JOURNAL OF VIROLOGY, Oct. 1972, p. 698-706

Vol. 10, No.
Printed in U.S.A.

Transcription by Infectious Subviral Particles of Reovirus

A. J. SHATKIN AND A. J. LAFIANDRA
Roche Institute of Molecular Biology, Nutley, New Jersey 07110

Received for publication 5 June 1972

Digestion of purified reovirus with chymotrypsin in the presence of 0.15 M NaCl converts virions to infectious subviral particles (SVP_i). The SVP_i have an active ribonucleic acid (RNA) polymerase and are similar in composition to the partially uncoated virions which have been isolated from infected L cells. SVP_i have a buoyant density of 1.40 g/ml in CsCl and sediment at 420*S* as compared to 1.37 g/ml and 630*S* for virions. They consist of 30% less protein and include the polypeptides of the inner structural layer, λ_1, λ_2, and σ_3, and a polypeptide derived by cleavage of μ_2, a constituent of the outer shell. The genome RNA is retained within SVP_i, but more than 60% of the "adenine-rich," single-stranded RNA is released by the proteolytic treatment. Infection of L cells with SVP_i or virions results in the transcription of all 10 genome segments. In cycloheximide-treated SVP_i-infected cells, transcription occurs predominantly from one medium and two small genome segments, the same pattern of early messenger RNA (mRNA) observed in virion-infected cells. In contrast, SVP_i incubated in vitro synthesize mRNA corresponding to all genome segments.

Transcription of the segmented, double-stranded ribonucleic acid (RNA) genome of reovirus is subject to temporal control in virus-infected L cells (13, 21). Viral messenger RNA (mRNA) synthesized 4 to 6 hr after infection, i.e., early mRNA, is copied predominantly from four of the ten genome segments, whereas at later times all duplex segments are transcribed. Only early mRNA is made in cycloheximide-treated infected cells, suggesting that its synthesis is catalyzed by the RNA polymerase which is present in parental virions (6, 15). The RNA polymerase can be activated in vitro by digestion of purified reovirus with chymotrypsin (15) or by brief exposure to 60 C (6). However, in contrast to early mRNA from infected cells, mRNA produced in vitro by the particle-associated polymerase is copied from all 10 genome segments (2, 10, 19). To study the basis for control of reovirus transcription, it is of interest to compare the species of mRNA synthesized by subviral particles in vitro versus those in infected cells. This comparison depends upon defining conditions for producing infectious subviral particles since degradation of reovirus with chymotrypsin under the conditions used in previous studies results in the formation of core particles which have a 10^4- to 10^5-fold lower specific infectivity than virions (2). We now report the in vitro preparation of infectious subviral particles which are very similar to partially uncoated virions isolated from infected cells (7, 18). The composition and biological properties of these infectious subviral particles are described.

MATERIALS AND METHODS

Cells and virus. Mouse L cells were grown in suspension culture in Eagle medium supplemented with 5% fetal bovine serum. For the preparation of purified virus, cells were concentrated to 10^7/ml, and reovirus type 3 Dearing strain was added at a multiplicity of infection of 10 plaque-forming units (PFU)/cell. After adsorption for 30 min, the cells were diluted to 10^6/ml and incubated at 34 C for 36 to 48 hr (20). The cells were then collected by centrifugation and homogenized with Genetron (1,1,2-trichlorotrifluoroethane), and the virus was purified by isopycnic sedimentation in CsCl as described previously (2). After dialysis against 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 8), the virus was purified further by sedimentation for 1 hr at 147,000 × *g* (SW41 rotor) in 20 to 40% linear density gradients of glycerol in 0.01 M phosphate buffer (*p*H 7.2), 0.15 M NaCl (PBS). The virus band was collected and dialyzed against Tris. Yields of 10 to 20 mg of virus/10^9 cells were obtained. ^{3}H-amino acid-labeled virus was similarly prepared, except that the medium contained one-fourth the usual concentration of amino acids, 1 μCi of ^{3}H-amino acid mixture per ml, and 0.25 μg of actinomycin per ml. ^{32}P-labeled virus was grown in phosphate-free medium containing actinomycin and 10 μCi of carrier-free ^{32}P-phosphoric acid per ml. ^{3}H-

uridine-labeled virus was purified from infected cells incubated in the presence of actinomycin and 0.5 μCi of ^{3}H-uridine per ml.

Preparation and analysis of subviral particles. Purified virus was diluted to the indicated concentration in PBS, Tris, or Tris-saline (0.01 M tris buffer [*p*H 8], 0.15 M NaCl). In all experiments, chymotrypsin was added at a final concentration of 200 μg/ml, and the virus was digested at 40 C for 1 hr. Virus concentrations expressed as milligrams of protein were assayed by the Lowry method with bovine serum albumin as a standard (12); purified reovirus contains 85% protein by weight (8). Infectivity was measured by plaque formation on L-cell monolayers.

For base composition analysis, ^{32}P-labeled virions and chymotrypsin-treated particles were resedimented in 20 to 40% glycerol gradients. Fractions were collected from the bottom of the gradients, and samples were counted directly in a mixture of 3 ml of Methyl Cellosolve (ethylene glycol monomethyl ether) and 5 ml of toluene-diluted Spectrafluor (Amersham/Searle, Arlington Heights, Ill.). Appropriate fractions were pooled together with 0.25 mg of L-cell RNA as carrier and precipitated with an equal volume of 2.5 N perchloric acid at 0 to 4 C. The precipitates were hydrolyzed in 0.3 N KOH for 18 hr at 37 C, the samples were neutralized with Dowex-50 (1), and the nucleotide compositions were determined by high-voltage paper electrophoresis (14).

