Reovirus-Specific Ribonucleic Acid from Polysomes of Infected L Cells

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Received for publication 31 August 1971

Polysomes from reovirus-infected L cells were analyzed. Although all 10 transcription products of the viral genome were represented in polysomes and presumably can be translated, fewer than 10 reovirus-specific polypeptides were detected in infected cells. The 5'-terminal sequence of all species of reovirus ribonucleic acid (RNA) from polysomes was ppGpYp, as previously found for the genome double-stranded RNA and the *in vitro* single-stranded products of the virion-associated RNA polymerase. RNA isolated from both heavy (>30 ribosomes) and light (5 to 8 ribosomes) polysomes includes similar amounts of large, medium, and small classes of reovirus single-stranded RNA, suggesting that linkage of the genome segments observed in virions may occur at the single-strand stage of RNA replication.

During the reovirus growth cycle in infected L cells, all 10 double-stranded (ds) ribonucleic acid (RNA) segments comprising the viral genome are transcribed into single-stranded (ss) RNA products (20). A portion of the ss RNA associates with ribosomes to form polysomes which actively synthesize viral proteins (21, 24). Only six to seven new proteins have been found in reovirus-infected cells (31). From a comparison of the molecular weights of the viral genome segments and these proteins, it is assumed that they are coded for by 6 or 7 of the 10 ss RNA species acting as monocistronic messengers (28). In an attempt to learn more about the relationship between ss RNA and viral protein synthesis, we analyzed reovirus-infected cells to determine whether all or only a limited number of the genome segments are represented in polysomes. In addition, we compared the 5'-termini of polysome-associated ss RNA with the ends of the genome RNA (A. K. Banerjee and A. J. Shatkin, *J. Mol. Biol.*, *in press*) and ss RNA synthesized *in vitro* by the virion-associated polymerase (2).

MATERIALS AND METHODS

Cells and virus. Mouse L cells were grown in suspension culture in Eagle's medium containing 5% fetal calf serum. For infection, cells were concentrated to 10^7 per ml, and 5 to 15 plaque-forming units (PFU) per cell of reovirus type 3 Dearing strain were added. This time was taken as the start of infection. After adsorption at 37°C for 30 min, the cells were diluted to 10^6 per ml in growth medium containing 0.25 µg of actinomycin per ml.

Preparation of reovirus-directed polysomes. In-

fecting cells were incubated at the indicated times with a radioactive precursor of RNA and then quickly cooled by stirring with one-fourth volume of frozen Earle's balanced salt solution ($1 \times$ BSS) in an ice bath. All subsequent steps were conducted at 4°C. The infected cells were pelleted at $2,000 \times g$ for 5 min, washed three times with $1 \times$ BSS, resuspended in RSB [10 mM tris(hydroxymethyl)aminomethane (Tris), pH 8, 10 mM NaCl, 3 mM MgCl₂] at a final density of 10^8 cells per ml, and lysed by gentle shaking for 10 min with 0.5% NP40 (5). After removal of the unbroken cells and nuclei by centrifugation for 5 min at $500 \times g$, the cytoplasmic fraction (3.0 to 3.5 ml) which contained 70 to 80% of the acid-precipitable radioactivity was layered on a preformed 30-ml 15 to 30% glycerol gradient in RSB. After centrifugation at 25,000 rev/min in an SW-27 rotor for the indicated times, 0.5-ml fractions were collected from the bottom of the gradient. Sodium dodecyl sulfate (SDS) was added (0.05 ml of 10%) to each fraction to inhibit cytoplasmic ribonuclease. Samples of each fraction were then precipitated with 10% trichloroacetic acid, collected on membrane filters (Millipore Corp.), and counted (1).

Analysis of polysomal RNA. Fractions from the polysome gradients were pooled as shown in each figure and extracted with an equal volume of water-saturated, redistilled phenol at 4°C. After alcohol precipitation, the RNA was dissolved in Tris-ethylenediaminetetraacetic acid (EDTA) buffer (10 mM Tris (pH 8), 1 mM EDTA) and layered onto 12-ml 5 to 30% glycerol gradients in 0.1 M NaCl, 20 mM Tris, (pH 8), and 5 mM EDTA. After centrifugation (13 hr, SW-41 rotor, 31,000 rev/min, 4°C), 0.5-ml fractions were collected and assayed for acid-precipitable radioactivity (1).

