials and Methods* (Fig. 5). The annealed products displayed essentially the same distribution pattern as the carrier double-stranded ^{14}C-RNA. Two additional ^{3}H-labeled components were also observed; these were not further characterized.

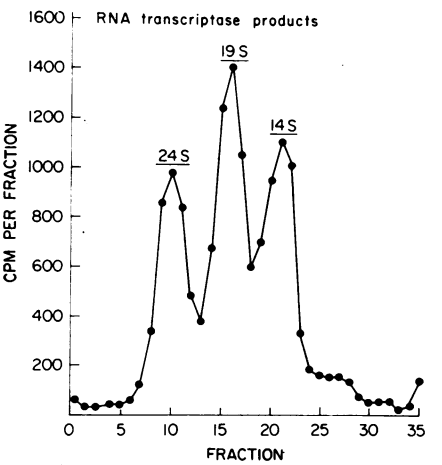


FIG. 4.—Sucrose gradients of *in vitro* RNA transcriptase products. Tritium-labeled single-stranded RNA products were prepared as described in *Materials and Methods*. Approximately 3 μg of ^{3}H-single-stranded RNA containing 16,430 cpm were layered on a 5–20% sucrose gradient and analyzed as described in *Materials and Methods*.

(4) *Mechanism of transcription:* Table 2 demonstrates that during the course of RNA synthesis ^{3}H-labeled double-stranded RNA was fully conserved. When RNA synthesis took place in the presence of labeled or unlabeled ribonucleoside triphosphates, the template RNA label was neither diluted nor rendered susceptible to hydrolysis by RNase. In other experiments, it was found that template RNA, which could be released from the particles by urea treatment, remained double-stranded in the particle throughout the course of synthesis. Hence the RNase-resistance of template RNA, the RNase-sensitivity of newly synthesized RNA, and the inability of these RNA products to displace template RNA indicated a conservative mechanism of transcription.

Discussion. Reovirus transcriptase is an integral part of the intact virion. The viral genome which serves as a template for the transcriptase is double-stranded RNA[23] and comprises 10 distinct segments.[5,17,27] The transcriptase activity is contained within the virion core and can be isolated directly from infected cells as a subviral particle with a buoyant density in CsCl of 1.43–1.44 g/cm^3. The purification procedure provides a simple method for the preparation of a relatively stable particulate enzyme in high yield and purity which rapidly synthesizes high molecular weight messenger RNA *in vitro*. As much as 66 pmol of nucleotide are incorporated per microgram protein/min. Enzyme activity requires all four ribonucleoside triphosphates. RNA synthesis is linear for short periods only, unless a ribonucleoside triphosphate regenerating system

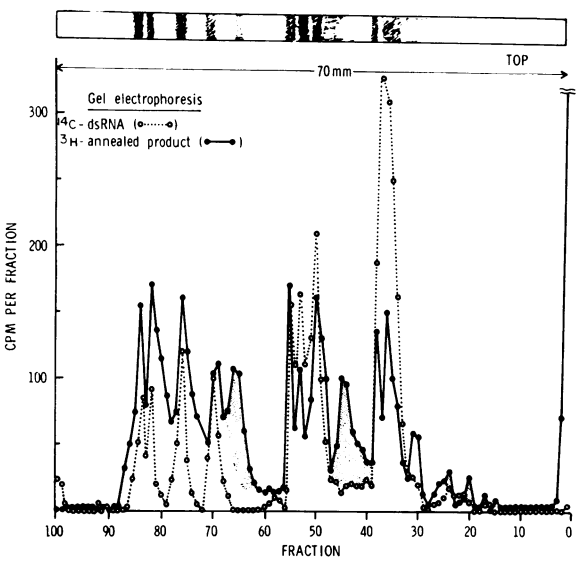


FIG. 5.—Polyacrylamide gel electrophoresis of the annealed product of double-stranded RNA template and single-stranded RNA. Purified unlabeled double-stranded RNA (100 μg) was annealed with 10 μg of ^{3}H-single-stranded RNA products containing 121,000 cpm and the annealed product purified on a Whatman CF11 cellulose column (*Materials and Methods*). An aliquot of annealed product containing 14,600 cpm was applied to a 5% polyacrylamide gel with 0.1 A_{260} unit of carrier ^{14}C-double-stranded RNA containing 12,230 cpm and electrophoresed as described in *Materials and Methods*. The gel was stained as previously described[17] and then analyzed for ^{3}H and ^{14}C as described in *Materials and Methods:* ^{14}C-double-stranded RNA (. . .); ^{3}H-annealed product (——). A calibrated diagram of the stained gel at the top of the figures demonstrates the correlation between the nine stained double-stranded RNA segments and the radioactive peaks.

is present. This requirement is due to nucleoside triphosphatases associated with the subviral particles which degrade all four triphosphates to their corresponding diphosphates and inorganic phosphate.[9] In addition, a third enzymic activity, which catalyzes an exchange between pyrophosphate and ribonucleoside triphosphates, is present in the particles.[10]

Electron microscopic data indicate that the double-stranded RNA template of the subviral particles lies within a protein shell. This observation may explain the insensitivity to nucleases of template RNA in the particles and the inability of added double-stranded RNA, synthetic RNA polymers, or RNA transcriptase products to affect RNA transcription. During RNA synthesis the template RNA is fully conserved and completed RNA chains are released from the subviral

TABLE 2. *Conservation of double-stranded RNA template during transcription.*

Incubation period (min)	Time of RNase addition	Acid-Precipitable CPM after Synthesis with ^{3}H-UTP	UTP
0	—	7,242	8,687
30	—	25,102	8,770
30	0	7,640	9,200
30	30	7,140	8,490

Incubation mixtures (0.1 ml) contained all of the components of the assay and either 1 mM ^{3}H-UTP (30,000 cpm/nmole) or 1 mM unlabeled UTP. The enzyme utilized in these assays was fraction 4 of a preparation labeled with ^{3}H-uridine in its double-stranded RNA (12,350 endogenous cpm/enzyme unit). Tubes containing ^{3}H-UTP had 0.6 enzyme unit; tubes with unlabeled UTP contained 0.7 enzyme units. Incubation was for 30 min at 37°C. Where indicated, 2 μg of RNase were added and incubation allowed to proceed further for 20 min at 37°C.

particles. A net RNA synthesis of 40-fold in excess of input template RNA indicates that a given enzyme molecule makes many copies of each template. The inability of the single-stranded RNA products to self-hybridize demonstrates that only one strand of each double-stranded RNA segment is copied *in vitro* as shown previously for the reovirus mRNA synthesized *in vivo*.[3-6]

The transcriptase preparations are free of nuclease activity and produce at least 10 single-stranded RNA species, which separate into three size classes in sucrose density gradients similar to those found *in vivo*.[3,6] More than 90% of the RNA products anneal to purified double-stranded RNA. After electrophoresis in polyacrylamide gels, the annealed products display a pattern similar to that of purified double-stranded RNA template, indicating a highly efficient *in vitro* transcription. These data are in accord with results recently reported by Skehel and Joklik[7] for enzyme preparations obtained directly from purified virus preparations after chymotrypsin treatment.

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