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Reovirus Progeny Subviral Particles Synthesize Uncapped mRNA

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Reovirus progeny subviral particles were isolated from L-cells at late times postinfection. It has been shown (D. Skup and S. Millward, J. Virol. **34:** 490–496, 1980) that these progeny subviral particles have masked capping enzymes, indicating that mRNA synthesized by these particles should be uncapped. When progeny subviral particles were used for mRNA synthesis in vitro, they failed to incorporate the β-phosphate of [β-^{32}P]GTP into the 5′ terminal. Direct analysis of reovirus mRNA synthesized by progeny subviral particles in the presence of either [α-^{32}P]GTP or [α-^{32}P]CTP indicated that the 5′ terminal was uncapped, having the structure pGpC... The implications of this finding to the reovirus replicative cycle are discussed.

The capsid of reovirions is composed of an outer protein shell and an inner ribonucleoprotein core, which contains the genomic segments of double-stranded RNA (dsRNA) (13, 19, 21, 28). Treatment of reovirions with chymotrypsin removes the outer capsid, leaving intact cores. Among the enzyme activities associated with core particles are an RNA polymerase (27, 31) and the capping activities, consisting of a guanyl transferase (9, 38) and an RNA methylase (5, 26). The enzymes associated with cores synthesize mRNA in vitro that terminates at the 5′ end with the capped structure, m7G(5′)ppp(5′)-G^{m}pCpUpAp ... (4, 7, 14).

During reovirus infection, two distinct populations of subviral particles (SVPs) are responsible for transcription of the 10 dsRNA genome segments. The first of these populations consists of parental SVPs, which result from the uncoating of viral particles in the cell (2, 29). Several lines of evidence indicate that capped structures may also be found at the 5′ end of mRNA synthesized by parental SVPs (3, 12). Parental SVPs isolated from infected cells incorporate the methyl group of *S*-adenosyl methionine into reovirus mRNA (12). Furthermore, capped reovirus mRNA can be isolated from infected cells labeled with [*methyl*-^{3}H]methionine from early times postinfection (3).

mRNA's transcribed from parental SVPs are translated into viral polypeptides at early times postinfection, initiating the synthesis of progeny SVPs. At later times postinfection, the bulk of the mRNA present in the infected cell is transcribed from progeny SVPs. In the accompanying paper (34), we reported that these progeny SVPs have masked capping enzymes. This finding suggested that mRNA synthesized by progeny SVPs is uncapped. In the present report we show that reovirus progeny SVPs synthesize uncapped mRNA and that this mRNA terminates at the 5′ end with pGpC...

MATERIALS AND METHODS

Cells, reovirus, and reovirus cores. Reovirus type 3 (Dearing strain) was propagated in suspension cultures of mouse fibroblasts (L-cells), extracted with freon as previously described by Smith et al. (35), and purified by repeated isopycnic banding in CsCl density gradients (36). Reovirus cores were prepared by digestion of virions with α-chymotrypsin (Mann Research Laboratories) as described previously (17).

Preparation of progeny SVPs from L-cells infected with reovirus. Progeny SVPs were isolated from L-cells infected with reovirus according to the procedure in the accompanying paper (34). Briefly, a large-particle fraction containing a mixture of mature virions, parental SVPs, and progeny SVPs was isolated at late times postinfection and fractionated on a glycerol gradient. Progeny SVPs, which are present in the slow-sedimenting region of the gradient, were recovered by pelleting and suspended in 0.1 M Tris-hydrochloride (pH 8.0) for use in standard transcriptase reactions (see below).

Synthesis and purification of reovirus mRNA. Viral mRNA was synthesized in vitro as previously described (5). Reaction mixtures for the synthesis of reovirus mRNA contained either viral cores or progeny SVPs at a final concentration of 2 to 3 absorbancy units at 260 nm per ml, 2 mM each of ATP, CTP, GTP, and UTP, 9 mM $MgCl_2$, 0.1 M Tris-hydrochloride (pH 8.0), 10 μM *S*-adenosyl methionine, 0.5 mg of bentonite per ml, 2 μg of actinomycin D per ml, 20 mM phosphoenolpyruvate, and 80 μg of rabbit muscle pyruvate kinase (Sigma Chemical Co.) per ml. RNA terminating at the 5′ end with ppGp ... was synthesized by reovirus cores in a standard transcriptase reaction with the following modifications: *S*-adenosyl methionine was not added, and the reaction contained 250 μM *S*-adenosyl homocysteine and 0.5 mM inor-

ganic sodium pyrophosphate (10). When radioactive precursors were used, the concentration of the corresponding nucleoside triphosphate was lowered to 0.4 mM. [β-^{32}P]GTP was prepared according to the method of Furuichi and Shatkin (11) from commercial [γ-^{32}P]ATP (New England Nuclear Corp.). At the end of the incubation period, aggregated SVPs were removed by centrifugation at 2,000 rpm for 10 min in an IEC clinical centrifuge. Reovirus mRNA synthesized in vitro was extracted with phenol, partially purified by precipitation in high salt (32), and then suspended in buffer containing 0.3 M NaCl, 50 mM Tris-hydrochloride (pH 8.0), 10 mM EDTA, and 1.0 M urea for further purification by velocity sedimentation in a sucrose density gradient. The solution was first heated at 70°C for 3 min and chilled quickly to reduce aggregation, and then it was layered onto 15 to 30% (wt/vol) sucrose density gradients prepared in STES buffer (0.3 M NaCl, 50 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA, and 0.5% sodium dodecyl sulfate) and centrifuged in an SW27 rotor at 25,000 rpm for 18 h. Gradients were collected, and radioactivity was determined in an Intertechnique liquid scintillation counter. mRNA was recovered from the sucrose gradients by precipitating the pooled fractions with ethanol overnight at −20°C.

