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Blocked and Unblocked 5′ Termini in Reovirus Genome RNA

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Uniformly ^{32}P-labeled, double-stranded genome RNA isolated from purified reovirus contains two types of 5′-terminal sequences. One strand contains a phosphatase-resistant 5′-terminal structure, XpppG*pCpU, which is also present in the viral mRNA. The 5′ blocking group, X, is removed by β-elimination indicating that it is a nucleoside containing free 2′,3′-hydroxyls. G*pC is an alkaline-resistant, 2′-*O*-methylated sequence. The other strand contains a phosphatase-sensitive 5′ sequence, ppGpPupPyp. The results are discussed in relation to blocked 5′-terminal structures in other viral and cellular RNAs.

Double-stranded (ds) RNA isolated from purified reovirus type 3 by phenol extraction consists of ten genome segments (22). One strand of each segment is transcribed in virus-infected cells and in vitro by an RNA polymerase present in virions (4, 13, 21, 23). The single-stranded RNA synthesized in vitro corresponds to the intracellular viral mRNA (10) and can be translated into 10 primary gene polypeptide products by cell-free extracts of wheat germ (5). Many rounds of viral mRNA synthesis are catalyzed by the virion polymerase in vitro indicating that it terminates and reinitiates RNA chains with fidelity for long periods (3). In virus-infected cells, the ss transcripts also are precursors of the ds RNA (19), consistent with their formation by end-to-end transcription of the template. These observations suggest that the ds genome RNA segments contain a common terminal sequence that is recognized as the initiation signal for transcription by the virion polymerase.

In the course of the present studies, ds viral RNA labeled in vitro at the 5′ termini by incubation with polynucleotide kinase and [γ-^{32}P]ATP was found to contain two 5′ sequences: ^{32}P-labeled pGpApUp in one strand and alkali-resistant ^{32}P-labeled pG*pCp in the other (15). The ds RNA was efficiently labeled only after pretreatment by sequential periodate oxidation and β-elimination with aniline (15). Since this procedure is known to remove, from RNA, nucleosides containing free 2′,3′-hydroxyl groups (24), it was suggested that reovirus genome RNA contains blocked 5′ termini (15). In an effort to understand better the structure-function relationship of the reovirus genome RNA segments, we have analyzed the 5′ termini of ds RNA uniformly labeled with ^{32}P. The results confirm the presence of two types of 5′-terminal sequences in reovirus genome RNA and indicate that the strand containing the alkali-resistant dinucleotide is also blocked at the 5′ end as in viral mRNA synthesized in vitro (9, 20).

MATERIALS AND METHODS

Preparation of radioactive reovirus ds RNA. For preparation of uniformly ^{32}P-labeled RNA, BHK-21 cells (kindly supplied by D. Dubin, Rutgers Univ.) were grown in suspension culture in Eagle modified medium containing nonessential amino acids and 5% fetal calf serum. For infection, 2×10^9 cells were concentrated to 10^7/ml in phosphate-free medium, and reovirus type 3 Dearing strain (multiplicity of infection of 20 PFU/cell) was adsorbed for 60 min at 37 C. Cells were then diluted fivefold in phosphate-free medium containing 0.3 μg of actinomycin D per ml. After 2 h at 37 C, 20 mCi of carrier-free [^{32}P]phosphoric acid was added, and the incubation continued for 32 to 38 h. Cells were harvested by centrifugation, and the supernatant fluid was used to resuspend a second lot of 2×10^9 infected cells. Actinomycin (0.3 μg/ml) was added, and after 32 h at 37 C the cells were harvested. The cell pellets were combined for virus purification by genetron extraction and centrifugation in CsCl solutions (1). The viral ds RNA was isolated from purified virions by phenol extraction and separated from the oligonucleotides by gel filtration in Sephadex G-100 (2). From 4×10^9 cells and 20 mCi of [^{32}P]phosphoric acid, approximately 0.7 mg of ds RNA containing 7×10^7 counts/min was obtained. For preparation of ^{3}H-labeled viral RNA, L cells were infected with 20 PFU of reovirus type 3 per cell, as described previously (2). [^{3}H]guanosine and [^{3}H]cytidine (1 mCi each) were added at 2 h after infection and virus was purified after 40 h as above.

DEAE-cellulose column chromatography of alkaline digestion products. ds RNA was digested with 0.3 N KOH for 20 h at 37 C, neutralized with Dowex-50 ($^+$H form), and applied to a column (1 by 20

cm) of DEAE-cellulose (DE-52) equilibrated with 0.05 M Tris-hydrochloride buffer, pH 8, containing 7 M urea. As optical density markers, at 260 nm, for the elution positions of the oligonucleotides, a pancreatic RNase digest of yeast tRNA was added (1 mg hydrolyzed with 0.1 mg of RNase for 6 h at 37 C). Samples were eluted with two different, consecutive gradients of NaCl in 0.05 M Tris buffer (pH 8) and 7 M urea: (i) 110 ml each of 0.0 M and 0.15 M NaCl; (ii) 90 ml each of 0.15 M and 0.3 M NaCl. Fractions of 1.5 ml were collected and 0.3 ml of each was counted in 5 ml of methyl-cellosolve and 7 ml of toluene-based scintillation fluid.