Buoyant densities in CsCl were determined with ^{3}H-uridine-labeled virions and subviral particles. After incubation with chymotrypsin, samples of 0.2 ml were layered onto preformed gradients of CsCl (density 1.35–1.48 g/ml) in Tris and centrifuged for 20 hr at 165,000 $\times$ *g* at 5 C in the SW50L rotor. Samples were collected from the bottom of the gradient, and fractions precipitated with 5% trichloroacetic acid were collected on membrane filters (Millipore Corp.) and counted in Spectrafluor. Densities were determined from refractive index readings in an Abbé refractometer.

The protein compositions of solubilized virions and subviral particles were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Particles were solubilized by heating for 5 min at 100 C in 2% SDS-1% mercaptoethanol. Urea (8 M) was added to the samples which were then dialyzed for 1 to 2 hr against 0.002 M phosphate buffer (*p*H 7.2), 0.1% SDS, 0.1% mercaptoethanol, and 8 M urea. Samples were applied to 10% polyacrylamide gels and subjected to electrophoresis for 20 to 24 hr at 4 mA/gel and 20 C in 0.1 M sodium phosphate buffer (*p*H 7.2), 0.1% SDS. Gels were fixed in 10% trichloroacetic acid overnight, stained for 18 hr with 0.025% Coomassie blue in trichloroacetic acid, and destained in 7% acetic acid. Gels containing radioactive proteins were frozen, sliced, and counted (11, 17).

Synthesis and extraction of viral RNA from cells infected with subviral particles. Duplicate cultures of L cells were infected with 50 PFU/cell of infectious subviral particles prepared by digestion of virions (285 μg/ml in Tris-saline containing 200 μg of chymotrypsin per ml for 1 hr at 40 C). After adsorption for 30 min, the unabsorbed infectious subviral particles (SVP_i) were removed by washing the cells in medium, and the cells were resuspended at 5 $\times$ 10^5/ml for incubation at 37 C. One of the cultures contained 20 μg of cycloheximide per ml during adsorption and throughout infection; 0.5 μg of actinomycin per ml was added to both cultures after adsorption. At 90 min after infection, 2 μCi of ^{3}H-uridine per ml was added together with enough unlabeled uridine to give a final concentration of 10^{-6} M.

At 12 hr after infection, the cells were chilled, collected by centrifugation, suspended at a density of 10^7/ml in 0.01 M acetate buffer (*p*H 5.1), 0.1 M NaCl, 0.001 M ethylenediaminetetraacetic acid (EDTA), and 0.5% SDS and shaken with an equal volume of water-saturated redistilled phenol for 5 min at 60 C. After cooling to 0 to 4 C, the mixture was centrifuged, the aqueous phase was reextracted with phenol at room temperature, and the RNA was precipitated by addition of two volumes of ethanol at −20 C. The RNA was dissolved in Tris-EDTA (0.01 M Tris buffer [*p*H 8], 0.001 M EDTA), washed four times with ether, reprecipitated by adding 0.1 volume of 20% potassium acetate and two volumes of ethanol, and dissolved in Tris-EDTA.

In vitro transcription by subviral particles. Infectious subviral particles were concentrated after incubation by layering 1 ml of virus-chymotrypsin-Tris-saline mixture onto 4 ml of 30% glycerol in 0.02 M Tris buffer (*p*H 8), 0.005 M EDTA, and 0.1 M KCl and centrifuging at 4 C for 2.5 hr at 150,000 $\times$ *g* in an SW50L rotor. The pellet was resuspended in Tris containing 0.15 M KCl. For the in vitro synthesis of RNA, the resuspended pellet, 120 μg, was added to 1 ml of reaction mixture containing 70 mM Tris-hydrochloride buffer (*p*H 8); 7.5 mM $MgCl_2$; 2 mM each of adenosine triphosphate, cytidine triphosphate, and uridine triphosphate; 1 mM ^{3}H-guanosine triphosphate (^{3}H-GTP) (specific activity: 4 $\times$ 10^3 counts per min per nmole); 2.5 mM phosphoenol pyruvate; 20 μg of pyruvate kinase; and 0.15 M KCl. KCl was added to stabilize the infectious subviral particles, and their protein composition by gel analysis was found to be unchanged after RNA synthesis. KCl was used because 0.15 M NaCl completely inhibited the in vitro synthesis of RNA. After incubation for 1 hr at 40 C, the mixture was chilled and shaken with an equal volume of phenol, and the RNA was precipitated, washed with ether, and reprecipitated as above. Analysis by centrifugation for 18 hr at 75,000 $\times$ *g* (SW41) in 5 to 30% glycerol gradients (0.02 M Tris, 0.005 M EDTA, 0.1 M NaCl) has been described (2).

Hybridization of mRNA with genome RNA. ^{3}H-labeled RNA isolated from infected cells or in vitro reaction mixtures and 30 μg of double-stranded RNA extracted from purified reovirus with phenol (2) were denatured in Tris-EDTA solution by adding nine volumes of dimethyl sulfoxide and heating at 37 C for 30 min. The samples were precipitated at −20 C by adding 200 μg of yeast transfer RNA, 0.1 volume of 20% potassium acetate, and two volumes of ethanol. The denatured RNA was annealed for 18 hr at 72 C in Tris-EDTA-0.3 M NaCl and analyzed by electrophoresis in 5% polyacrylamide gels as described

previously (2, 17). ^{32}P-labeled genome RNA (kindly provided by C. M. Stoltzfus and A. K. Banerjee) was added as a marker at the time of application of the sample to the gels.