Hybridization of mRNA to reovirus dsRNA. The procedure was similar to that described previously (14). Reovirus dsRNA was extracted from virions with phenol and purified by gel filtration on Sephadex G-100 (15). A 10-fold excess of dsRNA was added to labeled mRNA in 50 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA. Nine volumes of dimethyl sulfoxide was added with mixing, and the solution was incubated at 45°C for 30 min. NaCl was added to a final concentration of 0.3 M, and the denatured RNA was precipitated by the addition of 5 volumes of cold ethanol and left overnight at −20°C. The precipitate was collected by centrifugation, dried in air, and suspended in sufficient 0.3 M NaCl–50 mM Tris (pH 8.0)–1 mM EDTA to give a final RNA concentration of 200 μg/ml. Annealing was allowed to proceed for 6 h at 72°C, whereupon the RNA was again precipitated with ethanol. The precipitated RNA was redissolved to a final concentration of 2 mg/ml in 50 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA. Unlabeled reovirus mRNA (200 μg/ml) was added (as carrier), and the solution was made 1 M with respect to LiCl. After incubation overnight at 4°C, the suspension was clarified by centrifugation, and the supernatant fraction was diluted threefold with water before precipitation of the dsRNA with ethanol.

Labeling the 5′ terminal of mRNA using polynucleotide kinase (20). Purified mRNA was treated with bacterial alkaline phosphatase (100 μg/ml) in 50 mM Tris-hydrochloride (pH 8.0). Alkaline phosphatase was removed by extracting the digest with phenol, followed by precipitation with ethanol. The resulting precipitate was dissolved in a final volume of 125 μl containing: 70 mM Tris-hydrochloride (pH 8.0), 10 mM mercaptoethanol, 10 mM $MgCl_2$, 1 mM spermidine, 0.4 mM ATP (containing [γ-^{32}P]ATP to a final specific activity of about 1,000 Ci/mmol), and 5 U of polynucleotide kinase (Boehringer Mannheim). Each reaction was incubated for 1 h at 37°C and was then made 1 mM in EDTA and 1% in sodium dodecyl sulfate; finally, an equal volume of 10 mM ATP was added. The reaction mixtures were then put into collodion bags (no. 100, Schleicher and Schuell), dialyzed against 50 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA for 36 h to remove unreacted [γ-^{32}P]ATP, and then concentrated into a small volume by vacuum dialysis in the same collodion bags.

Determination of the nucleotide labeled by polynucleotide kinase. RNA labeled with polynucleotide kinase was hybridized to reovirus dsRNA according to the procedure described above. Purified, hybrid RNA was boiled for 3 min and quickly chilled to effect denaturation. The resulting single-stranded reovirus RNA was then digested for 2 h (37°C) with *Penicillium* nuclease (Calbiochem) at a final concentration of 400 μg/ml in 20 mM sodium acetate buffer (pH 6.0). After 2 h, the RNA was again subjected to denaturating conditions (as above), and another 400 μg of *Penicillium* nuclease per ml was added. The digests were incubated for an additional 1 h at 37°C and then desalted by loading onto a DEAE-cellulose (triethylammonium bicarbonate form) column (Whatman DE-11) and washing with 5 mM triethylammonium bicarbonate at pH 8.0. The labeled products were eluted from the columns with 1.3 M triethylammonium bicarbonate (pH 8.0) and evaporated to dryness under vacuum at 45°C. The labeled nucleoside monophosphates were suspended in water and chromatographed on PEI-cellulose thin-layer plates (Polygram CEL 300 PEI, Machery-Nagel) developed with 1 M acetic acid–3 M LiCl (9:1, vol/vol) (23). Unlabeled marker nucleoside monophosphates were detected under UV light, and labeled nucleoside monophosphates were located by radioautography using Kodak X-Omat-R1 film (Picker X-Ray Engineering Ltd.).