Reovirus proteins from infected cells. Reovirus-infected and mock-infected L cells were resuspended

in Eagle's medium containing 5% fetal calf serum, 0.25 μg of actinomycin D per ml, and one-fourth the usual concentration of amino acids. Between 10.5 and 12.5 hr after infection, both cultures were incubated with 5 μCi of ^3H -labeled reconstituted protein hydrolysate per ml. The cell suspensions were then quickly chilled, washed once with $1 \times \text{BSS}$, and resuspended in an equal volume of RSB containing 0.5% Nonidet P-40. After lysis, the cytoplasmic fractions were made to 14 mM β -mercaptoethanol (ME) and 4 M urea. The samples were then dialyzed for 3 hr at room temperature against 2 liters of 4 M urea, 0.2% SDS, and 14 mM ME. The dialyzed samples were analyzed by electrophoresis in 10-cm 8% polyacrylamide gels (24 hr, 4.5 ma/gel) in 0.1 M sodium phosphate (pH 7.2) and 0.1% SDS. The gels were frozen, sliced, dissolved in H_2O_2 , and counted in 10 ml of toluene-based scintillation fluid containing 1 ml of Nuclear-Chicago Solubilizer (NCS).

Identification of the reovirus structural proteins in infected cells was determined from the electrophoretic profile of the proteins from radioactive purified virions (1, 28).

Hybridization of polysomal RNA. ^3H -labeled reovirus RNA extracted with phenol from polysome gradients was annealed with an excess of dimethyl sulfoxide-denatured unlabeled RNA from purified virions and analyzed by electrophoresis in 5% polyacrylamide gels as described previously (1). To identify the 10 RNA segments, ^{32}P -labeled ds RNA extracted from purified virus (1) was mixed with the hybridized samples before electrophoresis.

Analysis of the 5'-termini. ^{32}P -labeled RNA extracted from reovirus-directed polysomes was hydrolyzed with pancreatic ribonuclease, and the digestion products were analyzed by diethylaminoethyl (DEAE) cellulose chromatography as described in detail previously (2; Banerjee and Shatkin, *J. Mol. Biol.*, *in press*). Selected column fractions were combined, dialyzed, lyophilized, hydrolyzed with 0.3 N KOH, and rechromatographed on DEAE cellulose.

Reagents. ^3H -guanosine and ^3H -uridine were purchased from New England Nuclear Corp., Boston, Mass., and carrier-free ^{32}P -orthophosphate from E. R. Squibb and Sons, Inc., New York, N.Y. ^3H -adenosine and reconstituted protein hydrolysate (Schwarz mixture) were from Schwarz BioResearch, Orangeburg, N.Y. Pancreatic ribonuclease was obtained from Worthington Biochemical Corp., Freehold, N.J.

RESULTS

Characteristics of reovirus-directed polysomes.

It has been shown previously (14, 23) that RNA synthesis in mouse L cells is inhibited by more than 95% after exposure for 2 hr to low concentrations of actinomycin (0.2 to 0.5 $\mu\text{g}/\text{ml}$). Reovirus type 3 multiplication in these cells is unimpaired. At 11 to 13 hr after infection with 10 PFU/cell, the time of maximal RNA synthesis under these conditions, at least 85% of the RNA produced is virus-specific. At least one-half of the newly formed RNA is in polysomes and presumably functions as messenger RNA

(mRNA). Reovirus-infected, actinomycin-treated cells were incubated with ^3H -uridine from 11.5 to 12.5 hr after infection. The cells were then gently lysed by treatment with Nonidet P-40, and the cytoplasmic fraction was analyzed by centrifugation in a 15 to 30% glycerol gradient as described above. Virus-specific polysomes were separated into a series of radioactive peaks which contained an increasing number of ribosomes (Fig. 1). This pattern is reproducible and independent of the RNA precursor used during the labeling period. However, the radioactivity profile does not correspond to the optical density tracing, especially in the lighter polysome region of the gradient. Since there is a relatively greater contribution of mRNA to the sedimentation rate of the lighter polysomes, the additional peaks of radioactivity may be due to the separation of distinct classes of disomes and trisomes, each containing an l, m, or s molecule of reovirus ss RNA. (1, 20).

