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Differential synthesis of blocked and unblocked 5′-termini in reovirus mRNA: Effect of pyrophosphate and pyrophosphatase

(methylation/7-methylguanosine)

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ABSTRACT **Viral mRNA synthesized *in vitro* by reovirus-associated enzymes contained 5′-terminal ppG, GpppG, or m^7GpppG^m. Selective synthesis of one type of 5′-end was obtained by adjusting the composition of the transcription reaction mixture based on a series of reactions proposed for formation of blocked, methylated 5′-terminal sequences of mRNA (cap formation). The presence of inorganic pyrophosphate increased the proportion of ppG 5′-ends, while addition of pyrophosphatase yielded mRNA with 5′-terminal $GpppG_2$ which in the presence of a methyl donor was converted to m^7GpppG^m.**

Many eukaryotic cellular and viral mRNAs contain blocked and methylated 5′-terminal "cap" structures of the general type, $m^7G(5')ppp(5')N$ (1). m^7G-Containing caps are also present in the high-molecular-weight, heterogeneous nuclear RNA of HeLa (2) and L cells (3), as well as in simian virus 40-specific nuclear RNA (4) and adenovirus-specific nuclear RNA which is larger than cytoplasmic viral mRNA (5). These results suggest that cap formation takes place in nuclei together with transcription and that caps may be conserved during maturation of mRNA (6).

The details of cap synthesis may be difficult to elucidate in complex cellular systems or fragile nuclear fractions. More amenable to study are the simpler viral systems in which virion-associated enzymes synthesize capped mRNA *in vitro* (7–10). Although capped mRNAs may be formed by different mechanisms in eukaryotic cells and viruses (11), functional mRNAs from both sources contain similar 5′-terminal structures. Consequently, the viral systems are of considerable interest for analyzing the mechanism of mRNA cap synthesis. Human reoviruses are useful for such studies because purified viral cores contain an RNA polymerase and several other enzymes that catalyze the synthesis of large amounts of functional mRNA *in vitro* (12). Reovirus mRNA made in the presence of *S*-adenosylmethionine (AdoMet) contains mainly 5′-terminal m^7GpppG^m-C (9), a structure also found in some cellular mRNAs (13). Recently, a mechanism was proposed (14, 15) for reovirus mRNA cap formation that involves the following five steps (AdoHcy = *S*-adenosylhomocysteine).

$$pppG + pppC \xrightarrow[\text{polymerase}]{\text{RNA}} pppG\text{-}C + PP_i \quad [1]$$

$$pppG\text{-}C \xrightarrow[\text{phosphohydrolase}]{\text{nucleotide}} ppG\text{-}C + P_i \quad [2]$$

$$pppG + ppG\text{-}C \xrightleftharpoons[\text{transferase}]{\text{guanylate}} GpppG\text{-}C + PP_i \quad [3]$$

$$GpppG\text{-}C + AdoMet \xrightarrow[\text{methyltransferase 1}]{} m^7GpppG\text{-}C + AdoHcy \quad [4]$$

$$m^7GpppG\text{-}C + AdoMet \xrightarrow[\text{methyltransferase 2}]{} m^7GpppG^m\text{-}C + AdoHcy \quad [5]$$

Abbreviations: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; m^7G, 7-methylguanosine; G^m, 2′-*O*-methylguanosine; cap, blocked, methylated 5′-terminal sequence of mRNA.

The availability of methylated and unmethylated mRNAs from reovirus and other viruses has facilitated studies on the role of caps in mRNA function (16–19). The 5′-terminal m^7G appears to be important for translation, apparently as part of a recognition mechanism for initiation complex formation (20–27). In previous studies on the effect of 5′-m^7G on reovirus mRNA translation, a fraction of the molecules made in the presence of AdoMet contained unblocked 5′-terminal ppG-C. mRNA synthesized in the absence of the methyl donor also contained a mixture of 5′-terminal ppG-C and GpppG-C (20). For future investigations on the relationship between mRNA 5′-terminal structure and function, it was desirable to develop conditions that permitted the preparation of mRNA with a single type of 5′-end. We have found that addition of inorganic pyrophosphate to transcription reaction mixtures yields reovirus mRNA with ppG-C as the only 5′-sequence. Furthermore, mRNA made with inorganic pyrophosphatase in the mixture contained predominantly 5′-GpppG-C which, in the presence of AdoMet, was converted almost quantitatively to m^7GpppG^m-C.