Analysis of the 5′-terminal structure of mRNA synthesized in vitro by progeny SVPs. mRNA was synthesized in the standard transcriptase reaction using progeny SVPs. The mRNA was labeled uniformly with ^{32}P using either [α-^{32}P]GTP or [α-^{32}P]CTP (New England Nuclear Corp.). The mRNA was extracted from the reaction mixture, purified, and hybridized to reovirus dsRNA as described above. After annealing, the hybrid RNA was precipitated from ethanol at −20°C overnight to remove residual salt. The hybrid RNA was recovered by centrifugation and suspended in 200 μl of 50 mM sodium acetate (pH 4.5) containing approximately 10^5 cpm of *methyl*-^{3}H-labeled mRNA to provide markers for cap structures in the subsequent analysis. The hybrid RNA was denatured by heating as described above and digested at 37°C with RNase T_2 (Sankyo) at a final concentration of 80 U/ml. After 6 h of incubation, the digest was subjected again to the same denaturing conditions; an additional 80 U of RNase T_2 per ml was added, and incubation was continued for another 12 h to ensure complete digestion.

The products of digestion were then analyzed by DEAE-cellulose column chromatography (Whatman DE-11) in 50 mM Tris-hydrochloride (pH 8.0) and 7 M urea (5). A sample of wheat germ rRNA, previously

hydrolyzed with pancreatic RNase A (Worthington Biochemical Corp.), was added as chain length markers. The columns were eluted with linear gradients of NaCl, and fractions of 5.5 ml each were collected. The optical density of the effluent was monitored constantly at 254 nm with an LKB 8303 A Uvicord II. Radioactivity in each fraction was determined as described above.

Fractions eluting from the column in the region of the −4 charge marker were pooled and desalted on DEAE-cellulose (triethylammonium bicarbonate form) columns. The labeled products were eluted with 1.3 M triethylammonium bicarbonate and evaporated to dryness under vacuum at 45°C. The dried samples were suspended in 10 μl of water, spotted on PEI-cellulose thin-layer plates, and chromatographed in 0.75 M potassium phosphate buffer (pH 3.5) (1). Unlabeled nucleoside diphosphate markers were located under UV light, and ^{32}P-labeled products were detected by radioautography.

Standard system for in vitro protein synthesis. Preparation of S-10 extracts and treatment of these extracts with micrococcal nuclease have been described elsewhere (32). In vitro protein synthesis was carried out in a final volume of 50 μl containing the following components: 30 μl of nuclease-treated S-10 extract, 1 mM fructose 3,6-diphosphate, 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.5), 90 mM potassium acetate (unless otherwise specified), 2.5 mM magnesium acetate, 1 mM dithiothreitol, 0.5 mM GTP, 5 μM of each amino acid in a mix minus methionine, 400 μCi of [^{35}S]methionine (New England Nuclear Corp.; specific activity, 700 to 800 Ci/mmol) per ml, reovirus mRNA, and water or other additions to a final volume of 50 μl. Incubation was carried out at 30°C for 60 min. At the end of the incubation, EDTA and RNase A (Worthington) were added to the reaction mixtures to a final concentration of 10 mM and 0.4 mg/ml, respectively. Samples were then incubated for 30 min at 37°C. At this point, 5-μl aliquots were removed, spotted on Whatman 3MM filter paper disks, and washed for 10 min in ice-cold 10% trichloroacetic acid containing unlabeled methionine. The filters were transferred to hot (90°C) 5% trichloroacetic acid for another 10 min, then washed twice in ice-cold 5% trichloroacetic acid, once in ethanol, once in ethanol-ether (3:1), and finally in ether alone. Filters were dried and placed in 5 ml of toluene-base scintillation fluid, and the radioactivity was determined in an Intertechnique SL30 liquid scintillation spectrometer.

The remaining portion of the incubation mixtures was made 10% to trichloroacetic acid and 3% to Casamino Acids and placed on ice for 10 min. The pellet obtained by centrifugation was suspended in 5% trichloroacetic acid and 3% Casamino Acids, heated at 90°C for 10 min, centrifuged again, washed three times with 5% trichloroacetic acid containing Casamino Acids and once with acetone, dried, and dissolved in sample buffer for analysis by polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out according to Laemmli (18). All S-10 extracts from infected cells were prepared from L-cells that had been infected with reovirus at a multiplicity of 5 PFU/cell and incubated for 30 h at 31°C. Under these conditions, the infected cells showed no signs of virus-induced cytopathic effects, and over 95% of the cells excluded trypan blue stain.

RESULTS

Velocity sedimentation analysis of mRNA synthesized by progeny SVPs. In view of the finding that progeny SVPs possess masked guanyl transferase and mRNA methylase activities (34), it became important to analyze the mRNA made by these particles. mRNA was synthesized in vitro using both progeny SVPs and progeny SVPs pretreated with chymotrypsin, since the latter treatment was shown to unmask the capping enzymes (34). The standard transcriptase reactions were carried out using [β-^{32}P]GTP to label specifically the 5′ terminal and [^{3}H]CTP as an internal label for the mRNA chain. The labeled mRNA was purified and analyzed by velocity sedimentation in sucrose density gradients. Figure 1 illustrates the sedimentation pattern obtained with mRNA synthesized by progeny SVPs before and after pretreatment with chymotrypsin (Fig. 1a and b, respectively). In both cases, the internal [^{3}H]-CMP label gave a pattern of three size classes to be expected for reovirus mRNA. The distribution of the ^{32}P label from [β-^{32}P]GTP, however, was very different in the two cases.

