Mechanism of Formation of Reovirus mRNA 5′-terminal Blocked and Methylated Sequence, m'GpppGm'pC

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YASUHIRO FURUICHI, S. MUTHUKRISHNAN, JENÔ TOMASZ*, AND AARON J. SHATKIN
From the Roche Institute of Molecular Biology, Nutley, New Jersey 07110, and the *Institute of Biophysics, Szeged, Hungary

Blocked and methylated 5′ termini of reovirus mRNA are formed by viral cores at an early stage of transcription. Cores incubated in a complete transcription reaction mixture for 30 s, or in a mixture lacking UTP and ATP for a longer time, synthesize the "cap" structure, m'GpppGm'pC. The dinucleotide ppGpC functions as substrate for a core-associated guanylyltransferase and is converted to GpppGpC by addition of pG from GTP. For optimal conversion, both the diphosphate terminus and phosphodiester bond are required. pGpC is not a substrate, but ppGpC is utilized after removal of the γ-phosphate by a core nucleotide phosphohydrolase. Methyltransferases also present in cores transfer methyl groups sequentially from S-adenosylmethionine (AdoMet) to the N′-position of the 5′-terminal guanosine followed by the 2′-OH of the penultimate guanosine. GpppGpC is hydrolyzed by cores in the presence of pyrophosphate to ppGpC, the predominant 5′-terminal structure of reovirus mRNA made in the absence of S-adenosylmethionine. N′-methylation prevents pyrophosphorolysis of m'GpppGpC, which may explain the increased proportion of blocked, methylated 5′ termini in viral mRNA synthesized in the presence of S-adenosylmethionine. On the basis of these findings, the following reaction series is proposed for the synthesis of reovirus mRNA caps. In the series, AdoHcy is the abbreviation for S-adenosylhomocysteine.

1. \[ \text{pppG} + \text{pppC} \xrightarrow{\text{RNA polymerase}} \text{pppGpC} + \text{PPi} \]
2. \[ \text{pppGpC} \xrightarrow{\text{Nucleotide phosphohydrolase}} \text{ppGpC} + \text{Pi} \]
3. \[ \text{ppG} + \text{pppGpC} \xrightarrow{\text{Guanylyltransferase}} \text{GpppGpC} + \text{PPi} \]
4. \[ \text{GpppGpC} + \text{AdoMet} \xrightarrow{\text{Methyltransferase 1}} \text{m'GpppGpC} + \text{AdoHcy} \]
5. \[ \text{m'GpppGpC} + \text{AdoMet} \xrightarrow{\text{Methyltransferase 2}} \text{m''GpppG''pC} + \text{AdoHcy} \]

Human reoviruses contain a double-stranded RNA genome consisting of 10 distinct segments (1). Like many other eukaryotic viruses, purified reovirions also contain several enzyme activities including RNA polymerase (2, 3). This enzyme transcribes one strand of each duplex genome segment both in vitro and in infected cells (4–9). The resulting single-stranded RNA products correspond to viral messenger RNAs capable of directing the synthesis of virus-specific polypeptides intracellularly and in cell-free systems (9–12). Other reovirion-associated enzymes modify specifically the 5′ ends of the polymerase products. A nucleotide phosphohydrolase activity converts 5′-terminal pppG . . . to ppG . . . (13–15) and in some molecules the 5′ ends are blocked G(5′)ppp(5′)G . . , presumably due to the action of a virion guanylyltransferase activity (16, 17). Methyltransferases are also present in reovirions (18). Consequently, mRNA synthesized in the presence of S-adenosylmethionine contains 5′-terminal m'G(5′)ppp(5′)G''pC . . . (19, 20). This same type of blocked methylated "cap" structure has been found in a wide variety of eukaryotic cellular and viral mRNAs (21). Caps are present in cellular mRNAs isolated from humans, monkeys, rabbits, hamsters, rats, mice, ducks, toads, silkworms, sea urchins, brine shrimp, slime molds, and yeast. Viral mRNAs with 5′-terminal m'GpppN include those from DNA viruses (pox, herpes, adenov, and SV40) and RNA viruses (reov, insect cytoplasmic polyhedrosis, vesicular stomatitis, Newcastle disease, influenza, Sindbis, Semliki Forest, avian sarcoma, murine leukemia, tobacco mosaic, wound tumor, rice dwarf, brome mosaic, and alfalfa mosaic).

The widespread distribution of caps in eukaryotic mRNAs is consistent with an important role in translation. Reovirus and vesicular stomatitis virus-capped mRNAs are translated more efficiently in wheat germ extracts than the unmethylated viral mRNAs (22). Furthermore, chemical removal of 5′-terminal 7-methylguanosine from rabbit globin mRNA results in a reduced ability to direct cell-free protein synthesis (23), and
7-methylguanosine 5'-monophosphate inhibits cell-free globin synthesis (24). The requirement for caps apparently occurs at the level of initiation, because reovirus mRNA containing m'GpppG,,. binds efficiently to wheat germ 40S ribosomal subunits, while mRNA with 5'-terminal pppG or GpppG, binds poorly or not at all (16). Since 5'-terminal m'GpppN may be a primary recognition signal for mRNA-ribosome initiation complex formation, it represents an important functional as well as structural feature of many eukaryotic mRNAs.

Caps are also present in heterogeneous nuclear RNA of mammalian cells (25, 26) and may be conserved during the maturation of various species of cytoplasmic mRNA (27). The unique 5'-3' linkage in the caps, which was first observed in low molecular weight RNA from nuclei of Novikoff hepatoma cells (28), suggests that unusual mechanism(s) are available for modification of 5' termini of eukaryotic RNAs. It was of interest to study the sequence of reactions involved in the synthesis of the blocked and methylated 5'-terminal structure of reovirus mRNA.