Radioactive compounds. Carrier-free ^{32}P-orthophosphate was from E. R. Squibb and Sons, Inc., New York. ^{3}H-GTP (14.5 Ci/mmole) and ^{3}H-labeled reconstituted protein hydrolysate were purchased from Schwarz/Mann, Orangeburg, New York. ^{3}H-uridine (26 Ci/mmole) was from New England Nuclear, Boston, Mass.

RESULTS

Protein composition of reovirus versus subviral particles. Purified reovirions contain seven polypeptides arranged in two structural layers (11 20). The outer shell comprises 70% of the viral protein and consists of four polypeptides: μ_1, μ_2, σ_1, and σ_3 of molecular weights 80,000, 72,000, 42,000 and 34,000, respectively (20). Polypeptide μ_1 which apparently is a precursor of μ_2 constitutes only 2% of the total protein (23). The inner protein layer contains polypeptides λ_1, λ_2, and σ_2 of molecular weights 150,000, 140,000 and 38,000, respectively (20). Treatment of virions with chymotrypsin in Tris degrades all of the outer shell proteins and converts the virions to core particles of 10^4- to 10^5-fold lower specific infectivity (2, 10, 15, 20). The remaining infectivity may be due to the presence of residual virions in the digested preparation. In contrast, digestion at 40 C for 1 or 2 hr in PBS or Tris-saline digests only 30% of the total viral proteins including μ_1, σ_3, and about 20% of the chain length of μ_2 (Fig. 1). The resulting subviral particles retain both λ polypeptides, σ_2, and a polypeptide with an apparent molecular weight of about 60,000, which is not present in untreated virions. Polypeptide μ_2 is quantitatively cleaved to yield the 60,000 molecular weight product because (i) the radioactivity in the larger λ polypeptides is not lost after chymotrypsin digestion and (ii) the reduction in molecular weight of μ_2 from 72,000 to about 60,000 (17%) is accompanied by a loss of about 20% of the radioactivity initially present in μ_2. σ_1 also appears to be retained, although its presence in small amounts makes quantitation difficult.

RNA composition of reovirus versus subviral particles. In addition to the double-stranded genome RNA, there is present in purified reovirus a mixture of single-stranded RNA oligomers (3–5, 16). This adenine-rich RNA includes oligomers of two to seven bases in length, each containing 5′-ppGp, and polyadenylic acid molecules of chain length 10–15 bases which are terminated by 5′-pAp (C. M. Stoltzfus and A. K. Banerjee, Arch. Biochem. Biophys., *in press*). Consequently, cell-associated and released reovirus purified as

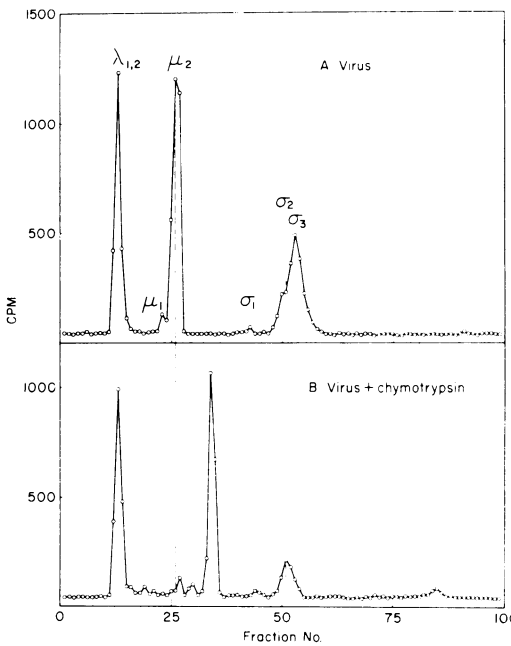

FIG. 1. *Polypeptide composition of reovirus and subviral particles. ^{3}H-amino acid-labeled reovirus before (A) and after (B) digestion for 1 hr at 40 C with 200 μg of chymotrypsin per ml in PBS was solubilized and analyzed on 10% polyacrylamide gels as described in Materials and Methods.*

described above contain 44.2 to 46.4% adenine (Table 1). Upon further purification by a second sedimentation in a glycerol gradient, the adenine content remains 42.7%. The single-stranded RNA is essentially all released when reovirus is degraded to core particles by chymotryptic digestion in Tris, and the remaining genome RNA has a base composition identical to purified reovirus double-stranded RNA (Table 1; reference 2).