Progeny SVPs in their native state incorporated [^{3}H]CMP but failed to incorporate significant amounts of ^{32}P from [β-^{32}P]GTP in the region of the gradient corresponding to reovirus mRNA (Fig. 1a). This result is in direct contrast to that obtained for the mRNA synthesized by progeny SVPs pretreated with chymotrypsin (Fig. 1b). Here the profile of the ^{32}P label paralleled that of the internal ^{3}H label, indicating that the β-phosphate of GTP was incorporated into the 5′ termini of the mRNA. As a control, mRNA was synthesized, using reovirus cores derived by chymotrypsin treatment of whole virions, under conditions known to give mRNA terminated at the 5′ end in ppGp . . . (10). The velocity sedimentation pattern of this mRNA preparation is shown in Fig. 1c. As expected, the ^{32}P label from [β-^{32}P]GTP paralleled the ^{3}H label from [^{3}H]CTP.

We conclude from these results that the mRNA synthesized in vitro by native progeny SVPs is composed of the normal three size classes of mRNA that are synthesized by virions treated with chymotrypsin. The principal difference between these two populations of mRNA clearly lies in the structure of their 5′ termini.

Identification of the 5′-terminal nucleotide labeled by polynucleotide kinase. To

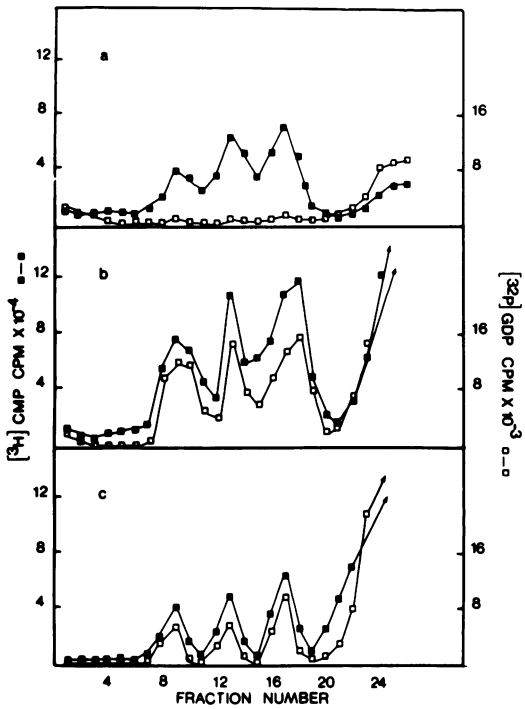

FIG. 1. *Sucrose density gradient sedimentation of reovirus mRNA labeled with [β-^{32}P]GDP and [^{3}H]-CMP. Reovirus mRNA was synthesized in a standard transcriptase reaction containing either progeny SVPs (a) or progeny SVPs pretreated with chymotrypsin (b). Uncapped mRNA was synthesized by reovirus cores in a reaction mixture containing S-adenosyl homocysteine and pyrophosphate (c). Purified mRNAs were sedimented in 15 to 30% sucrose density gradients. Sedimentation was from right to left.* (□) ^{32}*P Cerenkov radiation;* (■) ^{3}*H radioactivity.*

obtain more information on the 5′-terminal structure, we attempted to label mRNA synthesized by progeny SVPs in the polynucleotide kinase reaction. Both capped and uncapped mRNA's synthesized by reovirus cores were used as controls in the polynucleotide kinase reaction. Capped reovirus mRNA preparations could not be labeled using polynucleotide kinase either before or after treatment with alkaline phosphatase. This result suggested that these mRNA preparations did not contain detectable amounts of dsRNA. This was an important control because the minus strand of the dsRNA has the 5′-terminal structure ppGp . . . , which might serve as a substrate in the polynucleotide kinase reaction after treatment with alkaline phosphatase. Furthermore, this inability to label capped mRNA after treatment with alkaline phosphatase shows that the enzymes used in these experiments were free of the type of nuclease contaminant that might have exposed new 5′OH groups on G residues. As expected, ppGp . . . -terminated RNA could not be labeled with polynucleotide kinase unless the 5′ phosphates were first removed by phosphatase treatment. The 5′ nucleotide (HO-Gp . . .) could then be labeled with polynucleotide kinase using [γ-^{32}P]ATP (20). By this criterion, the mRNA synthesized by progeny SVPs was found to have at least one phosphate at the 5′ terminal, since the 5′ end of this mRNA could not be labeled in the polynucleotide kinase reaction unless the mRNA was first treated similarly with phosphatase. Accordingly, mRNA synthesized in vitro by progeny SVPs was labeled in the polynucleotide kinase reaction, then purified again by hybridization to reovirus dsRNA. The hybrid RNA was denatured by heating and reduced to nucleoside monophosphates with *Penicillium* nuclease. The resulting nucleoside monophosphates were analyzed by chromatography on PEI-cellulose thin-layer plates, followed by radioautography. All the ^{32}P radioactivity incorporated during the polynucleotide kinase reaction was associated with a single nucleoside monophosphate (Fig. 2). As expected, the nucleotide that was labeled using phosphatase-treated, ppGp . . . -terminated mRNA was pG (Fig. 2, lane A). Similarly, the only radiolabeled nucleoside monophosphate derived from progeny SVP mRNA was also pG (Fig. 2, lane B). This finding indicates that the 5′-terminal nucleoside of mRNA synthesized by progeny SVPs is guanosine. (Labeled material remaining at the origin was shown to be residual, unreacted nucleoside triphosphate.)