MATERIALS AND METHODS

Reovirus type 3 Dearing strain was grown in mouse L cells and purified as described (28). Virus cores prepared by chymotrypsin digestio were washed twice with 50 mM Tris·HCl (pH 8) containing 50 mM KCl (14). Viral mRNA, synthesized *in vitro* by washed cores as described in the legends, was extracted with phenol and separated from radioactive precursors by gel filtration through Sephadex G-100. The procedures for enzymatic digestion, isolation, and analysis of 5′-terminal structures have been described (9, 29). The sources and specific activities of radioactive materials were: [*methyl*-^{3}H]AdoMet = 10 Ci/mmol, the Radiochemical Centre (Amersham); [^{3}H]CTP = 17 Ci/mmol, [^{3}H]UTP = 41.4 Ci/mmol, [β,γ-^{32}P]GTP = 4.5 Ci/mmol, and [α-^{32}P]GTP = 29 Ci/mmol, New England Nuclear. Pyrophosphate phosphohydrolase (EC 3.6.1.1; inorganic pyrophosphatase) from yeast, 200 units/mg, was purchased from Boehringer Mannheim.

RESULTS

Effect of Pyrophosphate on Synthesis and Methylation of Viral mRNA. Previously it was found that pyrophosphate inhibits the guanylate transferase activity in reovirus cores and reduces the synthesis of GpppG-C from ppG-C and GTP (ref. 14, see Introduction, step 3). In addition, the back reaction is promoted by pyrophosphate (14). Since pyrophosphate is released from ribonucleoside triphosphates and accumulates in the transcription reaction mixture during reovirus mRNA

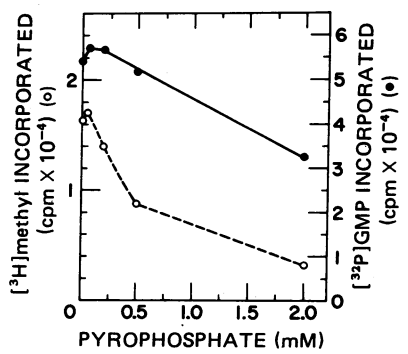

FIG. 1. Effect of pyrophosphate on the methylation and synthesis of reovirus mRNA *in vitro*. Synthesis of virus mRNA and methylation of the 5′-termini were studied in the presence of increasing concentrations of pyrophosphate. Reaction mixtures (100 μl) contained 100 mM Tris·HCl (pH 8), 10 mM $MgCl_2$, 2 mM each of ATP, CTP, and UTP, 0.4 mM GTP, 2 μCi of [α-^{32}P]GTP, 10 μCi of [*methyl*-^{3}H]AdoMet, 10 mM phospho*enol*pyruvate, 0.3 unit of pyruvate kinase, reovirus cores prepared from 200 μg of purified reovirus by chymotrypsin digestion (28), and the indicated amount of sodium pyrophosphate. Incubation was at 45° for 20 min, and reactions were stopped by the addition of an equal volume of water-saturated phenol. Acid-precipitable radioactivity in the aqueous layers was collected on Millipore filters and measured in toluene-based scintillant (Yorktown). (●) ^{32}P radioactivity; (○) [^{3}H]methyl radioactivity.