Treatment of the cytoplasmic extracts from reovirus-infected cells with 2 μg of pancreatic ribonuclease per ml for 10 min at 0 to 4 C before centrifugation resulted in a loss of 80% of the radioactivity from the polysome region of the gradients. In addition, the fractions obtained from the gradient were treated with 5 μg of pancreatic ribonuclease per ml for 30 min at 37 C in the presence of 0.3 M NaCl, a salt concentration

FIG. 1. Sedimentation pattern of ^3H -uridine-labeled polysomes from reovirus-infected cells. Infected cells (250 ml of $10^6/\text{ml}$) in the presence of 0.25 μg of actinomycin D per ml were pulse-labeled with ^3H -uridine (0.5 $\mu\text{Ci}/\text{ml}$, 26.4 Ci/mmol) 11.5 to 12.5 hr after infection. After lysis of the cells with Nonidet P-40 and centrifugation in a 15 to 30% glycerol gradient (120 min, SW-27, 25,000 rev/min), 0.5-ml fractions were collected and 0.05 ml of each was assayed for acid-precipitable radioactivity. For the optical density determination, 3×10^7 L cells were similarly lysed and centrifuged, and the OD_{260} was measured through a continuous-flow cell in a Gilford spectrophotometer. The 80S position was determined with purified L-cell ribosomes.

which renders ds RNA resistant to hydrolysis by ribonuclease. Less than 5% of the radioactivity remained precipitable in 5% trichloroacetic acid at 0 to 4 C. These results indicate that most of the newly formed RNA in reovirus-directed polysomes is ss RNA.

Transcription of the reovirus genome *in vitro* and *in vivo* results in the production of 10 distinct species of ss RNA. By centrifugation in glycerol density gradients, the ss molecules can be separated into three size classes which include three large (l), three medium (m), and four small (s) RNA species. They have sedimentation values of 25, 18, and 14S, respectively, and correspond to half the molecular weights of the ds genome segments. These three classes of ss RNA are found in reovirus-specific polysomes. Gradient fractions 12 to 19 (region A) in Fig. 1 were combined. Polysomes in this region contain 5 to 8 ribosomes as determined from the optical density profile at 260 nm (OD 260) of purified L-cell ribosomes and cytoplasmic fraction centrifuged in identical gradients. The RNA was isolated from the combined fractions by phenol extraction and ethanol precipitation and analyzed by centrifugation in a 5 to 30% glycerol gradient. Acid-precipitable radioactivity corresponding to the l, m, and s classes of ss RNA is present in a ratio of 1.0:0.8:0.8 (Fig. 2). The chain lengths of the l, m, and s molecules have been estimated to be 3,500, 2,000, and 1,000 nucleotides (2). Assuming that they are of similar specific radioactivity, it can be calculated that for each l molecule there are 1.4 molecules of m and 2.8 molecules of s RNA in polysomes containing 5 to 8 ribosomes. mRNA molecules of the chain lengths of l, m, and s RNA could bind a maximum of about 42, 25, and 12 ribosomes, respectively (10, 15). Therefore, the ss RNA molecules in the polysomes from region A of Fig. 1 are incompletely loaded with ribosomes.

In reovirus-infected cells and in preparations of purified reoviruses, a significant fraction of the viral RNA consists of ribonuclease-resistant, adenine-rich (A-rich) ss ribopolymers (3, 4, 25). The function of the A-rich RNA is unknown although it is apparently not required for infectivity (13). The messenger RNA species of adenovirus (L. Philipson et al., Proc. Nat. Acad. Sci. U.S.A., *in press*) and vaccinia virus (12) contain polyriboadenylic acid, and the rapidly labeled RNA in polysomes of HeLa (6, 7) and mouse sarcoma 180 cells (16) also has covalently bonded, ribonuclease-resistant polynucleotide sequences rich in adenine. It has been suggested that the A-rich sequences may be recognition sites for processing or translation of mRNA. We therefore examined the ribonuclease sensitivity of poly-