Direct analysis of the 5′ end of mRNA synthesized by progeny SVPs. The results presented so far on the analysis of mRNA synthesized by progeny SVPs can be summarized as follows: (i) the mRNA has a 5′-terminal structure which does not include the β-phosphate of GTP, (ii) the 5′-terminal structure contains at least one phosphate, (iii) the 5′-terminal nucleoside is guanosine. Taken together, the above findings imply that the most probable 5′-terminal structure of mRNA synthesized by progeny SVPs is pGp. . . This structure was confirmed by the following experiments. mRNA was synthesized by progeny SVPs in a standard transcriptase reaction, using either [α-^{32}P]GTP or [α-^{32}P]CTP as the labeled nucleoside triphosphate. Analysis of the labeled mRNA by velocity sedimentation in sucrose showed it to be composed of the expected three size classes (data not shown). The purified ^{32}P-labeled mRNA was mixed with [*methyl*-^{3}H]mRNA to provide cap markers, and then the mixture was hydrolyzed exhaustively with RNase T_2. Each digest was

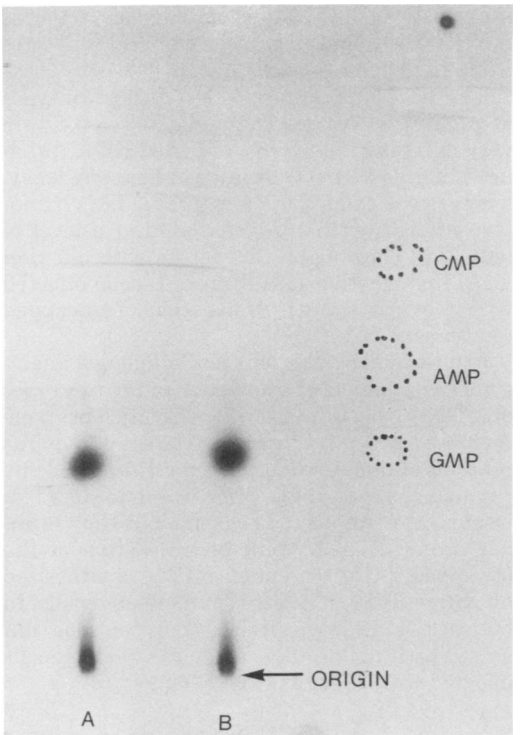

FIG. 2. *Nucleotide analysis of reovirus mRNA labeled by polynucleotide kinase with [γ-^{32}P]ATP. Reovirus mRNA was treated with alkaline phosphatase and subsequently labeled at the 5′ terminal with polynucleotide kinase. The labeled mRNA was digested with Penicillium nuclease, and the digest was analyzed by chromatography on PEI-cellulose thin-layer plates, followed by radioautography. (A) Analysis of ppGp . . . -terminated mRNA. (B) Analysis of mRNA synthesized by progeny SVPs. The dotted outlines show the positions of unlabeled nucleoside 5′-monophosphate markers.*

analyzed by DEAE-cellulose column chromatography in 7 M urea. The elution profiles for [α-^{32}P]GMP- and [α-^{32}P]CMP-labeled mRNA digests are presented in Fig. 3 and 4, respectively. In neither case did ^{32}P radioactivity elute in the position of the *methyl*-^{3}H-cap structures (type 0 and type 1), indicating that the mRNA is uncapped. Material eluting just before the −3 charge marker was found in both profiles. This material is of a charge not associated with any known 5′-terminal structure and probably represents the presence of linkages in the mRNA resistant to RNase T_2. The nature of this product was not investigated further. In both profiles, a ^{32}P-labeled product eluted in the region of the −4 charge marker. Nucleotides eluting in this region of the gradient are expected to have the structure pNp. When a similar analysis was done using mRNA labeled with [α-^{32}P]UTP or with [α-^{32}P]ATP, no radiolabeled material eluted in the −4 charge region of the chromatogram (data not shown).

The fractions coeluting with the −4 charge marker in each profile (Fig. 3 and 4) were pooled and desalted on DEAE-cellulose. Labeled products were analyzed by chromatography on PEI-cellulose thin-layer plates. Unlabeled nucleoside diphosphate markers were located under UV light, and the labeled RNase T_2 products were detected by radioautography (Fig. 5). The structure eluting at a charge of −4 on a DEAE-cellulose column cochromatographed with GDP, regardless of whether the mRNA was labeled with [α-^{32}P]CMP (lane A) or [α-^{32}P]GMP (lane B). These results clearly indicate that the mRNA synthesized in vitro by progeny SVPs terminates at the 5′ end with pGpC. . .