synthesis *in vitro*, the appearance of products with 5′-terminal ppG-C (20) probably resulted from pyrophosphate inhibition as well as pyrophosphorolysis of nascent GpppG-C ends. Pyrophosphorolysis was prevented by N^7-methylation of the terminal G, and m^7GpppG-C was not cleaved by reovirus cores in the presence of pyrophosphate (14). Furthermore, the proportion of mRNA molecules with unblocked 5′-terminal ppG-C was reduced from 75 to 25% by the addition of the methyl donor, AdoMet (12, 20). These findings, summarized in the proposed scheme for reovirus mRNA cap formation, suggested that mRNA with 5′-terminal GpppG-C, ppG-C, or m^7GpppG^m-C could be synthesized by appropriate selection of the incubation conditions *in vitro*. The effect of adding increasing amounts of pyrophosphate on the synthesis and methylation of mRNA by reovirus cores is shown in Fig. 1. Incorporation of both [^{32}P]GMP from [α-^{32}P]GTP and [*methyl*-^{3}H] from [^{3}H]AdoMet decreased progressively, but the inhibition was a differential one. At 0.5 mM pyrophosphate, RNA synthesis was inhibited by less than 5%. In contrast, [^{3}H]methylation to form 5′-terminal m^7GpppG^m-C was reduced by 45%. This finding indicates that in the presence of pyrophosphate the amount of GpppG-C 5′-ends decreases, resulting in a reduced number of blocked termini available for subsequent methylation by reovirus core-associated methyltransferases.

5′-Terminal Structures Synthesized in Presence of Inorganic Pyrophosphate Compared with Pyrophosphatase. A more direct test of the effect of pyrophosphate on the synthesis of 5′-terminal structures was made by using [β,γ-^{32}P]GTP to label nascent mRNA. [^{3}H]CTP was included in the incubation mixture as an internal label to monitor total RNA synthesis. The products were digested with *Penicillium* (P_1) nuclease and alkaline phosphatase under conditions that hydrolyze phosphodiester linkages to inorganic phosphate but leave intact blocked 5′-structures of the type GpppG and m^7GpppG^m (9). The digests were resolved into the constituent [^{3}H]cytidine, $^{32}P_i$, and ^{32}P-labeled enzyme-resistant structures by high voltage paper electrophoresis. As shown in Fig. 2A, mRNA synthesized with [β,γ-^{32}P]GTP and AdoHcy contained predominantly unblocked, phosphatase-sensitive 5′-ends. These were previously identified as ppG-C (20) and confirmed on the basis that P_1 nuclease digestion without phosphatase treatment yielded the expected amount of [^{32}P]ppG (data not shown). Only 9% of the incorporated ^{32}P was obtained as GpppG. Addition of