Analyses of pancreatic RNase digestion products. ds RNA (6×10^7 counts/min) was denatured by heating at 37 C for 30 min in 0.01 M Tris buffer (pH 8), 0.001 M EDTA, and 90% dimethyl sulfoxide (12). The RNA was precipitated in 2 volumes of ethanol and 0.15 M NaCl at −20 C, collected by centrifugation, washed with ethanol, dried in a stream of nitrogen, and dissolved in 1 ml of 0.1 M Tris buffer (pH 8) containing 1 mg of yeast tRNA and 0.1 mg of pancreatic RNase that had been heated for 3 min at 90 C in 0.02 M Tris (pH 8), 0.001 M EDTA (1 mg/ml) to inactivate contaminating phosphatase activity (6). After digestion at 37 C for 7 h, the sample was applied to a column (1 by 20 cm) of DE-52 and eluted with a linear salt gradient (0.05 M to 0.3 M NaCl) in 0.05 M Tris (pH 8) and 7 M urea. Samples of 1.2 ml were counted by Cerenkov radiation.

High voltage paper electrophoresis of 5′-terminal nucleotides. Samples eluted from the DE-52 were pooled as described for individual experiments, dialyzed exhaustively against water, and lyophilized. Recoveries were greater than 95% in each case. The lyophilized samples were dissolved in 0.05 ml of 0.01 M Tris buffer (pH 8).

For alkaline phosphatase digestion, samples were incubated with 5 U of enzyme per ml for 60 min at 37 C. The same conditions were used for digestion with 50 μg of *Penicillium* nuclease per ml (7). Spleen phosphodiesterase was used at 1 U/ml in 0.01 M Tris (pH 7). The hydrolyzed samples were applied as thin lines of 2-cm length on Whatman no. 1 chromatography paper. After electrophoresis at 3,000 V (60 V/cm) for 80 to 90 min in pyridine acetate buffer, pH 3.5, the paper was dried, cut into 1-cm strips, and counted in 10 ml of toluene-based scintillator. Authentic marker compounds including the four 5′ mononucleotides and GTP were located by absorbance under UV light. Other markers included ^{32}P-labeled ppGpCp and GpC obtained from reovirion oligonucleotides (25) as follows. The oligonucleotides ppGpCpC and ppGpCpU were purified by column chromatography on DEAE-cellulose and digested with pancreatic RNase (60 min, 37 C, 5 μg/ml, 0.01 Tris buffer, pH 7.4, 0.001 M EDTA). The resulting ppGpCp was purified by paper electrophoresis at pH 3.5. Alkaline phosphatase digestion released 72% of the radioactivity as inorganic orthophosphate and 28% as GpC which was verified by base composition analysis using spleen and venom phosphodiesterases and *Penicillium* nuclease (14).

For β-elimination with aniline, the lyophilized, blocked 5′-terminal material, purified by DEAE column chromatography, was dissolved in 1 ml of 0.1 M sodium acetate buffer, pH 5.3, and 0.001 M EDTA. The RNA was oxidized with potassium periodate (0.2 mM, 2 h, 4 C, in the dark), treated with 1 mM glucose (30 min, room temperature), dialyzed, lyophilized, and treated with aniline (0.33 M, 3 h, room temperature) as described by Steinschneider and Fraenkel-Conrat (24). After dialysis to remove the aniline, the sample was lyophilized again. The recovered material (80% of the original) was analyzed by high voltage electrophoresis.

In vitro synthesis of viral mRNA. RNA was synthesized by incubating purified virions with chymotrypsin under the standard conditions of synthesis described previously (20). The radioactive precursors were: *S*-adenosyl-L-[*methyl*-^{3}H]methionine (SAM) (specific activity 8.5 Ci/mmol), [α-^{32}P]ATP (75 μCi/μmol), [α-^{32}P]UTP (specific activity 200 μCi/μmol), and [α-^{32}P]CTP (specific activity 37 μCi/μmol). After synthesis had proceeded for 1 h, the reaction mixture was extracted with an equal volume of water-saturated phenol, and the aqueous phase was passed through Sephadex G-100. The excluded volume containing the RNA was precipitated at −20 C in 2 volumes of ethanol and 0.15 M NaCl. The RNA was collected by centrifugation, hydrolyzed in 0.3 N KOH at 37 C for 20 h, and analyzed by DEAE-cellulose column chromatography. Samples were eluted with a linear gradient of 0.05 M to 0.3 M NaCl in 0.05 M Tris buffer, pH 8, and 7 M urea and counted in methyl-cellosolve and toluene-based scintillation fluid.