Analysis of the 5′ termini of mRNA synthesized by progeny SVPs treated with chymotrypsin. Results presented earlier (Fig. 1b) indicated that treatment of progeny SVPs with chymotrypsin altered the enzymatic properties of these particles (34). After treatment with chymotrypsin, the β-phosphate of [β-^{32}P]GTP was incorporated into the 5′ termini of the mRNA synthesized. Furthermore, the ^{32}P

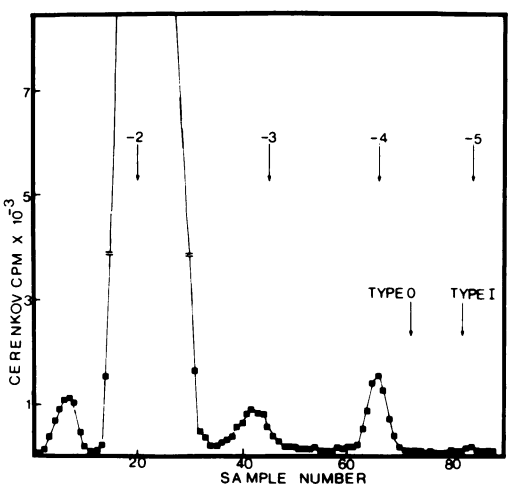

FIG. 3. *DEAE-cellulose column chromatography of RNase T_2 products derived from [α-^{32}P]GMP-labeled mRNA synthesized by progeny SVPs. Purified mRNA was hybridized to reovirus dsRNA, and [methyl-^{3}H]mRNA was added to provide markers for capped structures. The hybrid RNA was denatured and digested exhaustively with RNase T_2. The digest was loaded onto a DEAE-cellulose column and eluted with a linear NaCl gradient (see the text). The upper row of arrows indicates the positions of chain length markers. The lower arrows indicate the elution position of tritiated type 0 and type 1 cap markers.*

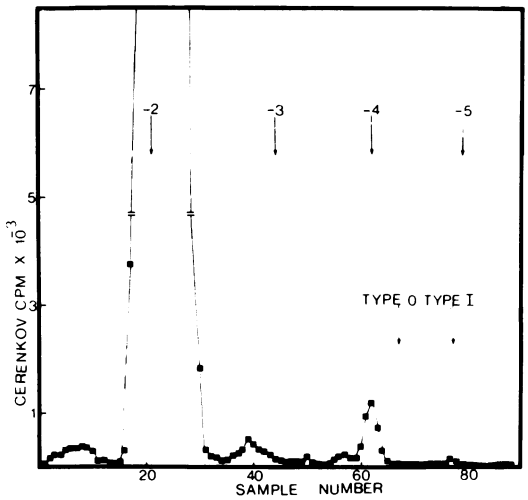

FIG. 4. *DEAE-cellulose column chromatography of RNase T_2 products derived from [α-^{32}P]CMP-labeled mRNA synthesized by progeny SVPs. Labeled mRNA was analyzed as described for Fig. 3. The upper row of arrows indicates the positions of chain length markers. The lower arrows indicate the elution positions for tritiated type 0 and type 1 cap markers.*

label was completely resistant to phosphatase digestion, indicating that the mRNA was probably capped. This was confirmed by the following analysis. mRNA was labeled with [β-^{32}P]-GTP and hybridized to reovirus dsRNA for final purification. The hybrid mRNA was denatured and digested exhaustively with RNase T_2 (along with reovirus [*methyl*-^{3}H]mRNA). The digest was then analyzed by DEAE-cellulose column chromatography in 7 M urea. The elution profile is presented in Fig. 6. Essentially all of the ^{32}P radioactivity from [β-^{32}P]GTP was found to coelute with the tritiated cap markers from [*methyl*-^{3}H]mRNA. These results indicate that progeny SVPs treated with chymotrypsin synthesize capped mRNA. This observation is consistent with the finding that chymotrypsin unmasks the capping enzymes in progeny SVPs as described by Skup and Millward (34).

Translation of the mRNA synthesized by progeny SVPs in cell-free extracts from normal and infected cells. The mRNA synthesized by progeny SVPs and by progeny SVPs digested with chymotrypsin was translated in vitro in lysates prepared from normal and infected L-cells (Table 1). We see that in the lysates from uninfected cells, the mRNA synthesized by chymotrypsin-treated SVPs (capped mRNA, Fig. 6) translated at high efficiency. The pGp ... -ended RNA (Fig. 5) synthesized by native progeny SVPs translated very poorly. This is in agreement with our results reported previously (33), where uncapped reovirus mRNA was found to translate poorly in lysates from uninfected L-cells.

The translational properties of the two types of mRNA in lysates from infected cells were very different. The pGp ... -ended RNA (product of native SVPs) translated at high efficiency, whereas the capped mRNA (product of chymotrypsin-digested SVPs) translated at a level of only 25%. Once again this agrees with our previous findings that lysates from L-cells infected with reovirus support translation of uncapped reovirus mRNA (33).