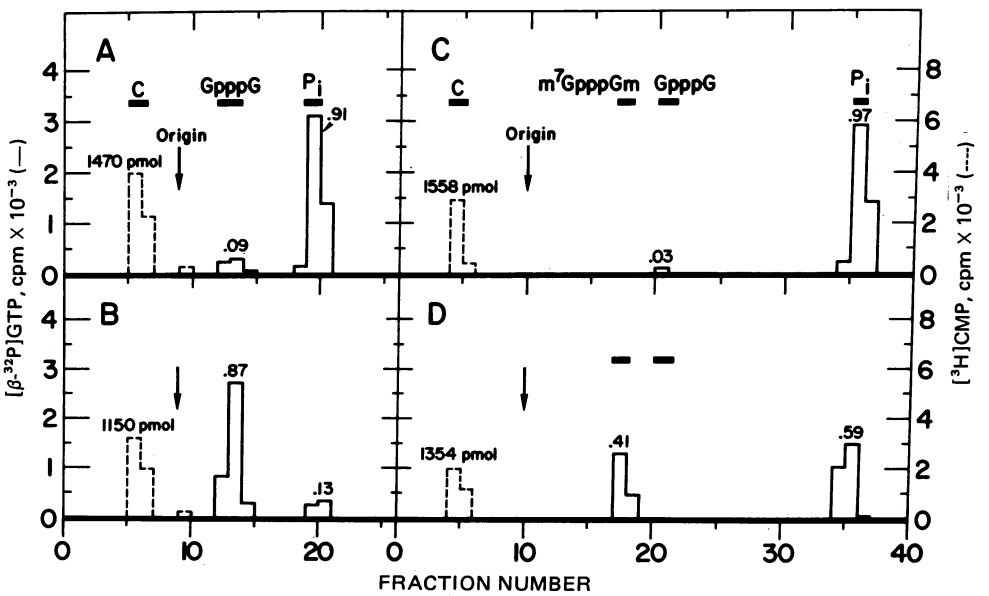

FIG. 2. Synthesis of blocked 5′-structures under different conditions. All reaction mixtures contained Tris·HCl buffer (100 mM), $MgCl_2$ (10 mM), ATP (1 mM), CTP (1 mM), UTP (1 mM), GTP (0.1 mM), phospho*enol*pyruvate (10 mM), pyruvate kinase (9 units/ml), and reovirus cores (5 mg/ml), and were incubated at 45° for 30 min. However, the four experiments differed as follows: (A) the incubation mixture (50 μl) included 21 μCi of [β,γ-^{32}P]GTP, 1 μCi of [^{3}H]CTP, and 0.5 mM AdoHcy; (B) as in panel (A) but with 0.2 unit of inorganic pyrophosphatase; (C) the reaction mixture (100 μl) contained 52 μCi of [β,γ-^{32}P]GTP, 4 μCi of [^{3}H]CTP, 0.5 mM AdoHcy, and 0.5 mM sodium pyrophosphate; and (D) as in panel (C) except AdoHcy was replaced with 0.5 mM AdoMet. In experiments (A) and (B), the entire samples of purified mRNA were analyzed for 5′-terminal structure by paper electrophoresis (45 V/cm, 30 min, pH 3.5) after digestion with P_1 nuclease and alkaline phosphatase (9). For (C) and (D), 25% of the newly synthesized mRNAs were similarly analyzed, but electrophoresis was at 53 V/cm for 60 min. For comparison of total mRNA synthesis, values for [^{3}H]CMP incorporated are expressed as pmol/50 μl.

Table 1. Effect of GTP concentration on the formation of blocked 5'-termini in reovirus mRNA

GTP (mM)	Amount (pmol) and distribution (%) of [^{32}P]phosphate incorporated into 5'-termini of mRNA		[^{3}H]CMP incorporated (nmol) into internal positions
	$G\overset{*}{p}\overset{*}{p}pG$	$\overset{*}{p}pG$	
0.1	0.26 (9%)	2.7 (91%)	1.47
0.5	0.63 (17%)	3.08 (83%)	1.88
1.0	1.39 (29%)	3.40 (71%)	2.24

Reaction mixtures (50 μl) contained 100 mM Tris·HCl (pH 8), 10 mM $MgCl_2$, 1 mM each of ATP, CTP, and UTP, 4 μCi of [^{3}H]CTP (specific activity 17 Ci/mmol), 5 mM phospho*enol*pyruvate, 0.45 unit of pyruvate kinase, and 250 μg of reovirus cores. Mixtures containing 0.1 mM, 0.5 mM, and 1.0 mM GTP included 21, 21, and 31.5 μCi of [β,γ-^{32}P]GTP, respectively. After incubation at 45° for 30 min, the products were separated from radioactive precursors by gel filtration through Sephadex G-100. Enzymatic digestion of the mRNA with P_1 nuclease and alkaline phosphatase and analysis by high voltage paper electrophoresis at pH 3.5 was as described (9).

0.5 mM pyrophosphate to the transcription reaction mixture reduced the yield of 5'-terminal GpppG in mRNA to 3% of the ^{32}P without affecting total RNA synthesis, as measured by [^{3}H]cytidine incorporation (Fig. 2C). This reduction in the yield of blocked 5'-ends by pyrophosphate was overcome by including 0.5 mM AdoMet in the incubation mixture (Fig. 2D). Under these conditions, 41% of the incorporated ^{32}P was resistant to digestion by P_1 nuclease plus phosphatase. The unhydrolyzed ^{32}P-labeled compound corresponds to 5'-terminal m^7GpppG^m, which is derived from GpppG by methylation, a modification that renders blocked ends resistant to pyrophosphorolysis and thus reduces the number of phosphatase-sensitive ppG-C ends (14). To eliminate the formation of ppG-C 5'-termini by pyrophosphate-dependent hydrolysis of GpppG-C ends during transcription, we synthesized mRNA in the presence of inorganic pyrophosphatase. This enzyme hydrolyzes inorganic pyrophosphate to phosphate, which in contrast to pyrophosphate does not inhibit the reovirus guanylate transferase activity at low concentrations (14). The hydrolysis of pyrophosphate, released as mRNA chain elongation proceeded, resulted in a marked increase in the proportion of mRNA molecules with blocked 5'-termini; 87% of the incorporated ^{32}P migrated as GpppG (Fig. 2B).