Sources of enzymes and radioisotopes. Pancreatic RNase, alkaline phosphatase, and spleen phosphodiesterase were purchased from Worthington Biochemicals Corp. *Penicillium* nuclease was kindly provided by K.-I. Miura, National Institute of Genetics, Mishima, Japan.

[*methyl*-^{3}H]SAM (specific activity 8.5 Ci/mmol), carrier-free [^{32}P]phosphoric acid, [α-^{32}P]CTP (6.5 Ci/mmol), [α-^{32}P]ATP (8.5 Ci/mmol), and [α-^{32}P]UTP (9.2 Ci/mmol) were from New England Nuclear. [^{3}H]guanosine and [^{3}H]cytidine (specific activities 7 and 19 Ci/mmol, respectively) were from Schwartz/Mann, Orangeburg, N.Y.

RESULTS

Separation of two types of 5′ termini from reovirus genome RNA. Reovirus ds RNA labeled uniformly with ^{32}P was hydrolyzed with KOH and the digest was analyzed by column chromatography on DEAE-cellulose in 7 M urea. Previously, 5′-terminal ppGp was identified in alkaline digest of reovirus RNA by a similar procedure (2). In the present study a more shallow salt gradient was used for eluting samples from the DEAE-cellulose and fractions of smaller volume were collected for counting. As a result of these changes, two distinct peaks were resolved in the positions of high net negative charge expected for 5′-terminal nucleotides (Fig. 1). Peak 1 which eluted between the marker oligonucleotides of charge −4 and −5

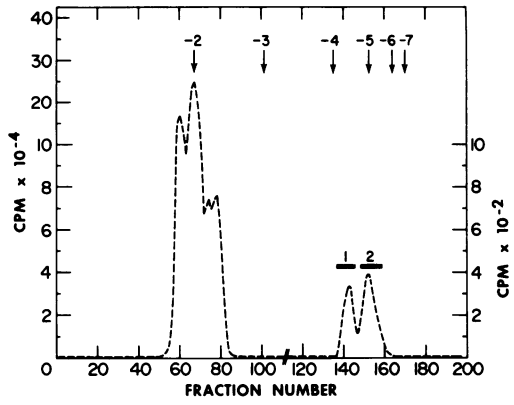


FIG. 1. *Chromatography of alkaline digest of ds RNA. ^{32}P-labeled RNA (1.4 × 10^7 counts/min) was isolated from purified reovirus, digested with 0.3 N KOH, and analyzed by DEAE-cellulose chromatography.*

contained 45% of the presumptive 5'-terminal ^{32}P; peak 2 contained 55% of the presumptive 5'-terminal radioactivity and eluted close to the position of net negative charge −5. The two peaks comprised 0.15% (4,200 counts/min) of the total ^{32}P (2.8 × 10^6 counts/min) recovered from the column, the proportion of 5'-terminal ^{32}P expected in uniformly labeled reovirus ds RNA (2).

Peak 1 was desalted and further analyzed by high voltage paper electrophoresis. All of the radioactivity migrated as a homogeneous component with authentic GTP (Fig. 2A). After digestion with alkaline phosphatase, the ^{32}P was recovered in the position of inorganic phosphate (Fig. 2B). These results indicate that peak 1 corresponds to the previously described 5'-terminal ppGp (2).

Peak 2 also migrated as a single component, slightly faster than pG, when analyzed by paper electrophoresis (Fig. 3A). However, in contrast to peak 1, treatment with alkaline phosphatase released only a fraction (26%) of its radioactivity as inorganic phosphate. Most of the remainder (71%) was in a phosphatase-resistant compound (peak 3) that migrated between pA and pG (Fig. 3B). The phosphatase digestion was complete because authentic ppGpCp, purified from the reovirus oligonucleotide mixture (25), was converted quantitatively to GpC and inorganic phosphate by enzyme treatment under the same conditions (Fig. 3B).

The phosphatase-resistant material in peak 3 was further analyzed by electrophoresis after digestion with *Penicillium* nuclease. This enzyme is a phosphodiesterase that produces 5' mononucleotides and can degrade sequences containing 3' phosphates and/or 2'-*O*-methylated residues, but does not hydrolyze pyrophosphate linkages (7, 14). *Penicillium* nuclease digestion hydrolyzed peak 3, yielding 30% of the radioactivity as pC and the remainder as a component that migrated in the position of pG but which was completely resistant to phosphatase digestion (Fig. 3C). The alkaline phosphatase-treated peak 3 was not hydrolyzed by incubation with spleen phosphodiesterase under conditions that resulted in the conversion of authentic ^{32}P-labeled GpCpC and GpCpU to Gp^{32} and Cp^{32} (data not shown). The resistance to 5'-exonucleolytic digestion by spleen phosphodiesterase after phosphatase treatment is consistent with the finding that the 5' phosphates in peak 3 are present in a blocked configuration.