Figure 7 shows the polyacrylamide gel analysis of the products of translation of the two types of mRNA whose translation was efficient. Lane A shows the typical three size classes of reovirus proteins obtained when capped mRNA made by chymotrypsin-treated SVPs was translated in lysates from uninfected cells. Lane B shows similar products as a result of translation of the uncapped, pGp ... -ended mRNA synthesized by native SVPs in lysates from infected cells. In both cases the synthesized polypeptides migrated with the proteins made in vivo in L-cells infected with reovirus (Fig. 7, lane C).

DISCUSSION

In the accompanying report (34), we assayed progeny SVPs for the enzyme activities known to be associated with reovirus cores. The results

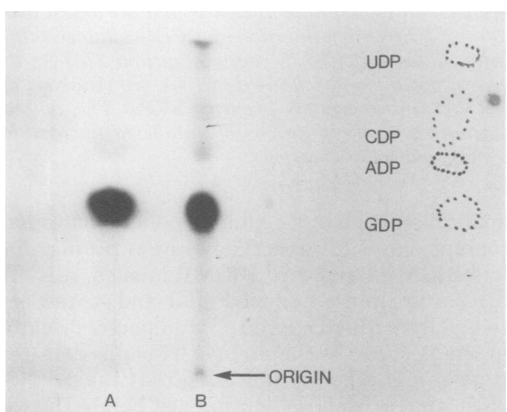

FIG. 5. *Analysis of the 5′-terminal nucleotide of mRNA synthesized by progeny SVPs. RNase T_2 products eluting with the −4 charge markers on DEAE-cellulose columns (Fig. 3 and 4) were pooled and desalted. The labeled products were then analyzed by chromatography on PEI-cellulose thin-layer plates. The labeled products derived from [α-^{32}P]-CMP (A) and [α-^{32}P]GMP (B) were detected by radioautography. The dotted outlines indicate the positions of unlabeled nucleoside 5′-diphosphate markers.*

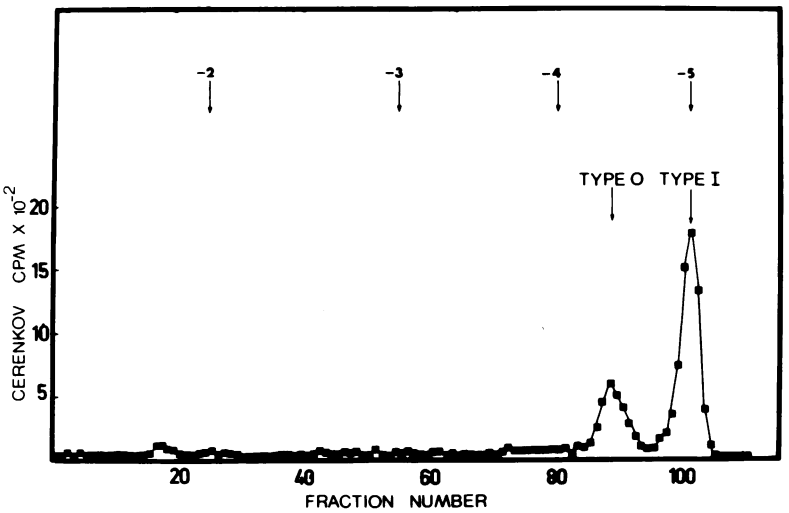

FIG. 6. *DEAE-cellulose chromatography of RNase T_2 products derived from [β-^{32}P]GDP-labeled mRNA synthesized by progeny SVPs treated with chymotrypsin. Labeled mRNA was synthesized in a standard transcriptase reaction containing [β-^{32}P]GTP. The mRNA was hybridized to reovirus dsRNA and analyzed as described for Fig. 3. The upper row of arrows indicates the elution positions of chain length markers. The lower arrows indicate the elution positions of tritiated type 0 and type 1 cap structures.*

TABLE 1. *Translation of mRNA synthesized by progeny SVPs*

Extract	Treatment of SVPs	Radioactivity (cpm × 10^{-1})[a]
Uninfected	Native	5
Uninfected	Chymotrypsin	369
Infected	Native	428
Infected	Chymotrypsin	104

[a] Counts per minute (cpm) of [^{35}S]methionine incorporated into trichloroacetic acid-precipitable material in a 5-μl sample of the translation mixture.

of this analysis revealed that both the guanyl transferase and the RNA methylase activities that are involved in capping of mRNA are masked in the progeny SVPs. This led to the prediction that mRNA synthesized by these particles should not be capped. Direct analysis indicated that the 5′ end of mRNA synthesized in vitro by progeny SVPs is monophosphorylated, having the structure pGpC... It seems unlikely that this monophosphorylated 5′ terminal is derived by specific cleavage of nascent mRNA molecules within the 5′-terminal noncoding region. The first two nucleotides at the 5′ end of progeny transcripts are pGpC, and these are the same two nucleotides at the 5′ end of both capped and uncapped mRNA synthesized by reovirus cores (4, 7, 10, 14). Furthermore, preliminary results indicate the existence of a unique enzyme in extracts from L-cells infected with reovirus (33; unpublished data). This enzyme