EXPERIMENTAL PROCEDURES

Preparation of Reovirus and Reovirus Cores—Reovirus was purified from infected L-cells as described previously (29). Virions were digested with 1 mg/ml of chymotrypsin by incubation at 45° for 30 min in 50 mM Tris/HCl buffer (pH 8) containing 50 mM KCl. The resulting cores were collected by centrifugation (10,000 x g, 15 min, 4°), resuspended in the same buffer, and re-centrifuged. This washing step was repeated twice to remove virus oligo(A) and short oligonucleotides from the core preparations (30, 31).

Assay of Capping Reaction—The standard reaction mixture (100 µl) contained 70 mM Tris/HCl buffer (pH 8), 4 mM MgCl₂, 200 µg of protein as washed reovirus cores, 1 to 2 µCi of [α-32p]GTP (specific activity 300 Ci/mmol, New England Nuclear), 0.8 mM GTP, 10 mM phosphoenolpyruvate, 0.6 unit of pyruvate kinase (Calbiochem), and 0.05 mM dGTP or the indicated nucleotides at the same concentration as GTP. AdoMet or ADOHcy were added where indicated.

Incubation was carried out at 45° for 60 min, and the reaction was stopped by addition of an amount of phenol equivalent to 1/2 volume of the incubation mixture. After phenol extraction at room temperature, the aqueous layer was extracted twice with ether, and the ether was removed by evaporation.

An aliquot of the deproteinized reaction mixture (50 µl) was incubated at 37° for 4 h with bacterial alkaline phosphatase (0.5 unit, Worthington) in order to dephosphorylate residual radioactive GTP. The reaction mixture was adjusted to the corresponding sodium or ammonium salts at pH 7.5, 1 mM MgCl₂. After 3.5 h at 37°, the samples were then lyophilized.

An equivalent amount of diphenylphosphoryl guanosine and pyridinium salt of nucleoside diphosphate were reacted in dry pyridine to give pyridinium salts of nucleoside monophosphates, ADP, CDP, UDP, and GDP were prepared by the corresponding sodium or ammonium salts at pH 7.5 (pH of pyridine form). The samples were then lyophilized.

An equivalent amount of diphenylphosphoryl guanosine and pyridinium salt of nucleoside diphosphate were reacted in dry pyridine/formic acid at room temperature. The yields from the reaction were 20 to 30%, and the resulting products, GpppN (N = A, C, U, and G) were purified by successive paper chromatographic separations in isobutyric acid/0.5 M NH₄OH (10/6 v/v) (solvent 1), followed by 1-propanol/concentrated NH₄OH/H₂O (55/10/35 v/v) (solvent 2). GpppG was obtained as a side product when the formation of active intermediate, diphenylphosphoryl guanosine, was incomplete and residual contaminating 5'-guanylic acid condensed with the intermediate. R₂ values for these compounds by paper chromatography in solvent 1 were: GpppA, 0.25; GpppC, 0.16; GpppU, 0.00; GpppG, 0.08; GpppG, 0.1. In solvent 2 the R₂ values were: GpppA, 0.28; GpppC, 0.28; GpppU, 0.26; GpppG, 0.17; GpppG, 0.21. These purified compounds were completely resistant to alkaline phosphatase digestion and showed ultraviolet spectra consistent with their structure (e.g. a 1:1 mixture of A and G for GpppA), at both acidic and neutral pH. GpppG prepared from Artemia salina was a gift from Dr. A. H. Warner, University of Wisconsin, Madison, Wisconsin.

Preparation of pGpC, ppGpC, and pppGpC—GpC (2 µmol Miles Laboratories) was incubated with 50 units of Escherichia coli polynucleotide kinase (Miles Laboratories) in 0.3 ml of incubation mixture containing 66 mM Tris/HCl buffer (pH 7.6), 10 mM MgCl₂, 16 mM β-mercaptoethanol, and 13 mM [α-32P]ATP (13.04 Ci/mmol) was incubated at 37° for 3.5 h. The methylated triphosphate was separated from unreacted GTP by paper electrophoresis at pH 3.5.

Preparation of pGpC, ppGpC, and pppGpC—GpC (2 µmol Miles Laboratories) was incubated with 50 units of Escherichia coli polynucleotide kinase (Miles Laboratories) in 0.3 ml of incubation mixture containing 66 mM Tris/HCl buffer (pH 7.6), 10 mM MgCl₂, 16 mM β-mercaptoethanol, and 13 mM [α-32P]ATP (final specific activity ~ 0.1 Ci/mmol). After 3.5 h at 37°, GpC was isolated by paper electrophoresis at pH 3.5; recovery of pGpC from GpC was about 80%. ppGpC and pppGpC were synthesized as described (37).

Preparation of pnApG—pnApG was prepared as described for pGpC by the addition of phosphate to the 5'-OH of ApG with polynucleotide kinase (38). Addition of a second phosphate to the 5' end of pnApG was accomplished by a modification of the method of Rapaport and Zamecnik (39). pnApG was phosphorylated at both 5' and 3' ends with the addition of [γ-32P]ATP (3.5 Ci/mmol) and [α-32P]ATP (13.04 Ci/mmol) purchased from Amerimed. The products were separated on a 5% polyacrylamide gel (31.38 Ci/mmole) and [α-32P]ATP (13.04 Ci/mmol) were purchased from I.C.N.