Similar results were obtained with ds RNA labeled with [^{3}H]guanosine and [^{3}H]cytidine. Presumptive 5'-termini were isolated from KOH digests of ^{3}H-labeled genome RNA by DEAE-cellulose chromatography as in Fig. 1. Fractions corresponding to the positions of net charge −4 to −6 were pooled, desalted and analyzed by paper electrophoresis. One component migrated with GTP (Fig. 4A) as observed in the electropherograms of ^{32}P-labeled peak 1

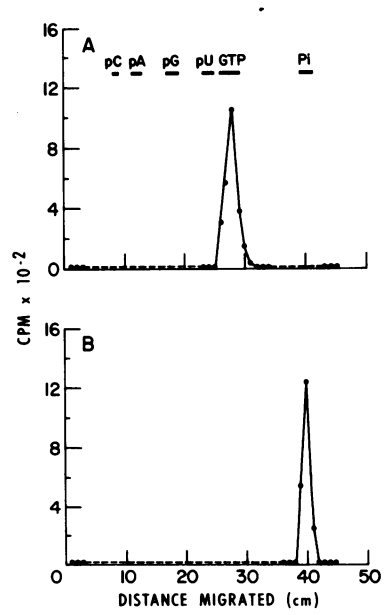


FIG. 2. *Electrophoresis of 5'-terminal structure, peak 1. Fractions corresponding to peak 1 in Fig. 1 (137–146) were pooled, dialyzed, lyophilized, and analyzed by high voltage paper electrophoresis before (A) and after (B) treatment with alkaline phosphatase.*

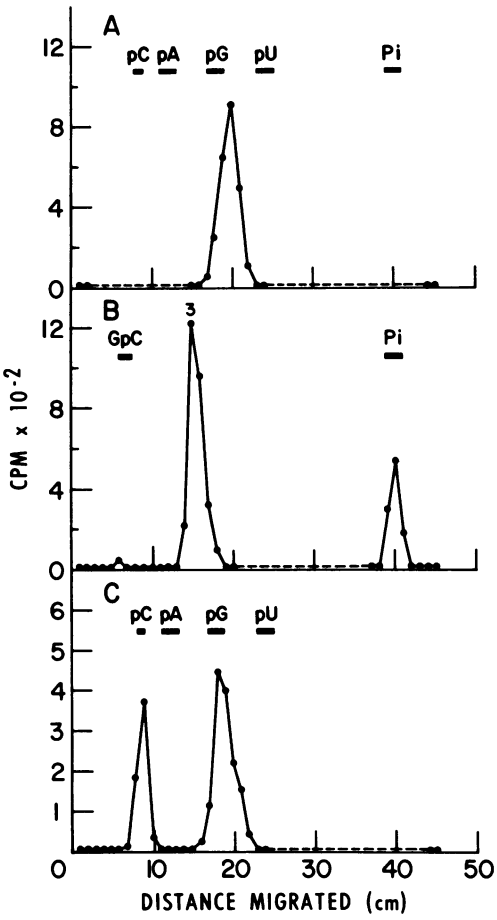


FIG. 3. *Electrophoresis of 5'-terminal structure, peak 2. Fractions corresponding to peak 2 in Fig. 1 (147–161) were combined, dialyzed, and lyophilized. The sample was divided and analyzed by paper electrophoresis before (A = 40% of total counts/min) and after (B = 60% of total counts/min) incubation with alkaline phosphatase. (C) The phosphatase-resistant material in (B) (14 to 18 cm) was eluted, lyophilized, digested with Penicillium nuclease, and reanalyzed.*