FIG. 2. Sedimentation pattern of reovirus-specific ^3H -labeled RNA isolated from polysomes. ^3H -uridine-labeled RNA was extracted with phenol from region A of the gradient shown in Fig. 1. The RNA in 0.1 ml of Tris-EDTA buffer was layered onto a 12-ml 5 to 30% glycerol density gradient. After centrifugation (13 hr, SW-41, 31,000 rev/min), the acid-precipitable radioactivity was measured in 0.5-ml gradient fractions. The positions of the three RNA size classes, l, m and s, were determined from the sedimentation pattern of reovirus mRNA synthesized *in vitro* (1) and centrifuged under the same conditions.

somes from ^3H -adenosine-labeled, actinomycin-treated, reovirus-infected cells. Cells were incubated with ^3H -adenosine (0.8 $\mu\text{Ci/ml}$, specific activity 27.4 Ci/mmol) from 11.5 to 12.5 hr after infection, and the polysomes separated from the cell extract as in Fig. 1. The resulting fractions were treated with pancreatic ribonuclease (20 $\mu\text{g/ml}$, 30 min, 37 C, 0.3 M NaCl), precipitated with 5% trichloroacetic acid at 0 to 4 C, collected on membrane filters (Millipore Corp.), and counted. As observed for ^3H -uridine or ^{32}P -labeled (*see below*) RNA in polysomes, more than 95% of the radioactivity was rendered acid-soluble, indicating that little, if any, of the reovirus A-rich RNA is present in polysomes.

Detection of all 10 ss RNA species in polysomes. Although all 10 reovirus genome segments are transcribed both *in vivo* and *in vitro*, only six or seven new proteins have been identified in infected cells (31). During the time of maximal reovirus RNA synthesis in L cells, we also observed that only a limited number of viral proteins are synthesized. They can be partially resolved by electrophoresis in a polyacrylamide gel (Fig. 3). As reported previously, the pre-

FIG. 3. Polyacrylamide gel electrophoresis pattern of proteins from reovirus-infected cells. L cells (20 ml of 10^6 /ml) in the presence of 0.25 μ g of actinomycin D per ml were incubated with 3 H-labeled reconstituted protein hydrolysate (5 μ Ci/ml) 10.5 to 12.5 hr after reovirus infection. A duplicate sample of mock-infected cells was treated and labeled in the same manner. The proteins from both samples were extracted and analyzed by gel electrophoresis as described in the text. The radioactivity profile of the proteins from uninfected cells was subtracted, and the difference is shown.

dominant newly formed proteins in extracts of pulse-labeled, actinomycin-treated infected cells correspond in molecular weight to the three size classes of viral structural proteins designated λ , μ , and σ according to the terminology of Smith et al. (28). However, the presence of small amounts of other viral proteins may be masked by the background of cellular proteins which is 50% or more of the total labeled proteins, even in cells pretreated for 16 hr with 0.25 μ g of actinomycin per ml to reduce cellular synthesis. It is also possible, in view of the similar molecular weights of mRNA species in each size class, that all viral proteins are not resolved by gel electrophoresis.

As an approach to the question of whether all 10 ss RNA species are translated in vivo, we examined the spectrum of ss RNA molecules present in polysomes of actinomycin-treated infected cells. Infected cells were incubated from 10.5 to 12.5 hr with 3 H-uridine (1.4 μ Ci/ml, specific activity 26.4 Ci/mmole), and the polysomes were combined from region A of a gradient identical to that shown in Fig. 1. After phenol extraction, the RNA was precipitated with alcohol and then annealed with an excess of reovirus genome RNA which had been denatured by treatment with dimethyl sulfoxide. The hybridized RNA was mixed with 32 P-labeled ds genome RNA and analyzed by electrophoresis in a 5% polyacrylamide gel. As shown in the lower pattern of Fig. 4, ss RNA formed duplexes with the L,

FIG. 4. Electrophoretic pattern of 3 H-labeled polyosomal RNA hybridized with genome RNA. Reovirus ss 3 H-RNA was extracted with phenol from region A of a gradient like that in Fig. 1 and annealed with an excess of unlabeled, denatured genome RNA. 32 P-labeled ds RNA from purified reovirus was added as a marker before electrophoresis in 10-cm 5% polyacrylamide gels for 23 hr at 4.5 ma/gel (lower) or 25.5 hr at 4.5 ma/gel followed by 15 hr at 4 ma/gel (upper) to resolve the L and M species of reovirus RNA. The gels were sliced and counted as described in the text.