RESULTS

Blocking and Methylation of Nascent Reovirus Uridygotonucleotides at an Early Stage in Transcription

Cytoplasmic polyhedrosis virus, another double-stranded RNA virus, synthesizes mRNA with 5'-terminal m'GpppA,. . . when incubated in a reaction mixture containing ribonucleoside triphosphates and AdoMet (40). Transcription is almost completely dependent on the presence of AdoMet, and caps are present in short nascent RNA chains suggesting that blocking and methylation are essential steps in the initiation of viral mRNA (41). By contrast, enzymes in wheat germ extracts (23) can convert the 5'-terminal ppG. . . of reovirus mRNA to m'GpppG,. . . , and guanylyltransferase and methylase activities from vaccinia virus can convert 5'-pppN . . . to m'GpppN . . . in vaccinia mRNA, synthetic poly(A) and poly(G) (42, 43). In order to determine if m'GpppG,. . . is synthesized as an initial or a final step in reovirus mRNA formation, nascent
RNA products of reovirus cores were examined for the presence of modified 5' termini.

RNA synthesis was initiated by addition of magnesium acetate to standard reaction mixtures containing chymotrypsin-digested, washed reovirus cores and [α-32P]GTP and [Me-3H]AdoMet as radioactive precursors. After incubation for the indicated intervals at 37\(^\circ\), reaction mixtures were extracted with phenol and the products were analyzed by filtration on calibrated columns of Sephadex G-75 (Fig. 1A). As shown in Fig. 1, B, and C, RNA of size sufficient to be excluded from the gel became apparent between 30 s and 1 min of incubation and subsequently increased in amount (Fig. 1D). The 32P/3H ratio was 0.3 in the products of a 1-min incubation as compared to 0.54 after 2 min, consistent with the presence of 5'-terminal [3H]methyl groups in the incomplete, internally 3P-labeled chains. Although no large RNA was synthesized in a 30-s reaction, the gel elution profile included 3H-labeled material in the position of an oligonucleotide marker of chain length 12 to 18 nucleotides (Fig. 1B). The [3H]methyl-labeled material which presumably corresponds to blocked and methylated nascent products was pooled as indicated and digested with P1 nuclease to produce 5'-mononucleotides and unhydrolyzed caps (19, 32). The digestion products were further incubated with bacterial alkaline phosphatase and analyzed by paper chromatography. Fig. 2 (panel I) shows that component I, which corresponds to oligonucleotides of average chain length (about 15 nucleotides) (44), contains all the \(^3\)H radioactivity in the cap structure, m\(^G\)pppG\(^m\). Component II, which is shorter in length than component I and elutes close to the position of GTP and AdoMet, consists of a mixture of oligonucleotides containing either the dimethylated cap, m\(^G\)pppG\(^m\) (93%) or the monomethylated cap, m\(^G\)pppG (7%) (Fig. 2, panel II). The results demonstrate that reovirus nascent mRNA of chain length 15 nucleotides or less already have blocked and methylated 5' termini. Some of the shorter nascent chains contain an incompletely methylated cap, m\(^G\)pppG, suggesting that methylation of the terminal guanosine precedes that of the penultimate residue.

In order to determine how early in their synthesis nascent chains are blocked and methylated, the products of a reaction mixture containing unwashed cores, [Me-3H]AdoMet, GTP, and CTP but no UTP and ATP were analyzed. Under these conditions, chain elongation is arrested at the sites of pU and

**Fig. 1.** Analysis of nascent mRNA products of reovirus cores by gel filtration. Synthesis of mRNA by reovirus cores was stopped at early stages of the transcription reaction (B, C, and D = 30, 60, and 120 s, respectively) by addition of phenol. The phenol extracts were applied to a calibrated column (1 x 70 cm) of Sephadex G-75. Elution was carried out with 0.02 M Tris/HCl buffer (pH 7.6), and aliquots (0.1 ml) of fractions (0.5 ml) were monitored for radioactivity. Each incubation mixture (0.5 ml) contained 70 mM Tris/HCl (pH 8), 50 mM KCl, 2 mM ATP, 2 mM CTP, 2 mM UTP, 0.5 mM GTP, 24 μCi of [α-32P]GTP (83 Ci/mmol, New England Nuclear), 40 μCi of [3H]-AdoMet, 7 mM MgCl\(_2\), and 600 μg of washed reovirus cores. Incubation mixtures without MgCl\(_2\) were warmed for 1 min at 35°C and transcription initiated by the addition of MgCl\(_2\). Incubation was at 35°C. ---, [3H]; ----, [32P].

**Fig. 2.** Analysis of \(^3\)H-methylated 5'-terminal structures of nascent reovirus mRNA. Fractions 35 to 45 (I) and 46 to 50 (II) were pooled from the 30-s and 60-s reactions (Fig. 1, B and C). The products in pools I and II were concentrated by adsorption to a small column (0.6 x 3 cm) of DEAE-cellulose, elution with 2 M NH\(_4\)HCO\(_3\), and lyophilization. Components I (upper) and II (lower) were each dissolved in 0.2 ml of 2 mM sodium/acetate buffer, pH 6.0, and digested with 20 μg of P1 nuclease at 37°C for 1 h. The reaction mixtures were adjusted to pH 8.0 and incubated with 1 unit of alkaline phosphatase (Worthington) at 37°C for 30 min. The digested compounds were analyzed by paper chromatography in isobutyric acid/0.5 M NH\(_4\)OH (10/6 v/v) with authentic markers, m\(^G\)pppG and m\(^G\)pppG\(^m\). \(^3\)P radioactivity (not shown in the figure) migrated in the position of P1, i.e. faster than the pG marker.
pA insertion, and short oligonucleotides accumulate. Electrophoresis on DEAE-cellulose paper (Fig. 3, panel I) separated several \(^{3}H\)metethyl-labeled components including the longer products of high negative charge near the origin (A and B), nascent chains of intermediate length (C), and material in the position of marker m'GpppGpC (D). These fractions were eluted, digested with P, nuclease, and analyzed separately by paper chromatography with marker compounds, m'GpppG and m'GpppGm. The larger nascent products (A and B) yielded both mono- and dimethylated caps, and more radioactivity was present as m'GpppGm in fraction A which contained the longest oligonucleotides (Fig. 3, panels II and III). The nascent chains of intermediate size (C) and very short products (D) contained almost exclusively monomethylated caps, m'GpppG (Fig. 3, panels IV and V). The minor components migrating in the position of nucleosides in panel IV were not identified. No structures of the type, GpppGm were detected under any conditions, again consistent with N'-methylation occurring before 2'-O-methylation. The results in Figs. 1 to 3 indicate that reovirus mRNA caps are formed by core-associated enzymes at the beginning of transcription.