When mRNA was synthesized in the absence of pyrophosphatase under the conditions shown in Fig. 2A, the concentration of pyrophosphate in the reaction mixture reached 0.1 mM after 30 min. This low concentration was apparently sufficient to drive the step 3 reaction in the reverse direction in the absence of N^7-methylation of the 5'-terminal G. In addition to the effect of pyrophosphate on the relative amount of blocked 5'-termini, it was important to consider possible effects of varying the concentration of GTP, which is a substrate in the step 3 blocking reaction. When the GTP concentration was raised from 0.1 to 1 mM, mRNA synthesis, as measured by either [β,γ-^{32}P]GTP or [^{3}H]CTP utilization, increased about 1.5-fold, and the amount of blocked 5'-termini ($G\overset{*}{p}\overset{*}{p}G$-C) increased 3-fold relative to unblocked $\overset{*}{p}pG$-C ends (Table 1).

On the basis of the preceding observations, transcription reaction mixtures can be designed to allow the synthesis *in vitro* by reovirus cores of viral mRNA with a single type (>90%) of 5'-terminal structure as follows: (*i*) 5'-terminal m^7GpppG^m-C: methyl donor (AdoMet), pyrophosphatase, and a high concentration (0.5 mM) of GTP; (*ii*) 5'-GpppG-C: methylation inhibitor (AdoHcy), pyrophosphatase, and 0.5 mM GTP; and (*iii*) 5'-ppG-C: AdoHcy, exogenous pyrophosphate, and a low concentration (0.1 mM) of GTP. Following these general principles, mRNA synthesized under condition (*i*) contained almost exclusively 5'-terminal m^7GpppG^m (Fig. 3A). Under similar conditions (*ii*), but where methylation was prevented by replacing AdoMet with its analog, AdoHcy, 93% of the products were terminated with the blocked structure, GpppG, and the remainder with ppG (Fig. 3B). In these experiments, an excess (4 units/ml; 1 unit hydrolyzes 1 μmol of pyrophosphate per min at 25° and pH 7) of inorganic pyrophosphatase was used relative to the calculated amount of pyrophosphate generated. However, the same results were obtained with a lower concentration (0.8 unit/ml) of enzyme (data not shown). By reducing the GTP concentration to 0.1 mM and replacing pyrophosphatase with 0.5 mM pyrophosphate (condition *iii*), the ratio of blocked to unblocked ends was reversed. Only 7% of the molecules had GpppG, and the predominant 5'-structure was phosphatase-sensitive ppG (Fig. 3C).