M, and S classes of ds genome RNA. The four S species of RNA were clearly resolved. By increasing the time of electrophoresis, all L and M segments were also found to be represented in ss RNA from polysomes (Fig. 4, upper pattern). When infected cells were similarly labeled with 3 H-adenosine and the polysome-associated ss RNA was hybridized with ds RNA, all 10 ss RNA species were again detected.

5'-Termini of polysome-associated reovirus RNA. It has been reported that the production of the double-stranded genome of reovirus takes place by a two-step mechanism (22). The first step involves the transcription of one strand of the genome by a conservative mechanism. In step two, the resulting ss RNA (plus strand) acts as template for the formation of ds RNA. Since only one strand of the duplex molecules is copied, i.e., all reovirus-specific ss RNA in infected cells is of the plus type (9), both template and messenger functions apparently can be performed by the plus RNA strand. It is not known, however, whether the same RNA molecule has both activities. It was recently shown that all 10 reovirus genome segments recovered from mature virions have ppGpYp (Y = pyrimidine, X = purine) at the 5'-termini of both strands (Banerjee and Shatkin, J. Mol. Biol., *in press*). The plus or template strand of the double-stranded segments would thus be expected to have a 5'-diphosphate in its ss form. Bacteriophage messenger RNA species contain a triphosphate at the 5'-terminus (18, 19, 29), and it seemed possible that the reovirus ss RNA molecules which served as

messengers might contain 5'-triphosphate in contrast to the 5'-diphosphate of template RNA. We therefore analyzed the 5'-termini of polysome-associated reovirus mRNA. Polysomes from actinomycin-treated, reovirus-infected cells were isolated by glycerol gradient centrifugation (Fig. 5). The RNA in the polysome fractions was labeled with ^{32}P by incubating the cell culture from 8.5 to 11.5 hr after infection with $10\ \mu\text{Ci}$ of ^{32}P -phosphoric acid per ml. Polysomes were divided into five portions as shown in Fig. 5 and extracted with phenol; samples of A, C, and E were sedimented in a 5 to 30% glycerol gradient. The sedimentation profiles of all three samples were similar to the pattern shown for E in the insert of Fig. 5. RNA samples A to E were then combined and treated with pancreatic ribonuclease, and the hydrolysis products were separated by DEAE cellulose column chromatography (Fig. 6A).

Pancreatic ribonuclease has been shown to release from all 5'-termini of the genome RNA

FIG. 5. Sedimentation patterns of ^{32}P -labeled polysomes and reovirus-specific mRNA. L cells ($10^6/\text{ml}$, 1 liter) in Eagle's medium without phosphate containing 5% fetal calf serum and $0.25\ \mu\text{g}$ of actinomycin D per ml incubated with $10\ \text{mCi}$ of ^{32}P -orthophosphate 8.5 to 11.5 hr after infection. The cytoplasmic fraction prepared as described in the text was centrifuged for 120 min at 25,000 rev/min in the SW-27 rotor, and 0.5-ml fractions were collected. Samples of 0.05 ml were precipitated with 10% trichloroacetic acid and counted. (Inset) Fractions in the bracketed region (32 to 40) were combined and phenol-extracted, and the resulting RNA was analyzed for the presence of l, m, and s classes of reovirus ss RNA by centrifugation in a 5 to 30% glycerol gradient (18 hr, SW-41, 26,000 rev/min).