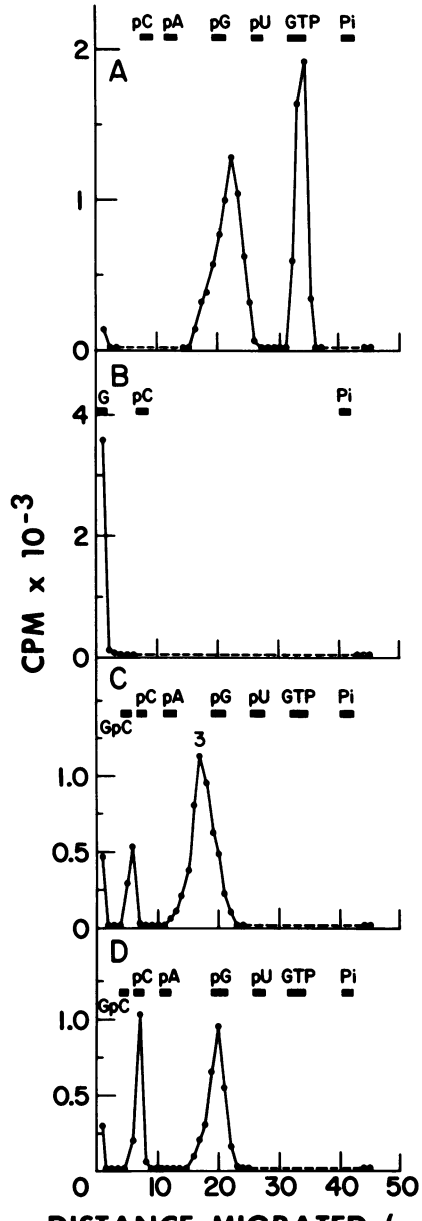


FIG. 4. *Electrophoresis of ^{3}H-labeled 5'-terminal structures. Genome RNA labeled with [^{3}H]guanosine and [^{3}H]cytidine was digested with KOH and the presumptive 5'-terminal structures were isolated by DEAE-cellulose chromatography as in Fig. 1. The material in the elution position corresponding to fractions 137–161 in Fig. 1 were pooled, dialyzed, lyophilized, and analyzed by high voltage paper electrophoresis (A). The peak corresponding to the position of GTP was eluted, treated with alkaline phosphatase, and reanalyzed (B). The component migrating between 16 and 25 cm from the origin was eluted, treated with alkaline phosphatase, and reanalyzed (C). Peak 3 of (C) was eluted, digested with Penicillium nuclease, and reanalyzed (D).*

material. After phosphatase treatment it moved to the position of guanosine (Fig. 4B) indicating that it corresponds to the 5'-terminal structure, ppGp, i.e., peak 1. The second component migrated in the position of pG (Fig. 4A), the same position found for ^{32}P-labeled peak 2 material (see Fig. 3A). After phosphatase treatment this component had the same electrophoretic mobility as peak 3 in Fig. 3B, i.e., between pA and pG (Fig. 4C). A small amount of the radioactivity (13% of the total) was converted to a material migrating with GpC, presumably due to degradation of the 5' blocking group. The ^{3}H-labeled peak 3 material was digested with *Penicillium* nuclease and reanalyzed. As ob-

served for ^{32}P-labeled peak 3, *Penicillium* nuclease digestion released pC and a phosphatase-resistant component migrating with pG (Fig. 4D). The results are consistent with two types of 5′ termini: ppGp and a 5′ structure of the type XpppG*pCp where X is a blocking nucleoside and G* is a modified G which renders G*pC resistant to hydrolysis with alkali.

Release of two types of 5′ termini by pancreatic RNase digestion. To confirm the presence of two types of 5′-terminal sequences, uniformly ^{32}P-labeled genome RNA was digested with pancreatic RNase. The resulting oligomers containing 3′ pyrimidine phosphates were analyzed by column chromatography (Fig. 5A). The individual oligonucleotide peaks of net charge −4, −5, −6, and −7 were collected, digested with 0.3 N KOH, and examined for the presence of 5′-terminal nucleotides by rechromatography. Only the peaks of net charge −5 and −6 from the RNase digestion products contained 5′-terminal nucleotides. The −5 peak from the RNase digest, after alkaline hydrolysis, yielded 0.08% of the total radioactivity as a peak eluting again in the −5 position (Fig. 5B). Digestion with alkaline phosphatase, *Penicillium* nuclease, and spleen phosphodiesterase followed by analyses as described for peak 2 confirmed that the terminal phosphates were blocked and in the 5′-terminal sequence, XpppG*pCp.

The −6 peak from the RNase digest, after alkaline hydrolysis, yielded 0.06% of the total radioactivity which eluted between the −4 and the −5 isopleths (Fig. 5C). It was further identified by enzymatic digestion and paper electrophoresis as ppGp as described for peak 1 in Fig. 1. The finding that the −6 isopleth obtained by pancreatic RNase digestion yielded 5′-terminal ppGp after treatment with alkali indicates that one set of the 5′-terminal sequences is ppGpPupPyp. Thus, reovirus genome RNA contains similar amounts of two 5′-terminal sequences: XpppG*pCp... and ppGpPupPyp....

Removal of the 5′-terminal blocking group by β-elimination. These results suggest that 10 of the 20 5′ termini in the 10 reovirus ds genome RNA segments are blocked and in a structure XpppG*pCp.... Recent studies by Miura et al. (15) also are consistent with the presence of blocked 5′ termini in reovirus ds RNA. When polynucleotide kinase and [γ-^{32}P]ATP were used in an attempt to label specifically the 5′ ends of phosphomonoesterase-treated reovirus ds RNA, low levels of radioactivity were obtained indicating that some of the 5′ termini were protected. The ds segments could be highly labeled at all 5′ termini by the kinase technique after sequential oxidation, β-elimination, and phosphomonoesterase digestion (15, 24). It was therefore of interest to test if the 5′ blocking moiety could be removed from the 5′-terminal XpppG*pCp by the β-elimination reaction.