The single-stranded RNA content of infectious subviral particles was also measured. In contrast to core particles digested in Tris which form readily sedimentable aggregates (2,000 rev/min; 3 min), particles degraded with chymotrypsin in PBS or Tris-saline sediment more slowly (~420S) in glycerol gradients than virions (~630S) (Fig. 2; reference 8). The adenine content of the RNA from banded subviral particles (sample B in Fig. 2) is decreased to 32.6% which is consistent with the loss of more than 60% of the adenine-rich RNA (Table 1). The released RNA is of low molecular weight and sediments near the top of a glycerol gradient (Fig. 2, sample A). This RNA has a high adenine content but also small amounts of the other three bases which are present in the short oligomers of the single-stranded RNA mix-

TABLE 1. *Base composition analysis*[a]

Determinations	Moles percent composition				
	Cytidylic acid	Adenylic acid	Guanylic acid	Uridylic acid	AG/CU[b]
Virus					
Cell-associated	17.0	46.4	16.3	20.2	1.6
Released	17.4	44.2	17.0	21.4	1.6
Gradient-banded two times	17.9	42.7	16.7	22.7	1.5
Virus + chymotrypsin					
Gradient—sample A	12.8	59.2	9.3	18.7	2.2
Gradient—sample B	20.7	32.6	17.9	28.8	1.0
Core particles	23.6	26.8	23.4	26.2	1.0
Double-stranded RNA	23.4	26.6	23.8	26.2	1.0

[a] ^{32}P-labeled virus was purified from infected cells (cell-associated) or medium (released). Purified virus was also banded a second time in 20 to 40% glycerol (gradient-banded two times). Virus was digested with chymotrypsin (200 μg/ml, 40 C, 1 hr) in PBS and rebanded in glycerol, as in Fig. 2, to separate infectious subviral particles (gradient-sample B) from single-stranded oligomers (gradient-sample A). Noninfectious particles were prepared by proteolytic digestion in Tris (2), and double-stranded RNA was extracted from purified virus with phenol and purified by passing through Sephadex G-100. The base compositions were determined by high-voltage paper electrophoresis of alkaline digests as described in Materials and Methods section.

[b] Adenine plus guanine/cytosine plus uridine.

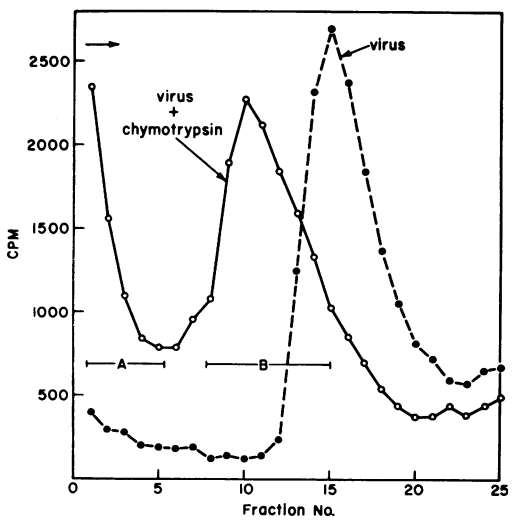

FIG. 2. *Sedimentation profiles of reovirus and subviral particles. ^{32}P-labeled reovirus and reovirus digested with 200 μg of chymotrypsin per ml (PBS, 1 hr, 40 C) were sedimented in separate linear density gradients of 20 to 40% glycerol as described in Materials and Methods. Samples of the collected fractions were counted directly in Methyl Cellosolve-Liquifluor, and the results are plotted together.*

ture (J. L. Nichols, A. R. Bellamy and W. K. Joklik, Bacteriol. Proc., p. 237, 1972; C. M. Stoltzfus and A. K. Banerjee, Arch. Biochem. Biophys., *in press*).

Infectivity of subviral particles. Incubation of virions in Tris containing 200 μg of chymotrypsin per ml reduced the specific infectivity of the resulting core particles from 4×10^{10} to less than 10^6 PFU/mg (2), with chymotrypsin levels as low as 10 μg/ml (Fig. 3). However, when PBS was present during the incubation, the infectivity was reduced by only about twofold at equal concentrations of virus and enzyme. Since the particle to PFU ratio for purified reovirus is high, the possibility was considered that the infectivity of the subviral particle preparation was associated with a small fraction of undegraded virions. To test this possibility, virions (200 μg/ml) were digested in PBS with chymotrypsin and sedimented in a glycerol density gradient as in Fig. 2. The specific infectivity of the fractions corresponding to subviral particles (region B) was 2.2×10^{10} PFU/mg. Thus the infectivity was associated with subviral particles and was similar to the specific infectivity of purified virions, 4×10^{10} PFU/mg. Similarly, the specific infectivities of ^{3}H-uridine-labeled virus and subviral particles prepared and purified as in Fig. 2 were 3×10^4 and 6×10^4 PFU per count per min, respectively.

When the virus concentration in PBS was increased to 0.3 mg/ml, there was a small decrease in infectivity to 1×10^{10} PFU/mg by chymotrypsin treatment (Fig. 3). At higher virus levels and 200 μg of chymotrypsin per ml, the specific infectivity decreased more than 10,000-fold. The

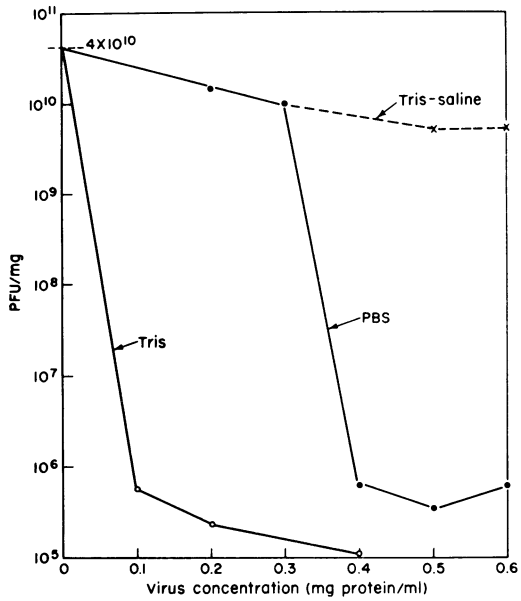

FIG. 3. *Specific infectivity of subviral particles. Purified virus with an initial titer of 4 × 10¹⁰ PFU/mg of protein was digested in Tris, PBS, or Tris-saline with 200 μg of chymotrypsin per ml for 1 hr at 40 C at the concentrations indicated. Titers were determined by plaque assay on L-cell monolayers.*

infectivity of subviral particles was more resistant to chymotrypsin in Tris-saline, the specific infectivity remaining at about 20% of the initial value even after the virus concentration was increased to 0.6 mg/ml, three times that of the chymotrypsin (Fig. 3). Thus the presence of 0.15 M NaCl during chymotrypsin digestion protects reovirus against loss of infectivity.