segments the dinucleotide, ppGpYp (Banerjee and Shatkin, J. Mol. Biol., *in press*), which has a net negative charge of -5 to -6 and elutes from DEAE cellulose between $(\text{Xp})_3\text{Yp}$ and $(\text{Xp})_4\text{Yp}$. To determine whether the mRNA segments isolated from polysomes also contain 5'-ppGpYp or similar 5'-terminal sequences, the eluted oligonucleotides of net charge -5 to -6 (Fig. 6A, fractions 42 to 54) were combined and hydrolyzed with $0.3\ \text{N}$ KOH; the hydrolysis products were analyzed on DEAE cellulose. If the 5'-terminal sequence is ppGpYp, the alkaline digestion products will be ppGp and Yp eluting at the positions of net charge -4 to -5 and -2 , respectively. Figure 6B shows that, in addition to the Yp, which elutes with the mononucleotides resulting from ^{32}P -labeled internal oligonucleotides, a second peak of radioactivity elutes between pppG and pppGp. This is consistent with the structure ppGp (Banerjee and Shatkin, J. Mol. Biol., *in press*). Since the approximate sizes of the l, m, and s RNA molecules are known and the relative amounts of each class were determined from centrifugation analyses (Fig. 5, insert), a calculation of the total expected 5'-termini can be made. On the basis of the theoretical value, 98% of the 5'-termini is recovered as ppGp.

To confirm that the purine in the 5'-terminal position of the mRNA segments is G, polysomes were recovered from reovirus-infected cells labeled with ^3H -guanosine from 11.5 to 12.5 hr after infection. The RNA in the combined polysome fractions was again extracted with phenol and digested with pancreatic ribonuclease. These digestion products were separated by DEAE cellulose chromatography as shown for the ^{32}P -labeled polysomal RNA in Fig. 6A; fractions corresponding to 42 through 54 were combined and hydrolyzed with alkali. The hydrolysis products were separated on a DEAE cellulose column, and all of the expected 5'-termini were recovered as ^3H -labeled ppGp (net charge -4 to -5). An additional small peak of radioactivity was observed in the position of pGp and is probably derived from newly formed transfer RNA present in polysomes (11). These findings indicate that the 5'-termini of the ss RNA in polysomes is ppGpYp, i.e., identical to the 5'-ends of the reovirus genome segments (Banerjee and Shatkin, J. Mol. Biol., *in press*), and to ss RNA synthesized *in vitro* by the virion-associated RNA polymerase (2).

Distribution of reovirus mRNA in heavy polysomes. The 10 segments of reovirus mRNA have been shown to be composed of three size classes, l, m, and s. As noted above, a fully loaded s molecule could accommodate a maximum of

FIG. 6. DEAE cellulose chromatography of digests of ^{32}P -labeled polysomal RNA. (A) Polysomal RNA was extracted with phenol from the pooled fractions A to E in Fig. 5. The resulting RNA was mixed with 1 mg of yeast transfer RNA and digested with 100 μg of pancreatic ribonuclease at 37 C for 7 hr (2). The digest was loaded onto a DEAE cellulose column (16 by 1 cm) previously equilibrated with 0.05 M Tris-hydrochloride (pH 8.0), 7 M urea, and 0.05 M NaCl. The oligonucleotides were eluted with a linear gradient of 0.05 to 0.5 M NaCl. Fractions of 2 ml were collected, and a sample (0.2 ml) was counted (2). The OD was continuously monitored at 260 nm to obtain the position of the oligonucleotides, Yp through $(\text{Xp})_5\text{Yp}$. Recovery of ^{32}P was essentially 100%. (B) The fractions comprising 42 to 54 in A were collected, desalted, lyophilized, and hydrolyzed with 0.3 N KOH at 37 C for 18 to 20 hr (2; Banerjee and Shatkin, *J. Mol. Biol.*, in press). The hydrolysate was neutralized with Dowex 50 H^+ resin and rechromatographed as in (A). Samples (1 ml) were counted. Authentic ^3H -pppG and ^{32}P -pppGp were also chromatographed under identical conditions as markers (Banerjee and Shatkin, *J. Mol. Biol.*, in press). Initial counts digested with ribonuclease = 0.6×10^6 counts/min. Counts recovered as ppGp = 864 counts/min. Expected counts/minute as 5'-ppGp = 900 counts/min.

about 12 ribosomes, and m and l molecules could bind as many as 25 and 42 ribosomes, respectively. Consequently, if each ss RNA segment is translated individually as a monocistronic messenger, the s species of RNA would be absent from polysomes containing more than about 12 ribosomes. Similarly, polysomes heavier than 25 ribosomes would contain only 1 RNA. The ss RNA shown in Fig. 2 and 5 was derived from polysomes containing less than 12 ribosomes and would be expected to contain all three classes of reovirus