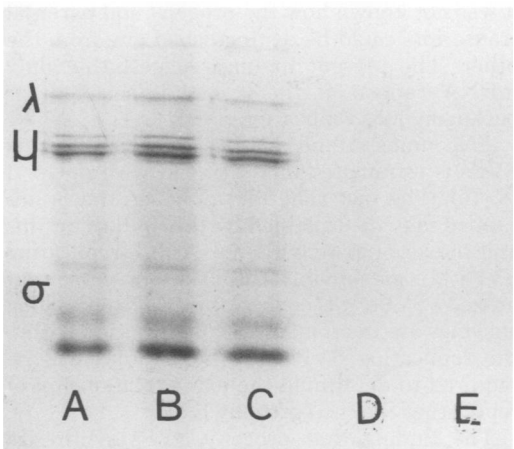

FIG. 7. *Radioautogram of polyacrylamide gel electrophoresis of ^{35}S-labeled translation products. Standard in vitro translation was carried out as described in the text. Incubation was for 60 min at 30°C. Samples prepared as described in the text were run on a 12.5% polyacrylamide slab gel. Electrophoresis is from top to bottom. (A) Polypeptides synthesized in lysate from uninfected cells, using mRNA synthesized by progeny SVPs treated with chymotrypsin. (B) Polypeptides synthesized in lysate from infected cells using mRNA synthesized by native SVPs. (C) Polypeptides synthesized in vivo in L-cells infected with reovirus. In this case, infected cells were labeled with [^{35}S]methionine from 10 to 15 h postinfection at 37°C. (D and E) Polypeptides synthesized in lysates from uninfected cells or from infected cells, respectively, with no exogenous mRNA added.*

specifically removes the β-phosphate of ppGp-terminated mRNA, thereby generating a monophosphorylated 5′ end, and is not present in extracts prepared from uninfected L-cells. Studies are in progress to characterize this reovirus-induced pyrophosphatase activity.

The finding that the 5′ terminus of the mRNA synthesized in vitro by progeny SVPs is uncapped clarifies several aspects of the reovirus replicative cycle. During reovirus replication, viral mRNA provides the plus strand of the progeny dsRNA genome by serving as a template for minus-strand synthesis in replicating SVPs (25). The plus strand of the genome has the same 5′-terminal cap structures found at the 5′ end of reovirus mRNA synthesized by reovirus cores (8), and additional evidence indicates that mRNA synthesized by parental SVPs has the same 5′-capped structures (3, 12). The finding that mRNA synthesized by progeny SVPs is uncapped suggests that only capped mRNA synthesized by parental SVPs is used as template for minus-strand synthesis in replicating SVPs. Although this was inferred from previous pulse-labeling (25, 30) and genetic experiments (6, 37), it was not known how the progeny and parental transcripts could be distinguished one from the other. The present findings suggest that only mRNA capped at the 5′ end is selected for packaging into replicating SVPs.

The minus strand synthesized in replicating SVPs is terminated at the 5′ end with ppGp... (8, 15). The fact that the minus strand is uncapped may be explained by the findings in this and the accompanying report. Since replicating SVPs are presumably the direct precursors of progeny SVPs, it is conceivable that the capping enzymes are in an inactive conformation within the replicating SVPs. Further studies will be required to determine the exact relationship of replicating SVPs to progeny SVPs.

The finding that progeny SVPs synthesize uncapped mRNA is of great interest in view of recently published work (33). We found that, as a result of reovirus infection, a transition occurs in the cap-dependent translational machinery of L-cells. Cell-free extracts prepared at late times postinfection preferentially translate uncapped reovirus mRNA, indicating that changes in the capping pattern of mRNA may be involved in takeover of protein synthesis in cells infected with reovirus. The data presented in this paper support this hypothesis. In addition to being uncapped, mRNA synthesized by progeny SVPs fails to translate in extracts from uninfected L-cells. This mRNA, however, does translate efficiently in extracts from reovirus-infected cells to yield apparently normal viral polypeptides (Fig. 7). Further support for this hypothesis comes from work to be published elsewhere, in which we have shown that mRNA isolated from polysomes at late times postinfection is also uncapped. Thus, after infection with reovirus, the translational machinery of the L-cell is modified, the end result being preferential translation of uncapped reovirus mRNA. At the same time the bulk of the viral mRNA is made by progeny SVPs and hence, as shown here, is uncapped. This leads to preferential translation of viral mRNA's.

The nature of the modification to the translational machinery following reovirus infection is as yet unknown. Preliminary results indicate that the modification occurs in the post-ribosomal fraction of the cell-free protein-synthesizing system (unpublished data). A recent report by Rose et al. (24) implicates a modified initiation factor in the takeover of protein synthesis by poliovirus. It is of interest that the 5′ structure of picornavirus mRNA is also pNp... (16, 22; P. Fellner, D. Frisby, J. Goodchild, A. Porter, and N. H. Carey, Abstr. 3rd Int. Congr. Virol., abstr. no. 161, 1975). Studies are in progress to determine whether a similar mechanism may be involved in the takeover of protein synthesis in L-cells infected by reovirus.

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