The decrease in specific infectivity is correlated with the extent of digestion of the outer shell polypeptides. Digestion in PBS at a virion concentration of 0.2 mg/ml, conditions which reduced the infectivity only twofold, resulted in the loss of μ_1 and σ_3 and in the loss of 20% of μ_2 polypeptide (Fig. 4, middle). At 0.5 mg/ml, the outer shell including μ_2 was completely digested, and the infectivity decreased by a factor of 10^5 (Fig. 3 and 4). The proteins remaining were identical to those in the core particles prepared in Tris-chymotrypsin (2, 20).

Buoyant density of particles in CsCl. The extent of degradation of the outer shell polypeptides is also correlated with particle buoyant density in CsCl. ^{3}H-uridine-labeled reovirus has a density of 1.37 g/ml (Fig. 5). After conversion to infectious subviral particles by digestion with 200 μg of chymotrypsin per ml in Tris-saline or in PBS at a virus concentration of 100 μg/ml, the value increases to about 1.40 g/ml. Proteolytic digestion at a virus concentration of 400 μg/ml, conditions which completely degrade the outer shell structural proteins (70% of the total), converts virions to core particles of buoyant density 1.45 g/ml (Fig. 5) (2, 20). The specific infectivity of the subviral particles (ρ = 1.40 g/ml) obtained from CsCl gradients identical to those in Fig. 5 was 2×10^{10} PFU/mg as compared to 2×10^{10} and $<10^6$ PFU/mg for virions (ρ = 1.37 g/ml) and core particles (ρ = 1.45 g/ml), respectively. The protein composition of CsCl-banded SVP_i was also examined and found to be identical to that shown in Fig. 4 (middle).

Transcription by subviral particles in infected cells versus in vitro. Watanabe et al. reported that viral mRNA is transcribed predominantly from four of the ten genome segments during the early period of reovirus replication in L cells (21). As the replicative cycle proceeds, the remaining six segments are also copied. The same four species of mRNA are made in cycloheximide-treated cells, and it was suggested that a pre-

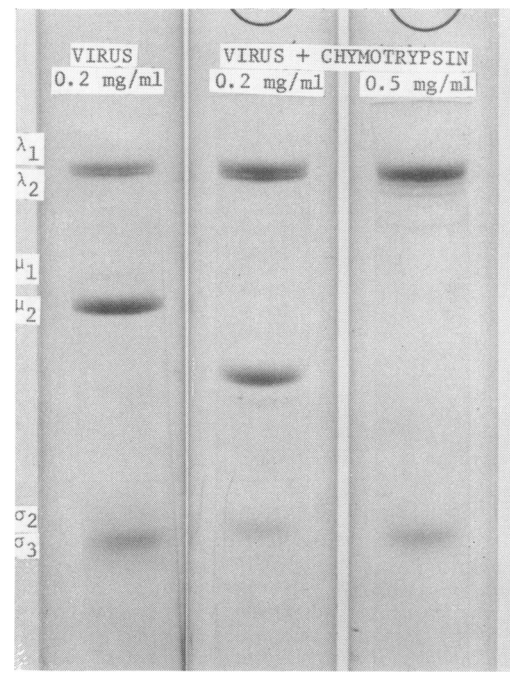

FIG. 4. *Electropherograms of virus, infectious subviral particles and cores. Reovirus at a concentration of 0.2 or 0.5 mg/ml was digested with chymotrypsin in PBS, solubilized, and analyzed by electrophoresis in 10% polyacrylamide gels and stained with Coomassie blue as described in Materials and Methods.*

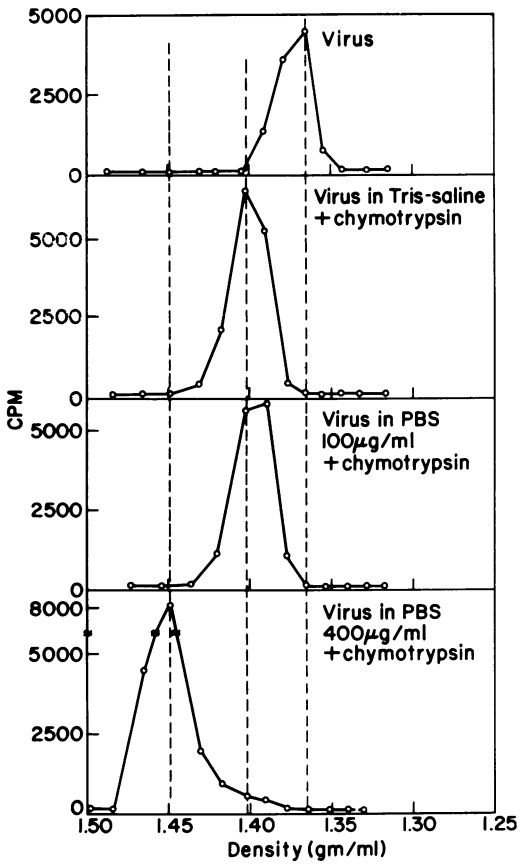

FIG. 5. *Buoyant densities of virus, subviral particles and cores in CsCl.* ^{3}*H-uridine-labeled reovirus was sedimented to equilibrium in CsCl before and after digestion with chymotrypsin in Tris-saline or PBS as described in Materials and Methods. Samples were collected, and fractions were precipitated with 5% trichloroacetic acid and counted. Density was determined from the refractive index.*