FIG. 7. Sedimentation patterns of heavy polysomes from reovirus-infected cells. L cells ($10^6/\text{ml}$, 500 ml) were incubated with ^3H -uridine (1 $\mu\text{Ci}/\text{ml}$, 26.4 Ci/mole) 11 to 12.5 hr after infection. The cell lysate prepared by Nonidet P-40 treatment was equally divided, and one of the resulting samples was brought to 0.3% sodium deoxycholate. After 10 min at 4 C, the samples were centrifuged in 15 to 30% glycerol gradients for 40 min (SW-27, 25,000 rev/min). Fractions of 0.5 ml were collected, and 0.05-ml samples were counted after precipitation with 10% trichloroacetic acid. A second 0.05-ml sample of the fractions from the deoxycholate-treated lysate was diluted in 2 ml of Tris-EDTA buffer containing 0.3 M NaCl and digested with 20 μg of ribonuclease per ml at 37 C for 30 min, and acid-precipitable radioactivity was measured. The positions of purified reovirus (630S) and ribosomes (80S) were determined under identical conditions. Fractions were combined from regions 1 to 4, extracted with phenol, and analyzed for l, m, and s RNA content by gradient centrifugation as in Fig. 2; the results are shown in Table 1. Deoxycholate-treated sample ($\square$).

mRNA. It was, therefore, of interest to analyze the ss RNA from heavier polysomes.

The cytoplasmic fraction from actinomycin-treated L cells labeled with ^3H -uridine 11 to 12.5 hr after reovirus infection was centrifuged in a density gradient identical to that shown in Fig. 1. However, the time of centrifugation was reduced threefold to 40 min. As shown in Fig. 7, this centrifugation time permits the separation of heavier polysomes from both virions (630S) and lighter polysomes. The fast sedimenting polysomes apparently are not membrane-bound since there is little change in the sedimentation profile when the cytoplasmic fraction is treated with 0.3% sodium deoxycholate prior to centrifugation (Fig. 7).

The fractions containing heavy polysomes were combined to obtain samples 1 to 4 (Fig. 7). The RNA in each sample was then extracted with phenol and precipitated with alcohol. Since reovirus ds RNA has a sedimentation value similar to s molecules of ss RNA (14S), a portion of each sample was tested for ribonuclease resistance before determining the relative amounts of l, m, and s RNA by sedimentation analysis. The ribonuclease resistance of the radioactive RNA in sample 4 was 11% (Table 1), presumably due to the presence of virions or virus precursors in this region of the gradient; this fraction was not studied further. In contrast, only 2% or less of the ^3H -labeled RNA in samples 1 to 3 remained acid-precipitable after ribonuclease treatment, indicating that these fractions were essentially free of radioactive ds RNA. Accordingly, the l, m, and s molecules in these fractions were separated by gradient centrifugation as described for Fig. 2. All three samples derived from heavy polysomes were found to contain essentially the same relative percentages of each of the three classes of reovirus mRNA (Table 1). Similar values were obtained for RNA extracted from sodium deoxycholate-treated polysomes (Fig. 7).

The approximate *S* values of pooled samples 1 to 3 (Fig. 7) were calculated from the positions of single ribosomes (80S) and purified reovirus (630S) (8) and are shown in Table 1. It has been estimated that polysomes of animal cells which sediment at 200S and 300S contain 7 and 23 ribosomes, respectively (15). Since the polysomes from sample 2 (320 to 360S) contain at least 20 ribosomes, no monocistronic s molecules would be expected to sediment in this region of the gradient. Similarly, polysomes derived from sample 3 (380 to 440S) contain more than 30 ribosomes and should, therefore, have no monocistronic m or s species of mRNA. Since the

TABLE 1. *Distribution of single-stranded RNA in heavy polysomes^a*

Sample	$\sim S$ value	Per cent ribonuclease resistant	Relative per cent		
			l	m	s
1	240	1	32	35	33
2	340	1	37	30	33
3	410	2	34	34	32
4	480	11			

^a RNA from regions 1 to 4 of the gradient (Fig. 7) was tested for ribonuclease resistance as described in the legend to that figure. The relative per cent of l, m, and s was determined by gradient analysis, and the fractions included for each size class are as shown in Fig. 2. The approximate sedimentation values are based on the positions obtained for purified reovirus (630S) and ribosomes (80S).