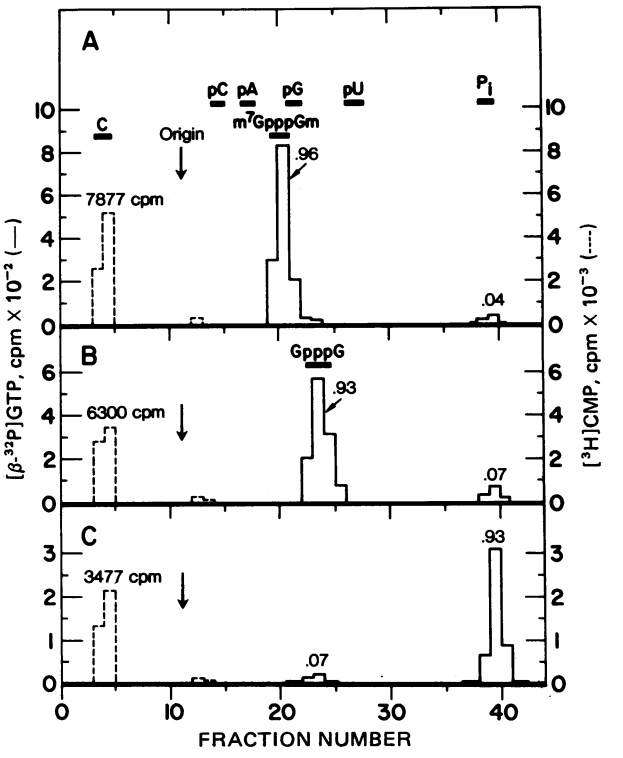

FIG. 3. Conditions for preferential synthesis of mRNA with blocked methylated, blocked unmethylated, or unblocked 5'-termini. Reaction conditions were selected for synthesis of (A) blocked and methylated mRNA, (B) blocked but unmethylated mRNA, or (C) mRNA with unblocked 5'-termini. (A) The incubation mixture (50 μl) contained 100 mM Tris·HCl (pH 8), 10 mM $MgCl_2$, 2 mM each of ATP, UTP, and CTP, 0.5 mM GTP, 21 μCi of [β,γ-^{32}P]GTP, 1 μCi of [^{3}H]CTP, 0.4 mM AdoMet, 10 mM phospho*enol*pyruvate, 0.9 unit of pyruvate kinase, 0.2 unit of inorganic pyrophosphatase, and virus cores prepared from 400 μg of reovirus by chymotrypsin digestion. (B) As in (A) except the AdoMet was replaced by 0.5 mM AdoHcy. (C) as in (B) but inorganic pyrophosphatase was replaced with 0.5 mM pyrophosphate, and a lower concentration (0.1 mM) of GTP was used. After 30 min at 45°, the RNA was isolated (9, 29) and digested with P_1 nuclease and alkaline phosphatase; the products were analyzed by paper electrophoresis as in the legend of Fig. 2. The proportion of ^{32}P incorporated is shown above the peak samples.

Analysis of Transcription Products Made under Different Conditions. All of the double-stranded RNA genome segments

of reovirus are transcribed by the core-associated RNA polymerase (12). The resulting single-stranded viral mRNAs can be separated by sedimentation on glycerol density gradients into three size classes: *l*, *m*, and *s* (12). In order to determine whether pyrophosphate selectively inhibits the synthesis of one class of viral mRNA, unmethylated products were synthesized in reaction mixtures containing [β,γ-^{32}P]GTP and [^{3}H]UTP in the absence or presence of 2 mM pyrophosphate. Analysis by sedimentation in glycerol gradients demonstrated that addition of pyrophosphate reduced by 50% the yield of each of the three reovirus mRNA classes as measured by ^{32}P or ^{3}H incorporation. However, the size and relative amounts of each mRNA class were not changed (data not shown).

Reovirus-associated guanylate transferase required a 5′-terminal diphosphate as substrate for the formation of blocked structures (14). Consequently, if the inorganic pyrophosphatase contained contaminating phosphatase activity, its use for increasing the synthesis of reovirus mRNA with blocked 5′-termini, or intact ppG-C ends, would be limited. mRNA synthesized with [β,γ-^{32}P]GTP and [^{3}H]CTP under conditions that yielded molecules with predominantly 5′-terminal $\overset{*}{p}$pG-C (as in Fig. 3C) was used to assay for contaminating phosphatase(s). Incubation of the ^{32}P-labeled mRNA with inorganic pyrophosphatase for 30 min at 45° under the same conditions as used for mRNA synthesis (Fig. 3) released 3% of the ^{32}P from the 5′-terminal $\overset{*}{p}$pG, as determined by sedimentation analysis. In addition, on the basis of an unaltered sedimentation profile, it appeared that the level of any contaminating RNase activity was below that which would interfere with the use of pyrophosphatase for the preparation of intact, "capped" mRNA. A more stringent test for the synthesis of intact, capped mRNA was made by synthesizing methylated mRNA in the presence of pyrophosphatase and bentonite to minimize cleavage by any contaminating RNase. The mRNAs labeled with [^{32}P]GMP and [^{3}H]methyl were resolved by gradient analysis into *l*, *m*, and *s* classes (Fig. 4A). The ratio of 5′-terminal [^{3}H]methyl radioactivity to internal ^{32}P increased with decreasing chain length. The distribution of ^{3}H-labeled, 5′-terminal m^{7}GpppG^m-C among *l*, *m*, and *s* classes was 7, 28, and 65% of the total ^{3}H radioactivity, respectively. A similar distribution was obtained after denaturation (Fig. 4B). These results confirm that the inclusion of pyrophosphatase in the incubation mixture is useful for the synthesis of intact, capped mRNA.