formed RNA polymerase, later shown to be associated with parental virions, catalyzes early viral mRNA formation in infected cells. Since early mRNA transcription by parental virus can be regulated in the presence of an inhibitor of protein synthesis, it seemed possible that control is mediated by virus structural protein(s). To test whether polypeptides μ_1, σ_3, and intact μ_2 are required for early mRNA regulation, cells were infected in the presence and absence of cycloheximide with virions or with subviral particles prepared in chymotrypsin-Tris-saline. The virus yield increased from 4 PFU/cell at 90 min to 360 PFU/cell at 24 hr after infection in the untreated samples. The cycloheximide-treated cultures decreased from 4 PFU/cell to 0.2 PFU/cell during the same interval. Viral mRNA labeled between 90 min and 12 hr after infection was isolated, hybridized to genome RNA, and analyzed by polyacrylamide gel electrophoresis. The mRNA from cells productively infected with SVP_i gave the same gel pattern as mRNA from virus-infected cells, consistent with the transcription of all genome segments (reference 13; Fig. 6A). However, early mRNA formed in the presence of cycloheximide hybridized predominantly with one M (medium) and the two smallest S (small) genome segments and to a very limited extent with the L (large) and other two S segments (Fig. 6B). The same gel profile was obtained with mRNA isolated from cycloheximide-treated cells infected with undigested virions as reported previously (13, 21). The results indicate that the pattern of virus mRNA synthesis is unaltered in cells infected with SVP_i, particles treated with chymotrypsin under conditions which digest polypeptides μ_1 and σ_3 and cleave one-fifth of polypeptide μ_2.

Core particles which lack all the polypeptides of the outer shell synthesize all 10 mRNA species in vitro (2, 10, 19). It was of interest to compare the pattern of in vitro mRNA synthesis by infectious subviral particles since they transcribe predominantly from a limited number of segments in cycloheximide-treated infected cells (Fig. 6B). Subviral particles were prepared by chymotrypsin digestion of virions in Tris-saline and concentrated by sedimentation as described above. After incubation in the reaction mixture for RNA synthesis with or in the reaction mixture without cycloheximide, a sample was analyzed by polyacrylamide gel electrophoresis. The polypeptide pattern of these particles was unchanged by the incubation (Fig. 4, middle), indicating that the particles were not further degraded during RNA synthesis in vitro. The in vitro mRNA products included similar amounts of the three classes of reovirus mRNA: l, m, and s equals large, medium, and small, respectively (Fig. 7). In contrast to the early mRNA pattern from subviral particle-infected, cycloheximide-treated cells (Fig. 6B), the in vitro products resembled the mRNA from productively infected cells shown previously to be copied from all 10 genome segments (reference 13; Fig. 6C).

DISCUSSION

Purified reovirus digested with chymotrypsin in dilute Tris buffer is converted to core particles which lack the four structural proteins comprising the virion outer shell and have a low specific infectivity (15, 20). However, by adding 0.15 M NaCl to the buffer, the extent of degradation is reduced and infectious subviral particles are