Uniformly ^{32}P-labeled ds RNA was digested with pancreatic RNase and the resulting oligonucleotides were separated by column chromatography as described for Fig. 5. The blocked 5′ sequence, XpppG*pCp, which was present in the −5 isopleth, was further purified from the

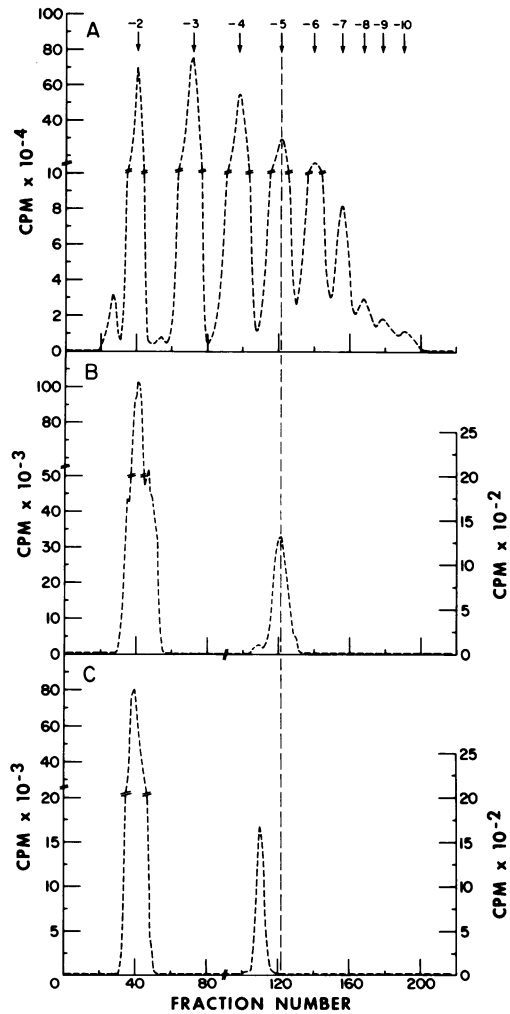


FIG. 5. *Chromatography of digestion products of ds RNA. (A) ^{32}P-labeled RNA (6 × 10^7 counts/min) was digested with pancreatic RNase and analyzed by DEAE-cellulose chromatography. (B) The peak fractions of net negative charge −5 (108–130) were pooled, dialyzed, lyophilized, digested with KOH, and rechromatographed. (C) The fractions of net charge −6 (131–151) were dialyzed, lyophilized, incubated with KOH and reanalyzed.*

internal tetranucleotides, $(Pup)_3Pyp$, by KOH hydrolysis and column chromatography as in Fig. 5B. Approximately 80% of the expected radioactivity was recovered after these procedures. The purified material was oxidized with periodate, treated with aniline, and incubated with alkaline phosphatase to determine if its constituent phosphates had become sensitive to enzymatic digestion. Figure 6 shows the electrophoretic analysis of the oxidized, aniline-treated, 5'-terminal material before and after phosphatase treatment. The blocked 5'-terminal XpppG*pCp that migrates near pG (Fig. 3A and 4A), after β-elimination, moved as a peak near authentic ppGpCp (Fig. 6A). In addition, 75% of the radioactivity in this peak was released as inorganic phosphate by phosphatase digestion (Fig. 6B). The remaining radioactivity moved with GpC. These results indicate that the blocking moiety, X, in the 5'-terminal structure XpppG*pC contains free 2',3'-hydroxyl groups and can be removed by β-elimination.

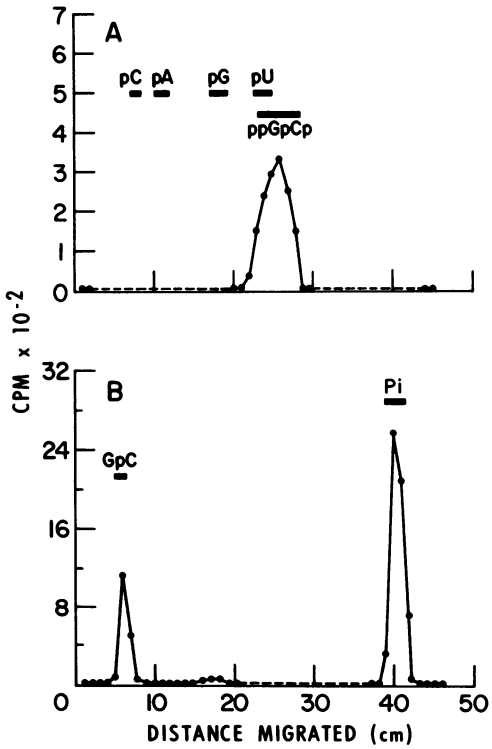


FIG. 6. *Electrophoresis of blocked 5' termini after β-elimination. The blocked 5'-terminal structures eluting from DEAE-cellulose in the position of net negative charge −5 were oxidized with periodate and treated with aniline. An aliquot (15% of total counts/min) was analyzed by electrophoresis (A) and the remainder was treated with alkaline phosphatase (B) before electrophoretic analysis.*