FIG. 3. Analysis of methylated cap structures synthesized by reovirus cores in incomplete reaction mixtures. A transcription reaction mixture without UTP and ATP was used to obtain nascent 5'-oligonucleotides of reovirus mRNA. Incubation mixture (150 ~1) containing 70 mM Tris/HC1 (pH 8.0), 10 mM KCl, 2 mM CTP, 2 mM GTP, 5 mM phosphoenolpyruvate, 100 mM of reovirus, and 50 mM of chymotrypsin was incubated at 45° for 30 min to convert virions to cores. RNA synthesis was started by the addition of 5 mM magnesium acetate together with 30 ~M [\(^{3}H\)]AdoMet and 0.6 unit of pyruvate kinase in a final volume of 0.2 ml. After 1 h at 45°, the mixture was phenol-extracted. The residual phenol was removed by ether extraction, and the products were analyzed by DEAE-cellulose paper electrophoresis in 7% formic acid at 50 V/cm for 5 h. Strips (1 cm) were cut and counted in toluene-based scintillation fluid (A). Strips from the bracketed regions A to D were extracted with fresh 1 M triethylammonium bicarbonate solution and the eluted material lyophilized. Each was then digested with P, nuclease and alkaline phosphatase, and the digests were analyzed by paper chromatography with authentic marker compounds in isobutyric acid/0.5 M NH_2OH (10/6 v/v) solvent.

Cap Formation in Reovirus Cores

When the partial incubation mixture contained [\(\alpha-^{32}P\)]GTP, CTP, and AdoMet, but no UTP or ATP, caps were found in trinucleotides (see Table III, Experiment 2). However, in a reaction mixture containing [\(\alpha-^{32}P\)]GTP as the only ribonucleoside triphosphate, the synthesis of blocked structures was reduced by more than 90%, suggesting that caps are added after the first phosphodiester bond is formed in nascent mRNA chains. Since restoration of ATP or UTP did not increase cap formation, while CTP had a marked stimulatory effect (see Table III, Experiments 1 and 2), the possibility that caps can be formed from GTP plus CTP was considered. Reovirus mRNA synthesized in the absence of AdoMet or in the presence of the competitive inhibitor of methylation, AdoHcy, consists of a mixture of molecules with 5' termini that are predominantly unblocked ppGpC. This suggested that blocked 5' termini in nascent mRNA are formed by the condensation of GTP with 5'-terminal ppGpC to yield GpppGpC. In order to define the blocking reaction more clearly, various substrates including ppGpC were tested as precursors of reovirus mRNA caps.

Conversion of ppGpC to GpppGpC by Transfer of pG from pppG—The dinucleotide, ppGpC, which corresponds in sequence to unblocked 5' termini of reovirus mRNA was incubated for 1 h with reovirus cores and [\(\alpha-^{32}P\)]GTP in the presence of AdoHcy. The incubation mixture was phenol-extracted, treated with alkaline phosphatase, and an aliquot was analyzed by paper electrophoresis (Fig. 4A). Most of the \(^{32}P\) was released from the residual [\(\alpha-^{32}P\)]GTP as \(^{32}P\)\(_{i}\), and the material at the origin did not correspond to blocked structures since it was also converted to P, by digestion with phosphatase and P, nuclease (data not shown). A peak of phosphatase-resistant radioactivity remained near pA, the migration position determined previously for GpppGpC. This \(^{32}P\)-labeled product had a net negative charge of about -4 by DEAE-cellulose chromatography and eluted between GpppG and m'GpppGm marker compounds, as expected for GpppGpC (Fig. 4B, inset). The \(^{32}P\)-labeled presumptive GpppGpC migrated by paper chromatography as a single component which yielded the slightly faster moving G'pppGpC (Fig. 4B, inset). The \(^{32}P\), nucleoside-alkaline phosphatase-resistant material was converted to \(5'G\) by digestion with nucleotide pyrophosphatase, the small amount of \(5'G\), presumably resulting from contaminating phosphatase (Fig. 4C). Thus, guanylyltransferase activity in reovirus cores can block ppGpC by transfer of \(5'G\) from ppGpC, producing GpppGpC. On the basis of the amount of \(^{32}P\) radioactivity incorporated into GpppGpC, about 20% (1 nmol) of the input ppGpC was "capped" under these conditions.

Modification of ppGpC to Form Methylated, Blocked Structures—Blocking of ppGpC by reovirus cores was also studied under conditions of methylation, i.e., in the presence of \(\alpha-^{32}P\)GTP and [\(\alpha-^{32}P\)]AdoMet. The alkaline phosphatase-resistant \(^{32}P\) and \(^{3}H\)-labeled products were again separated by paper electrophoresis (Fig. 5A). The material indicated by the bracket was eluted, digested with P, nuclease, and resolved into three radioactive peaks by paper chromatography (Fig. 5B). Peak I which comprised the predominant \(^{3}H\)-labeled product contained no \(^{3}H\)radioactivity and migrated with marker GpppG indicating that it was derived from the blocked, unmethylated GpppGpC. Further analysis of peak II by paper electrophoresis after treatment with nucleotide pyrophosphatase revealed the presence of \(^{3}P\)- and \(^{3}H\)-labeled 7-methy-
Formation of m°GpppG