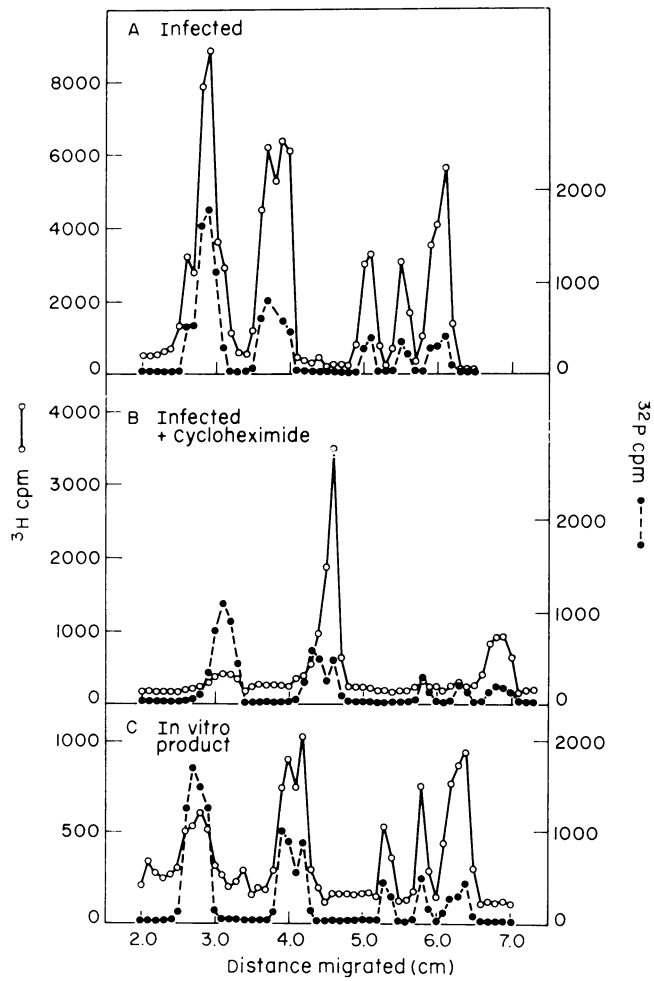

FIG. 6. *Electrophoretic patterns of hybridized reovirus mRNA. Cells were infected with subviral particles (50 PFU/cell) and labeled with ^{3}H-uridine from 1.5 to 12 hr after infection in the presence (B) or absence (A) of 20 μg of cycloheximide/ml. RNA was extracted with phenol, denatured with dimethyl sulfoxide, and annealed with reovirus genome RNA. ^{32}P-labeled genome RNA was added to the annealed RNA as a marker, and the mixture was analyzed by electrophoresis in 5% polyacrylamide gels as described in detail in Materials and Methods. The infectious subviral particles were also concentrated and incubated for 1 hr in a reaction mixture designed for the synthesis of viral mRNA. The in vitro product was extracted, hybridized, and analyzed.*

formed. The infectious particles prepared in vitro are very similar to the partially uncoated virions isolated from infected cells early in the infectious cycle (7, 18). They retain about 80% of the total molecular weight of polypeptide μ_2, suggesting that a portion of μ_2 must be present if the subviral particle is to be infectious. They also have a highly active RNA polymerase. These findings are consistent with reports that the other major polypeptide of the outer shell, σ_3, is degraded before μ_2 and that loss of σ_3 manifests the virion-associated RNA polymerase activity (7, 18). Perhaps μ_2 is structurally integrated into both virion protein layers, and the presence of 0.15 M NaCl stabilizes that portion of the polypeptide which is part of the inner shell. Stabilization apparently is not strictly a salt effect, since μ_2 is completely degraded and virions are converted to core particles by chymotrypsin digestion in the presence of 0.15 M KCl. At higher virus concentrations, the loss of infectivity and μ_2 also occurs even in NaCl-containing buffers. This concentration dependence is probably related to the observation that core particles aggregate during chymotrypsin digestion in Tris (2). Aggregated particles may undergo a conformational

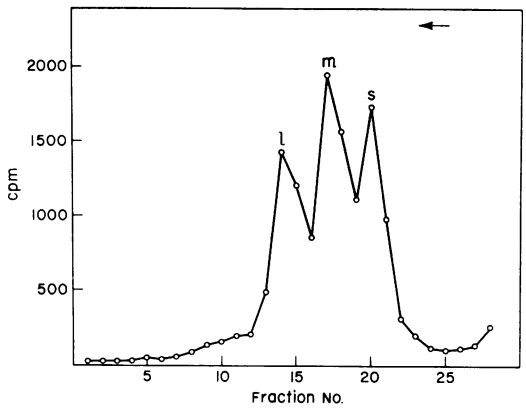

FIG. 7. *Sedimentation profile of RNA products made in vitro by subviral particles. RNA synthesized in vitro by infectious subviral particles was sedimented in a 5 to 30% glycerol gradient as described in Materials and Methods, and the resulting fractions were precipitated in 5% trichloroacetic acid and counted.*

change which renders μ_2 accessible to proteases. The presence of NaCl may protect particles against loss of infectivity by preventing aggregation below a certain concentration of virus. Alternatively, NaCl may directly affect the chymotrypsin since its activity is known to be influenced by various ions, i.e., inhibited by heavy metals and enhanced in buffers containing Ca^{2+} (22).

The RNA of purified reovirus includes both the double-stranded genome segments and a mixture of single-stranded RNA oligomers which are rich in adenine (3–5, 15; C. M. Stoltzfus and A. K. Banerjee, Arch. Biochem., Biophys., *in press*). In contrast to previous findings (9), the cell-associated and released virus contain similar amounts of the adenine-rich RNA, and this is reflected in their base compositions (Table 1). The single-stranded RNA is completely released during conversion of virions to core particles by chymotrypsin digestion (2). Most of the single-stranded RNA is also released from infectious subviral particles, a finding consistent with the report that adenine-rich RNA is not required for infectivity (9). However, about 40% of the adenine-rich RNA remains associated with digested infectious particles even after further purification by banding in glycerol gradients (Fig. 2, Table 1). Since the specific infectivity also decreases by twofold under conditions of chymotrypsin digestion which release about 60% of the adenine-rich RNA, the possibility cannot be excluded from these results that the single-stranded oligomers have a role in initiating virus replication and are required for infectivity.