Identification of Up in the blocked 5'-terminal sequence. The polymerase associated with purified reovirus transcribes one strand of each of the duplex genome segments, producing single-stranded RNA that functions as viral mRNA (5) and as a precursor of ds RNA (19). Thus, the single-stranded RNA species correspond to one complete strand of the genome RNA, the plus strand. Recently, it was found that single-stranded RNA synthesized in vitro in the presence of the methyl donor, SAM, is methylated specifically at its 5' ends (20). Because this structure contains an alkaline-resistant dinucleotide, G^mpCp, it can be isolated and used to identify the next nucleotide in the chain by nearest neighbor analysis (11). Figure 7 shows the results of the analyses when [α-^{32}P]ATP or [α-^{32}P]UTP were used as labeled precursors in the presence of [*methyl*-^{3}H]SAM. With [α-^{32}P]UTP as precursor, a prominent ^{3}H, ^{32}P-labeled peak was obtained eluting close to the position of the 5'-terminal structure with the sequence XpppG*pCp^{32} (Fig. 7B). Of the total radioactivity incorporated from [α-^{32}P]UTP, 0.15% was present in this peak. In contrast, only ^{3}H and no ^{32}P was associated with the alkaline-resistant termini when [α-^{32}P]ATP was used as precursor in the presence of [*methyl*-^{3}H]SAM (Fig. 7A). The results indicate that most of the RNA synthesized under these conditions contains the 5' sequence XpppG*pCpUp.

The same type of experiment was done with [α-^{32}P]CTP as precursor. RNA synthesized in vitro in the absence of SAM contains the alkali-sensitive 5' sequence, ppG^{32}pCp, and after KOH hydrolysis, 0.2% of the radioactivity was obtained in the position of ppGp^{32} (Fig. 8A). The alkaline digest of RNA synthesized in the presence of [*methyl*-^{3}H]SAM and [α-^{32}P]CTP contained double-labeled XpppG*p-^{32}Cp (Fig. 8B). The same proportion of total ^{32}P was present in the 5'-terminal structures indicating that both the unblocked and the blocked 5' termini of viral plus strands contain a single Cp. On the basis that the plus strands are precursors of genome RNA (19), the results suggest that the 5'-terminal sequence of the duplex plus strand of reovirus is XpppG*pCp-Up.

DISCUSSION

The genome of human reovirus type 3 consists of 10 segments of ds RNA. Analysis of the 5' termini of uniformly ^{32}P-labeled genome RNA

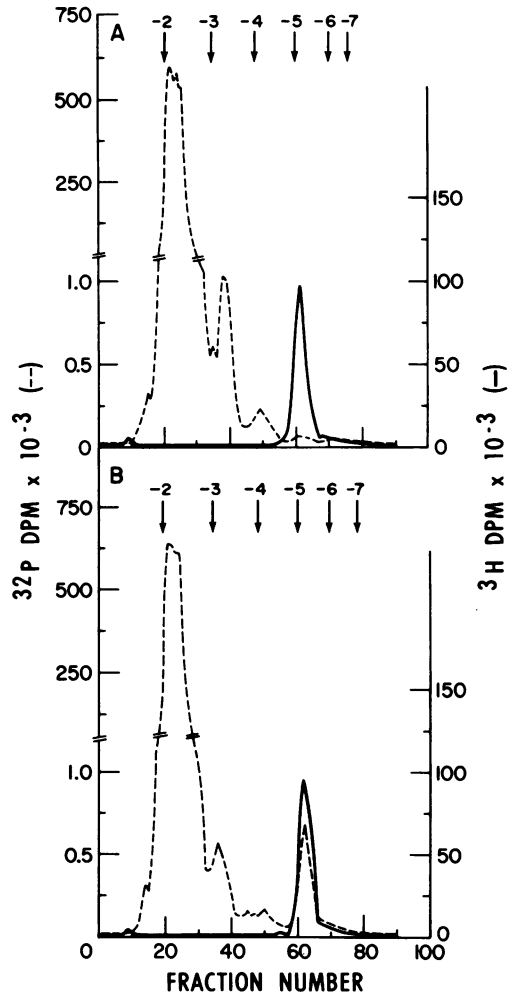


FIG. 7. *Chromatography of alkaline digests of mRNA synthesized in vitro. RNA was synthesized with S-adenosyl-L-[methyl-^{3}H]methionine and (A) [α-^{32}P]ATP or (B) [α-^{32}P]UTP as the labeled precursors. The products were hydrolyzed with KOH and analyzed by DEAE-cellulose chromatography.*

and RNA labeled with [^{3}H]guanosine and [^{3}H]cytidine revealed the presence of similar amounts of two types of structures: ppGpPup-Pyp ... and XpppG*pCp.... The results with uniformly-labeled ds RNA prepared by infecting cells in the presence of ^{32}P confirm recent findings with reovirus ds RNA labeled in vitro with polynucleotide kinase and [γ-^{32}P]ATP. Two 5′-terminal sequences were also identified in each genome segment labeled in vitro: ^{32}pGpApUp and alkali-resistant ^{32}P-labeled pG*pCp (15). Reovirus mRNA synthesized in vitro by the virion-associated transcriptase in the presence of SAM also contains alkali-resistant G^{m}pCp (20) indicating that the ^{32}P-labeled pG*pCp sequence is probably in the plus strand of the duplex segments and that G* is 2′-*O*-methylguanosine.