The standard reaction mixture for the capping reaction was supplemented with 0.1 mM AdoHcy, incubated, phenol-extracted, and treated with alkaline phosphatase as described under “Experimental Procedures.” A, an aliquot (20 μl) was analyzed by paper electrophoresis. B, the phosphatase-resistant material in another aliquot that migrated in the position of pA was extracted from the paper and divided into three fractions. The first was directly analyzed by chromatography (solid line), and the second fraction was digested with P1 nuclease before paper chromatography in isobutyric acid/0.5 M NH4OH (10/6 v/v) as solvent (shaded area). A third fraction was analyzed to determine its net charge by chromatography on a DEAE-cellulose column (0.6 × 25 cm) with 0.05 to 0.3 M NaCl linear gradient elution (100 ml of each in 50 mM Tris/HCl buffer, pH 8, containing 7 M urea). B, inset. Standard markers included pancreatic ribonuclease-digested tRNA (A260 = 10), [32P]GpppG, and [3H]m°GpppG·pC. C, the presumptive GpppG (B, shaded area) was extracted, digested with nucleotide pyrophosphatase, and the resulting products were analyzed by paper electrophoresis.

FIG. 4. Conversion of ppGpC to GpppGpC by reovirus guanylyltransferase. The standard reaction mixture for the capping reaction was supplemented with 0.1 mM AdoHcy, incubated, phenol-extracted, and treated with alkaline phosphatase as described under “Experimental Procedures.” A, an aliquot (20 μl) was analyzed by paper electrophoresis. B, the phosphatase-resistant material in another aliquot that migrated in the position of pA was extracted from the paper and divided into three fractions. The first was directly analyzed by chromatography (solid line), and the second fraction was digested with P1 nuclease before paper chromatography in isobutyric acid/0.5 M NH4OH (10/6 v/v) as solvent (shaded area). A third fraction was analyzed to determine its net charge by chromatography on a DEAE-cellulose column (0.6 × 25 cm) with 0.05 to 0.3 M NaCl linear gradient elution (100 ml of each in 50 mM Tris/HCl buffer, pH 8, containing 7 M urea). B, inset. Standard markers included pancreatic ribonuclease-digested tRNA (A260 = 10), [32P]GpppG, and [3H]m°GpppG·pC. C, the presumptive GpppG (B, shaded area) was extracted, digested with nucleotide pyrophosphatase, and the resulting products were analyzed by paper electrophoresis.

The digestion products included [3H]- and [32P]-labeled 7-methylguanosine monophosphate which migrated close to the origin and [3H]-labeled 2'-O-methylguanosine monophosphate; both compounds were also identified by paper chromatography. These are the components expected from m°GpppG·pC.
pyrophosphatase cleavage. It should also be noted that the ratio of [\(^{1}H\)]methyl to [\(^{32}P\)]radioactivity in the purified m\(^{7}G\)pppG\(^{m}\) was about twice that in the m\(^{7}G\)pppG, in agreement with the proposed structure. Thus, the structures in peaks I, II, and III are GpppG, m\(^{7}G\)pppG, and m\(^{7}G\)pppG\(^{m}\) derived from GpppGpc, m\(^{7}G\)pppGpc, and m\(^{7}G\)pppG\(^{m}\)pc, respectively.

In addition to GpppGpc and m\(^{7}G\)pppG\(^{m}\)pc synthesized from ppGpc, low levels of GpppG, and m\(^{7}G\)pppG were also detected in the phosphatase-treated products in Fractions 11 to 12 and Fraction 8, respectively, of Fig. 5A). They were identified by paper chromatography with authentic marker samples as in Fig. 5B (data not shown). These compounds presumably were formed in a limited reaction involving condensation of 2 molecules of ppGpG. The quantities of the different 5'-terminal structures synthesized by reovirus cores in the presence of AdoMet are summarized in Table I. Conversion of ppGpc to GpppGpc appears to be the most efficient reaction and is at least 20-fold more effective than ppGpG condensation for the formation of the blocked structure, GpppG. N'-methylation of the terminal guanosine in GpppGpc (or GpppG) was incomplete in these partial reaction mixtures, and only about 10% of the products were methylated. The second methylation, i.e. 2'-O-methylguanosine formation, of the blocked trinucleotide was even more limited (<1%), as observed for short nascent chains synthesized in an incomplete reaction mixture (Fig. 3).

Previously it was found that the initiation of transcription by purified cytoplasmic polyhedroa virus was almost completely dependent upon the presence of AdoMet in the incubation mixture (41). This was in contrast to viral mRNA synthesis by reovirus cores which was not increased by addition of AdoMet (18). Similarly, the formation of reovirus cores of \(^{32}P\)-labeled blocked structures from ppGpc and [\(^{32}P\)]GTP was not increased by AdoMet or AdoHcy. The incubation mixture (100 &mu;m) contained 0.2 &mu;m Tris/HCl (pH 8.0), 5 &mu;m MgCl\(_2\), 5 &mu;m MnCl\(_2\), 2 &mu;m GTP, 5 &mu;m ppGpC, 0.8 &mu;m GTP, 5 &mu;m phosphoenolpyruvate, 1 unit of pyruvate kinase, 100 &mu;g of washed reovirus cores, and 1 mm AdoMet or 1 mm AdoHcy. Incubations were at 45° for 45 min. Incubations were stopped by the addition of phenol. Analysis was performed by paper electrophoresis as in Figs. 4 and 5. Blocked structures (GpppGpc and m\(^{7}G\)pppG\(^{m}\)pc) separated in the electrophoreograms were counted and the radioactivity plotted versus reaction time. 