Recently subviral particles which are produced by the partial uncoating of parental virions have been isolated from infected cells (7, 18). Like the subviral particles prepared in vitro by proteolytic digestion in the presence of 0.15 M NaCl, they have a buoyant density in CsCl intermediate between that of virions and core particles. The partially uncoated particles and in vitro SVP_i also are similar in size (diameter = 66.0 ± 2.0 nm) and morphology (M. Boublik, *unpublished results*; reference 18). Both the partially uncoated particles from cells and the in vitro subviral particles contain the double-stranded genome segments, a reduced amount of adenine-rich RNA, core polypeptides λ_1, λ_2, and σ_2, about 60,000 daltons of μ_2, and a highly active RNA polymerase. The specific infectivity of particles from infected cells has not been reported, but their similarity to the in vitro particles suggests that they be infectious. Presumably early viral mRNA synthesis in infected cells is catalyzed by the partially uncoated virions, but the in vivo pattern of limited transcription was not maintained upon incubation of these particles in vitro. All 10 genome segments were transcribed (18). We have found that the RNA polymerase in chymotrypsin-treated infectious subviral particles also transcribes all 10 genome segments in vitro (Fig. 6C). Cells infected with particles partially uncoated in vitro by chymotrypsin digestion, on the other hand, synthesize early viral mRNA (Fig. 6B). These results indicate that the host cell plays a role in the regulation of reovirus transcription or that intracellular and in vitro conditions of mRNA synthesis are very different, or both. Since cycloheximide was present during infection with subviral particles, they further suggest that preformed host protein(s) may be involved in the control of early viral mRNA synthesis. It will be of interest to study the effect of cell proteins on the in vitro transcription of the genome segments.

ADDENDUM IN PROOF

It was recently reported that SVP from infected cells have one-fifth the specific infectivity of reovirions (C. Astell, S. C. Silverstein, D. H. Levin, and G. Acs. 1972. Virology **48**:648–654).

LITERATURE CITED

1. Banerjee, A. K., U. Rensing, and J. T. August. 1969. Replication of RNA viruses. IX. Isolation of a small self-replicating RNA from the Qβ RNA polymerase. J. Mol. Biol. **45**: 181–193.
2. Banerjee, A. K., and A. J. Shatkin. 1970. Transcription in vitro by reovirus-associated ribonucleic acid-dependent polymerase. J. Virol. **6**:1–11.
3. Bellamy, A. R., and L. V. Hole. 1970. Single-stranded oligonucleotides from reovirus type III. Virology **40**:808–819.
4. Bellamy, A. R., and W. K. Joklik. 1967. Studies on the A-rich RNA of reovirus. Proc. Nat. Acad. Sci. U.S.A. **58**:1389–1395.

5. Bellamy, A. R., L. Shapiro, J. T. August, and W. K. Joklik. 1967. Studies on reovirus RNA. I. Characterization of reovirus genome RNA. J. Mol. Biol. **29**:1–17.
6. Borsa, J., and A. F. Graham. 1968. Reovirus RNA polymerase activity in purified virions. Biochem. Biophys. Res. Commun. **33**: 895–901.
7. Chang, C. T., and H. J. Zweerink. 1971. Fate of parental reovirus in infected cells. Virology **46**:544–555.
8. Gomatos, P. J., and I. Tamm. 1963. The secondary structure of reovirus RNA. Proc. Nat. Acad. Sci. U.S.A. **49**:707–714.
9. Krug, R. M., and P. J. Gomatos. 1969. Absence of adenine-rich ribonucleic acid from purified infectious reovirus 3. J. Virol. **4**:642–650.
10. Levin, D. H., N. Mendelsohn, M. Schonberg, H. Klett, S. Silverstein, A. M. Kapuler, and G. Acs. 1970. Properties of RNA transcriptase in reovirus subviral particles. Proc. Nat. Acad. Sci. U.S.A. **66**:890–897.
11. Loh, P. C., and A. J. Shatkin. 1968. Structural proteins of reovirus. J. Virol. **2**:1353–1359.
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**:265–275.
13. Millward, S., and M. Nonoyama. 1970. Segmented structure of the reovirus genome. Cold Spring Harbor Symp. Quant. Biol. **35**:773–779.
14. Sebring, E. D., and N. P. Salzman. 1964. An improved procedure for measuring the distribution of $P^{32}O_4^{-3}$ among the nucleotides of ribonucleic acid. Anal. Biochem. **8**:126–129.
15. Shatkin, A. J., and J. D. Sipe. 1968. RNA polymerase activity in purified reoviruses. Proc. Nat. Acad. Sci. U.S.A. **61**: 1462–1469.
16. Shatkin, A. J., and J. D. Sipe. 1968. Single-stranded, adenine-rich RNA from purified reoviruses. Proc. Nat. Acad. Sci. U.S.A. **59**:246–253.
17. Shatkin, A. J., J. D. Sipe, and P. Loh. 1968. Separation of ten reovirus genome segments by polyacrylamide gel electrophoresis. J. Virol. **2**:986–991.
18. Silverstein, S. C., C. Astell, D. H. Levin, M. Schonberg, and G. Acs. 1972. The mechanisms of reovirus uncoating and gene activation *in vivo*. Virology **47**:797–806.
19. Skehel, J. J., and W. K. Joklik. 1969. Studies on the *in vitro* transcription of reovirus RNA catalyzed by reovirus cores. Virology **39**:822–831.
20. Smith, R. E., H. J. Zweerink, and W. K. Joklik. 1969. Polypeptide components of virions, top component and cores of reovirus type 3. Virology **39**:791–810.
21. Watanabe, Y., S. Millward, and A. F. Graham. 1968. Regulation of transcription of the reovirus genome. J. Mol. Biol. **36**:107–123.
22. Wilcox, P. E. 1970. Chymotrypsinogens-chymotrypsins, p. 64–108. *In* G. E. Perlmann and L. Lorand (ed.), Methods in enzymology, vol. 19, Academic Press Inc., New York.
23. Zweerink, H. J., and W. K. Joklik. 1970. Studies on the intracellular synthesis of reovirus-specified proteins. Virology **41**: 501–518.