In addition to 2′-*O*-methylguanosine, the plus strands of uniformly labeled reovirus RNA contain phosphatase-resistant, i.e., blocked, 5′-terminal phosphates. The presence of a 5′-terminal protecting group, X, was also suggested from the result of the studies with polynucleotide kinase (15). To label the 5′ termini of reovirus RNA in vitro, it was first necessary to treat the RNA sequentially with periodate, aniline, and phosphomonoesterase (15). The same sequence of reactions was also required before the 5′ ends of another ds RNA virus, cytoplasmic polyhedrosis virus, could be labeled with the kinase method (14). This reaction is presumably required to remove the blocking group, X, as demonstrated in Fig. 6. Thus, the 5′ terminus of reovirus plus strand is a blocked methylated

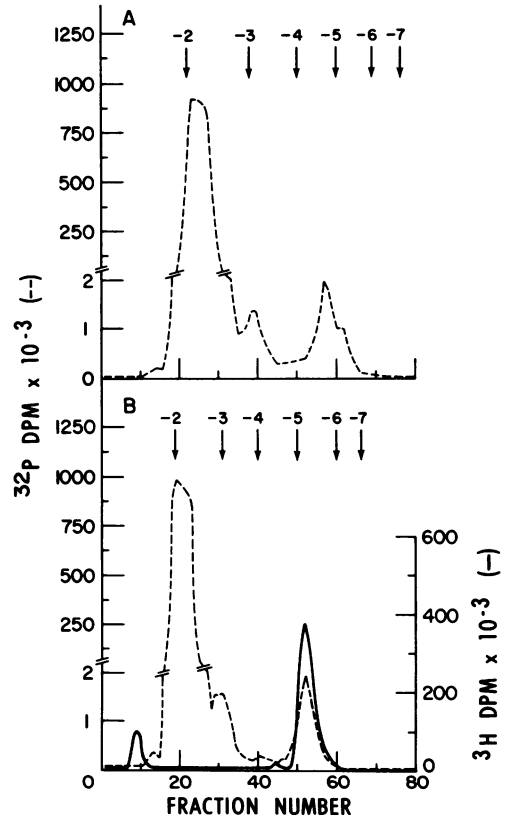
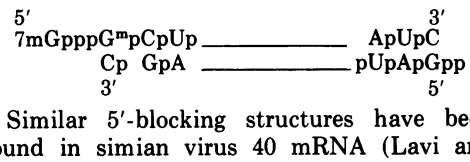


FIG. 8. *Chromatography of alkaline digests of in vitro mRNA synthesized in the presence and absence of S-adenosylmethionine. RNA was made in vitro in the presence of [α-^{32}P]CTP without (A) and with (B) S-adenosyl-L-[methyl-^{3}H]methionine. The purified products were digested with KOH and analyzed by column chromatography on DEAE-cellulose.*

structure, X$^{5'}$ppp$^{5'}$G^{m}pCpUp . . . and its complementary strand contains an unblocked, unmethylated sequence, ppGpApUp. The 3′ ends of the two strands comprising each reovirus genome RNA segment are . . . UpC and . . . GpC (16).

The presence of a 5′-terminal blocking group has been demonstrated in several other RNAs in addition to the genomes of two ds RNA-containing viruses. These include the single-stranded viral mRNAs transcribed in vitro in the presence of the methyl donor, SAM, by the virion transcriptases of cytoplasmic polyhedrosis virus (8), reovirus (20), vaccinia virus (Urushibara, Furuichi, Nishimura, and Miura, personal communication), and vesicular stomatitis virus (Banerjee and Rhodes, personal communication). In vaccinia mRNA X has been identified as 7 mG protecting two (Furuichi, personal communication) or three phosphates (Wei and Moss, personal communication) in the structures, 7mGp(p)pG^{m}p and 7mGp(p)pA^{m}p. In cytoplasmic polyhedrosis virus (8) and reovirus RNA (20), X is also 7mG in the 5′-terminal structures, 7mGpppA^{m}p and 7mGpppG^{m}p, respectively. On the basis of these results, a tentative structure for the terminal sequences of reovirus genome RNA is:



Similar 5′-blocking structures have been found in simian virus 40 mRNA (Lavi and Shatkin, unpublished data) and cellular mRNAs (18; Lavi and Shatkin, unpublished data) and the blocked 5′-sequence in the low-molecular-weight nuclear RNAs of Novikoff hepatoma cells has been identified as m$_3^{2,2,}$ ^{7}G$^{5'}$pp$^{5'}$ A^{m}p (17). The biological role of blocked 5′-termini in the formation and function of viral and cellular RNAs will be of interest for future studies.

LITERATURE CITED

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