heavy polysomes contain both m and s species (Table 1), mRNA molecules, in addition to binding ribosomes, must be linked to each other or to other components in the infected cells. For example, the fast-sedimenting polysomes may consist of subviral particles (26) containing both nascent mRNA segments and ribosomes. Under these conditions, transcription and translation would be coordinate processes. This model would require that the ds genome RNA in the subviral particles be made during an earlier labeling period since the ^3H -RNA derived from the heavy polysomes is almost entirely ribonuclease-sensitive (Fig. 7). The 10 ds genome segments apparently are linked within purified virions (30), and our results suggest that the viral RNA is already linked during the ss phase of replication.

DISCUSSION

When the reovirus genome is transcribed in infected cells or in vitro by the virion-associated RNA polymerase, ss RNA products are synthesized by a conservative mechanism (1, 17, 27) from the 10 unique segments comprising the viral genome. These transcription products fall into three size classes of RNA. They have molecular weights which are consistent with their being monocistronic messengers for the three classes of new polypeptides present both in reovirus-infected cells and in purified virions. However, in infected cells only six to seven viral polypeptides have been found, and their relative amounts are not correlated with the observed quantities of their corresponding mRNA species.

We have examined the ss RNA in reovirus-

specific polysomes and found that all 10 genome segments are represented. Thus, the failure to detect 10 viral proteins in infected cells is apparently not due to an inability of certain ss RNA species to bind to ribosomes to form polysomes. Since the efficiency of duplex formation by complementary strands of RNA decreases with increasing molecular weight, it is not possible to compute from the results in Fig. 4 the absolute amount of each species of mRNA which is in polysomes. However, the relative quantity of each species of ss RNA within any one size class synthesized between 11.5 and 12.5 hr after infection does not differ by more than two or three-fold. Furthermore, the l, m, and s classes of ss RNA are present in polysomes containing as many as 30 ribosomes in almost equal amounts (Fig. 2 and Table 1). In contrast to the ss RNA, virus-specific proteins are made in markedly unequal amounts during the same labeling period after infection (Fig. 3). The medium-sized polypeptides (μ class) comprise more than 55% of the newly formed proteins. Thus the efficiency of read-out among the polysome-associated l, m, and s classes of ss RNA apparently is highest for the m molecules. These results are consistent with regulation of protein synthesis taking place at the level of translation.

Several groups have reported that some mRNA species isolated from animal cells are covalently bonded to long sequences of adenine. It was suggested (6) that addition of the adenine residues to the termini of mRNA may provide a recognition mechanism for regulation of mRNA processing and for translation. Almost 30% of the RNA in infectious reovirus particles is composed of short sequences of ss, A-rich RNA. It is possible that the reovirus ss RNA which functions as messenger also may contain A-rich sequences at some point during the infectious cycle. However, few, if any, A-rich sequences are present in polysome-associated reovirus ss RNA synthesized 11.5 to 12.5 hr after infection, a time of maximal RNA production. In addition, the 5'-termini of the ss RNA species are all ppGpYp as reported previously for ds RNA and ss RNA formed *in vitro* (2; Banerjee and Shatkin, *J. Mol. Biol.*, *in press*). Since the viral genome segments and the ss RNA in polysomes have the same 5'-terminal dinucleotide, this ss RNA may serve both as messenger for protein synthesis and as template for ds RNA synthesis.

Although the genome of reovirus is segmented, the segments are probably linked within the virion (30). The necessity for correctly assembling 10 different molecules of ds RNA during virus maturation would be circumvented if the parental genome remained linked throughout replication.

It has been reported that during the infectious cycle the parental reovirus genome is conserved within the subviral particle or "core" (22). If the ss RNA molecules are precursors of the genome segments, linkage could occur at the ss phase of RNA replication. The presence of similar quantities of l, m, and s classes of reovirus mRNA sedimenting with polysomes containing 5 to more than 30 ribosomes is consistent with this possibility. Linkage between monocistronic mRNA species in polysomes may serve to regulate their translation and could account for the lack of correlation between the quantities of l, m, and s RNA molecules and their corresponding polypeptides. We are investigating further the basis for the presence of s and m RNA species in heavy polysomes.

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