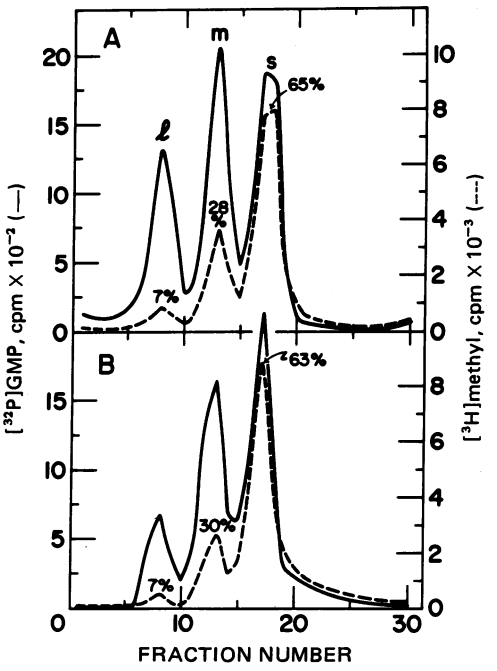

FIG. 4. Sedimentation of reovirus mRNA synthesized in the presence of inorganic pyrophosphatase. Reovirus mRNA labeled at the 5′-termini with [*methyl*-^{3}H] and internally with [^{32}P]GMP was synthesized in the presence of inorganic pyrophosphatase. Reaction mixtures (400 μl) contained 100 mM Tris·HCl (pH 8), 10 mM $MgCl_2$, 2 mM each of ATP, CTP, GTP, and UTP, 3.3 μCi of [α-^{32}P]GTP, 40 μCi of [*methyl*-^{3}H]AdoMet, 0.32 unit of inorganic pyrophosphatase, 12 mM phospho*enol*pyruvate, 3 units of pyruvate kinase, reovirus cores prepared from 500 μg of purified reovirus, and 2 mg of bentonite as a RNase inhibitor. After incubation at 45° for 30 min, virus cores and bentonite were sedimented at 10,000 rpm for 5 min at 4°. The supernatant containing the radioactive mRNA was extracted twice with phenol, and the mRNA was isolated by Sephadex G-100 gel filtration and ethanol precipitation. mRNA was characterized by glycerol gradient centrifugation (28) either before (A) or after (B) denaturation by heating at 68° for 2 min in 80% dimethylsulfoxide solution. The heated RNA solution was immediately diluted 10-fold with redistilled water, and placed onto a preformed 5–30% glycerol gradient. The same amount of unheated RNA was similarly analyzed.

DISCUSSION

Recently, we proposed (14) a mechanism for cap formation during reovirus mRNA synthesis in which (*i*) the formation of the first phosphodiester bond by the virion-associated RNA polymerase precedes cap formation; (*ii*) the resulting nascent oligonucleotide, pppG-C, is converted by another enzyme, nucleotide phosphohydrolase, to ppG-C, which acts as GMP acceptor for blocking by the guanylate transferase; (*iii*) the synthesis of the blocked structure, GpppG-C, from ppG-C and GTP with concomitant release of pyrophosphate is reversible by pyrophosphorolysis, unless the terminal G is subsequently methylated at the N^7 position; and (*iv*) 2′-*O*-methylation of the penultimate G in the cap structure occurs only after N^7-methylation of the terminal G and requires at least one phosphodiester linkage in the substrate oligonucleotide. A similar ability to transfer GMP and block RNA with a 5′-terminal diphosphate, followed by sequential methylation, has also been described for the DNA-containing vaccinia virus (10, 30) and another double-stranded RNA virus, insect cytoplasmic polyhedrosis virus (8, 31). In reovirus-infected L cells, a second 2′-*O*-methylation also occurs in a fraction of the viral mRNAs to form 5′-terminal m^{7}GpppG^m-C^m (Furuichi, LaFiandra, and Shatkin, unpublished results), a structure corresponding to the "cap 2" found in some cellular mRNAs (1).