**Modification of ppGpc for Synthesis of Blocked, Methylated Structures**—The formation of [\(^{1}H\)]methyl-labeled blocked structures was also studied with [Me-\(^{1}H\)]AdoMet, unlabeled GTP, and ppGpc (Table II). Like ppGpG, ppGpc was converted to a mixture of the capped structures, m\(^{7}G\)pppGpc and m\(^{7}G\)pppG\(^{m}\)pc. Although the yield of methylated, blocked compounds with ppGpG was 70% of that obtained with ppGpc, the distribution of mono- and dimethylated compounds produced from each was almost identical. These data indicate that the y-phosphate of pppGpc is removed by the core-associated phosphohydrolase (13-15), and the resulting ppGpc used for capping and methylation. Consistent with this suggestion, it was found that ppGpc was converted to ppGpG at a rate of about 0.02 nmol/mg of cores/h at 45°, under similar incubation conditions. 0.2 mmole of caps in mRNA were formed per mg of reovirus cores/h. Thus, the core-associated phosphohydrolase apparently is required to modify 5'-terminal ppGpc in nascent chains before blocked structures are synthesized.

**Substrate Utilization for Blocked Structure Synthesis**—A variety of different substrates were tested as precursors for the synthesis of blocked structures by reovirus cores. As shown in Table III, condensation of GTP to form GpppGpc occurred to a limited extent and was not affected by addition of GDP, UTP,
or ATP (Table III, Experiment 1). However, when cores were incubated with GTP plus CTP, the formation of blocked structures increased by 20 fold. The products were predominantly GpppGpC. Addition of GDP or UTP partially inhibited the reaction. GpppGpC formation from GTP was also decreased upon addition of CTP, i.e. under conditions that promoted GpppGpC synthesis (Table III, Experiment 2). The yield of GpppGpC as compared to GpppGpC is consistent with phosphodiester bond formation before transfer of the 5'-terminal pG in the synthesis of blocked structures (Fig. 4). Further evidence for this suggestion was obtained by comparing the dinucleotides, ppGpC, ppGpC, or pGpC as precursors of caps. The highest yield of blocked structures was obtained with GTP plus ppGpC (Table III, Experiment 3). The dinucleotide with a 5'-triphosphate end functioned to a lesser extent, presumably because prior conversion to ppGpC by the nucleotide phosphohydrolase was required. The 5'-monophosphate-containing pGpC was not utilized as a substrate for the blocking reaction. ppApG, which has the same general structure but differs in nucleotide sequence from the 5'-terminal ppGpC of unblocked reovirus mRNA, was not used as a substrate for the blocking reaction by reovirus cores (Table III, Experiment 4). When pGpC or pApG was added to the blocking reaction, only the former inhibited cap formation from ppGpC plus GTP (23% reduction in the presence of 2/1 molar ratio of pGpC to ppGpC). These results are consistent with the blocking of nascent mRNA chains that are base-paired with the template minus strand of reovirus genome RNA. Other compounds were also tested as substrates for the guanylyltransferase. [α-32P]GDP and [α-32P]m'GTP or [γ-32P]GTP were not active, confirming the requirement for GTP (Table III, Experiment 5). No 32P-labeled GpppGpC was synthesized when [β,γ-32P]GTP rather than [α-32P]GTP was used as donor, consistent with transfer of only the α-phosphate of ppG to the diprophosphate-terminated acceptor, ppGpC.

Effects of Pyrophosphate on Blocked Structure Synthesis
Pyrophosphate, which is formed by reovirus cores in both the RNA polymerase and guanylyltransferase reactions is an effective inhibitor of blocked structure synthesis. At concentrations of 0.05 and 0.5 mM the yield of GpppGpC from ppGpC

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Table III

Table III

| Experiment | Substrates | Blocked structures |
|------------|------------|--------------------|
|            |            | GpppGpC | GpppGpC' |
| 1          | GTP        | 0        | 12        |
| GTP + GDP  | 0          | 12        |
| GTP + UTP  | 0          | <10       |
| GTP + ATP  | 0          | <10       |
| 2          | GTP + CTP  | 280      | 8         |
| GTP + CTP + GDP | 292 | <10 |
| GTP + CTP + UTP | 218 | 6 |
| 3          | GTP + ppGpC | 910      | 8         |
| GTP + ppGpC' | 72       | <10       |
| GTP + ppGpC | 0          | 11        |
| 4          | GTP + ppGpC | 270      | 8         |
| GTP + ppGpC | 207       | 15        |
| GTP + ppGpC + pGpC | 261 | 7 |
| GTP + ppGpC + pApG | 215 | 15 |
| 5          | ppGpC + GDP | 0        | 0         |
| ppGpC + m'GTP | 0        | 0         |
| ppGpC + β,γ-32P-GTP | 0       | 0         |
| 6          | GTP + pyrophosphate | 0       | 0         |
| GTP + CTP + pyrophosphate | 0       | 0         |
| GTP + ppGpC + pyrophosphate | 0       | 0         |
| GTP + CTP + phosphate | 205 | <10 |

* The fractions containing GpppGpC also include smaller amounts of m'GpppGpC (~6% of total pmol formed) and m'GpppGpC (~1% of total); the GpppGpC' fractions contained m'GpppGpC (10% of total).

* Product identified only on basis of electrophoretic mobility at pH 3.5.

* GpppG.
and GTP was reduced by 70% and 95%, respectively (Fig. 7 and Table III, Experiment 6). At 2 mM pyrophosphate, a concentration which reduced mRNA chain elongation by only 50% (data not shown), no blocked structures were detected using ppGpC as substrate (Table III, Experiment 6). By contrast, a 50-fold higher concentration of inorganic phosphate (0.1 M) decreased GpppGpC synthesis by 25% and mRNA synthesis by 28%.

The products synthesized by the reovirus-associated RNA polymerase in the presence of AdoHcy included molecules with 5'-terminal GpppGpC (27%) and ppGpC (71%) (16). The ratio of RNA chains with blocked versus unblocked 5' termini was reversed when methylated RNA products were synthesized: m'GpppGmpC (75%) and ppGpC (25%). Some unblocked ends probably result from progressive inhibition of guanylyl transfer by pyrophosphate accumulated during polymerization (Fig. 7). An increase in the proportion of unblocked termini in unmethylated mRNA would also be expected if pyrophosphate promoted reversal of the capping reaction, (ppGpC + GTP = GpppGpC + PP,) but not the pyrophosphorylase of the methylated cap, m'GpppGpC. This possibility was tested by comparing GpppGpC and m'GpppGpC as substrates for pyrophosphorylase. GpppGpC was hydrolyzed by reovirus cores to the extent of 19% after incubation for 75 min in the presence of pyrophosphate, and the resulting cleavage products were partially resolved from the starting substrate by paper electrophoresis (Fig. 8A). The bracketed fractions were eluted and the products identified by paper chromatography as predominantly GDP (Fig. 8B) The GDP probably was derived from the GTP product by the action of the highly active core-associated nucleotide phosphohydrolase (1 pg of cores converts 20 pmol of GTP to GDP in 15 min at 45°C). In contrast, m'GpppGpC was not hydrolyzed under these conditions (Fig. 8C). GpppG also was not degraded by pyrophosphorylase (data not shown), consistent with the above results that (a) cap formation probably does not occur by GTP condensation, and (b) the substrate for reovirus mRNA cap synthesis includes a minimum of one phosphodiester bond, i.e. ppGpC. Thus, the N'-methylation of m'GpppGpC prevents pyrophosphorylation and may account for the greater proportion of capped molecules in methylated reovirus mRNA.

**Proposed Mechanism of Reovirus Cap Synthesis**

Together with previous findings (17), the data shown are consistent with cap synthesis occurring during mRNA formation on reovirus double-stranded template RNA as follows.

**Reovirus Transcription**

\[ (+)^{m'GpppG}pCpUp \rightleftharpoons ApUpC^3 \]

**Genome RNA.**

\[ (--) \]

\[ C_pGpAp \rightarrow UpApGpp_4 \]

**m'GpppGpC**

\[ (+)^{m'GpppG}pCpUp \rightleftharpoons C^3 \]

**m'GpppGpC + AdoMet Methyltransferase 1**

\[ m'GpppGpC + AdoHcy \]

\[ m'GpppGpC + AdoMet Methyltransferase 2 \]

\[ m'GpppGpC + AdoHcy \]

This proposed mechanism of reovirus mRNA cap synthesis implies that formation of the first phosphodiester bond on the genome double-stranded template RNA precedes guanylyl transfer to the 5'-diphosphate end, i.e. it requires that ppGpC, but not ppG, be a substrate for blocking by reovirus cores in the presence of GTP. As seen in Table III, a small amount of GpppG was formed by GTP condensation. However, it appears to be not a precursor of caps, but a side product formed in the incomplete reaction mixture because when caps were synthesized from ppGpC and GTP or from GTP plus CTP, GpppG formation decreased as compared to the quantity synthesized with GTP alone. Furthermore, the rate of GpppG formation is insufficient to account for the amount of cap structures made during mRNA synthesis. For example, in the experiment shown in Fig. 1C, 200 μg of reovirus cores produced 1.6 pmol of capped and methylated nascent RNA in 2 min. This amount of cores in the same time interval produced only 0.3 to 0.4 pmol of GpppG when incubated with GTP. In the presence of GTP plus CTP, 10 pmol of GpppGpC were formed. Since the rate of methylation is about one-tenth that of the capping reaction (Table I), the amount of GpppGpC synthesized from GTP plus CTP compares favorably with the quantity of capped and methylated nascent mRNA synthesized.

**Specificity of Reovirus Methyltransferases**

Although GpppG is not hydrolyzed by reovirus cores in the presence of pyrophosphate, it is methylated at the N'-position of a guanosine residue by core-associated methyltransferase (Table IV). The N'-methyltransferase activity requires as substrate 2 guanosine residues linked through 3 phosphates. GTP and blocked structures containing other nucleosides or a different number of phosphates in the bridge are methylated poorly or not at all. As expected on the basis of the proposed mechanism of synthesis of blocked structures, GpppGpC was severalfold better as a substrate for methylation than GpppG. The reovirus methyltransferase which modifies the N'-position of guanosine in caps (N'-G-methylase) has a strict specificity for the 5'-terminal guanosine since it does not methylate.

**Table IV**

| Compound | [1H]Methyl incorporated |
|----------|-------------------------|
|          | pmol                    |
| Gppp     | 0.3                     |
| GpppA    | 0                       |
| GpppC    | 1                       |
| GpppU    | 0                       |
| GppG     | 2                       |
| GpppG    | 28*                     |
| GpppGpC  | 0                       |
| GpppGpC  | 151*                    |

* m'GpppG.
* m'GpppGpC (80%) and m'GpppG*pC (20%).
internal nucleotides in nascent mRNA and recognizes only blocked structures consisting of 2 guanosine residues linked 5'-5' through 3 phosphates. Such a strict substrate recognition may be a property characteristic of the reovirus-associated enzyme. The N7-G-methylase solubilized and purified from vaccinia virus methylates GpppGp(Gp),G and GpppAp(Ap),A, producing m7GpppGp(Gp),G and m7GpppAp(Ap),A in the absence of viral genome DNA (42, 43). The purified vaccinia methylase does not require metal ions for activity. In contrast, the reovirus core-associated N'-G-methylase, one of the several activities in the transcriptional complex, apparently requires Mg2+ ion for activity (Table V). The cation blocked structures consisting of 2 guanosine residues linked 5'-5' through 3 phosphates. Such a strict substrate recognition may be a property characteristic of the reovirus-associated methylase.