In the present report, we have established conditions for the preparation of reovirus mRNA with a single type of 5′-terminal structure. We have taken advantage of the features of step 3 in the reaction series (see introduction), which permits control in the forward or backward direction by adjusting the concentration of GTP and pyrophosphate as well as by the subsequent methylation step. By reducing the GTP concentration and adding inorganic pyrophosphate and AdoHcy to the incubation mixture, products were obtained with unblocked 5′-ppG-C. Elimination of pyrophosphate, released during mRNA synthesis, by including inorganic pyrophosphatase in the transcription reaction yielded mRNA with blocked ends, GpppG-C. When the methyl donor, AdoMet, was also present, the 5′-termini were methylated to form m^{7}GpppG^m-C. The different experimental conditions required to obtain the three kinds of 5′-termini were developed on the basis of the reaction series proposed earlier (14, 15) for the synthesis of capped reovirus mRNA by core-associated enzymes *in vitro*. Pyrophosphate promotes hydrolysis of 5′-GpppG-C to 5′-ppG-C (Fig. 3C), and its removal by pyrophosphatase yields mRNA

with blocked ends (Fig. 3B). After N^7-methylation the 5'-structure, m^7GpppG-C, becomes resistant to pyrophosphorolysis, and essentially all molecules are capped in the presence of pyrophosphatase and the methyl donor, AdoMet (Fig. 3A). The present findings thus are consistent with the mechanism of formation of 5'-terminal structures of reovirus mRNA described previously.

In contrast to the situation *in vitro*, the pyrophosphate produced during mRNA chain elongation in reovirus-infected cells may be hydrolyzed by host cell pyrophosphatase(s), possibly enabling the subviral particle-associated enzymes to synthesize capped and methylated viral mRNA. Formation of the 5'-terminal cap apparently occurs at an early stage of reovirus mRNA synthesis (14), as observed for cytoplasmic polyhedrosis virus (32). However, both the blocking and methylation reactions involved in reovirus mRNA cap formation can also occur on nascent, partially completed, 10S mRNA chains (Y. Furuichi and A. J. Shatkin, unpublished results). These results suggest that the reovirus guanylate transferase and methylase activities are independent of the RNA polymerase. Apparently, they can remain at the site of mRNA initiation under conditions where the RNA polymerase has moved several hundred nucleotides away. Therefore, reovirus mRNA synthesis is not necessarily dependent upon cap formation. Under certain experimental conditions capping can occur post-transcriptionally, although under standard conditions caps are synthesized at the initiation stage of reovirus mRNA formation (14, 15). This mechanism may also be favored for cap formation on the 5'-ends of the primary transcripts of cellular DNA that are synthesized by RNA polymerase II, since heterogeneous nuclear RNA is known to contain caps (2, 3). However, if heterogeneous nuclear RNA is processed to smaller mRNA and cleavage is accompanied by capping by a reovirus type of mechanism, a phosphokinase activity would be required to form the 5'-terminal diphosphate at the cleavage site (33). Another type of capping mechanism that may serve as a model for cellular mRNA maturation has been described for vesicular stomatitis virus (7). The mRNA of this virus synthesized *in vitro* by virion-associated enzymes is modified by addition of ppG from GTP to 5'-terminal pA in nascent strands. The nascent chains with a 5'-terminal monophosphate appear after synthesis of a short "leader RNA" sequence (34). They may arise by cleavage of a primary transcript in a fashion analogous to production of phage T7 early mRNA (35) since the five viral mRNAs have the same 5'-sequence which is not complementary to the 3'-terminal sequence of the genome template RNA (36). It remains to be determined if cellular mRNAs are modified at the 5'-end by mechanisms of the reovirus and/or vesicular stomatitis virus types.

For future studies on the formation and function of eukaryotic mRNA caps, the ability to synthesize mRNAs with unique 5'-ends of different structure will be very useful. For example, reovirus 5'-terminal m^7GpppG^m-C, GpppG-C, and ppG-C have been incorporated into a variety of ribopolynucleotides by incubation with polynucleotide phosphorylase under primer-dependent conditions (37). The ribopolymers were then used to demonstrate an effect of both 5'-structure and base composition on the ability to bind to ribosomes. The availability of capped and uncapped viral mRNAs should continue to facilitate studies on eukaryotic mRNA maturation and translation.

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