Identification of the Intermediate ppGpC in the Synthesis of GpppGpC

Reovirus cores synthesize the 5'-terminal blocked structure of mRNA, GpppGpC, from GTP plus CTP (Table III). The proposed reaction series for its synthesis includes ppGpC and pppGpC as intermediates. Since 2 mM pyrophosphate almost completely inhibits reaction 3 in the series, i.e. the formation of GpppGpC from ppGpC plus GTP (Fig. 7), it was of interest to test for the accumulation of ppGpC as an intermediate when cores were incubated with [α-32P]GTP, [14C]CTP, and 2 mM pyrophosphate. As shown in Fig. 9A, electrophoretic analysis of a reaction mixture incubated under these conditions included several radioactive compounds in material in the position of ppGpC. However, because of the presence of residual radioactivity including [14C]CDP, it was difficult to determine directly if ppGpC was present. In order to assay for 14C-labeled, phosphodiester bond-containing compounds: pGpC, ppGpC, and pppGpC, samples were eluted from the electropherogram. An aliquot of each eluate was exclusively digested with alkaline phosphatase and re-analyzed by electrophoresis for the presence of 14C-labeled GpC (Fig. 9B). A small amount of radioactivity was obtained from the fractions corresponding to the positions of GpC and pGpC, but the bulk of the phosphatase-resistant 14C radioactivity was recovered in fractions corresponding to the position of marker ppGpC. The calculated amount of 14C-labeled ppGpC plus pppGpC synthesized in the presence of 2 mM pyrophosphate was 160 pmol as compared to the value of 280 pmol of GpppGpC synthesized under similar conditions but without added pyrophosphate (Table III, Experiment 2).

In order to identify further the major 14C-labeled product, another aliquot of the radioactive material from the region of ppGpC (Fig. 9A, bracket) was analyzed by Dowex 1 chromatography with marker compounds. Again, a peak of 32P-labeled material with coincident 14C radioactivity eluted in the position of ppGpC (Fig. 9C). After phosphatase digestion, the double-labeled material was converted to [14C]GpC plus 32P-labeled inorganic phosphate (Fig. 10A). In addition, treatment of the double labeled ppGpC with RNase yielded [14C]-labeled pG and 32P-labeled ppG and pG, the latter possibly due to contaminating pyrophosphatase (Fig. 10B).

**DISCUSSION**

Reoviruses consist of an outer protein shell enclosing an inner core which contains the segmented, double-stranded RNA genome. One strand ("minus") of each of the 10 duplex segments is transcribed by a core-associated RNA polymerase. Transcription is probably end to end since the resulting single-stranded ("plus") products function as precursors of double-stranded RNA as well as messenger for viral protein synthesis (44). Furthermore, the 5'-terminal sequences of the mRNA and the corresponding strand in genome RNA are the same: m7G(5')ppp(5')G(5')pC (45).

Synthesis of caps during transcription is catalyzed by several
This is accomplished for all reovirus mRNA classes by pG phosphates, only GTP (and not GDP) is used for capping of ppGpC. RNA is... Among the four ribonucleoside triphosphates, only GTP (and not GDP) is used for capping of ppGpC. This is accomplished for all reovirus mRNA classes by pG transfer from [α-32P]GTP. Furthermore, when [β,γ-32P]GTP was tested as substrate, the β-phosphate was incorporated into mRNA at the 5′ terminus of ppG, but conversion of unlabeled ppGpC to GpppGpC in the presence of [β,γ-32P]GTP did not yield radioactivity in the products. Among the in vitro mRNA products of reovirus cores are molecules with 5′-terminal ppGpC. They comprise 75% of the total in preparations of unmethylated RNA made in the absence of AdoMet or presence of AdoHcy, but only 25% under conditions of methylation. Some molecules could escape the blocking reaction if polymerization were faster than capping and if the RNA polymerase and guanylyltransferase both moved away from the transcription initiation site. The unblocked 5′ ends may also arise from one or more effects of pyrophosphorylase on the blocking reaction. First, the guanylyltransferase appears to be inhibited by pyrophosphate, as is the core-associated RNA polymerase. Because the transferase activity is more sensitive to the inhibitor, capping would be differentially and progressively reduced as polymerization proceeds with concomitant release of pyrophosphate. Second, pyrophosphorylase of GpppGpC 5′ ends, i.e. reversal of the capping reaction, would yield ppGpC plus GTP (Fig. 8). The back reaction would be prevented in 5′ termini that contain mG. Since AdoMet does not stimulate the capping reaction (Fig. 6), these results probably account for the 3-fold increase in the proportion of 5′-blocked molecules in preparation of methylated core unmethylated mRNA (16).

Like the guanylyltransferase, N7-G-methylase is also nucleotide-specific. It methylates the N7-position of only 1 of the guanosine residues in GpppG, the structure homologous to reovirus blocked ends. It does not significantly methylate GppA, GppC, or GppU. GpppGpC is a more effective substrate than GpppG for the N7-G-methylase, consistent with cap formation at the dinucleotide level (Table IV). Substrate specificity is also a feature of the core-associated 2′-O-methylating activity. It modifies only those oligonucleotides that already contain the 5′-terminal mG and also requires at least one phosphodiester linkage in the substrate, i.e. m7GpppGpC or GpppG are not 2′-O-methylated by cores.

Two RNA viruses, human reo and insect cytoplasmic polyhedrosis and the DNA-containing vaccinia virus contain guanylyltransferases that in each case add pG to the 5′ termini of mRNA to form caps. For reovirus and cytoplasmic polyhedrosis virus, caps clearly are synthesized at the initiation step of transcription and are not formed during processing of precursors to smaller RNA products. The inability of the vaccinia virus guanylyltransferase to use 5′-monophosphate termini and its requirement for diphasosphate 5′ ends suggests that initiation-related capping may also occur in vaccinia mRNA synthesis. However, the solubilized vaccinia enzyme, in contrast to the reovirus or cytoplasmic polyhedrosis virus virion-associated activity, can also modify the 5′ termini of long RNA chains (43). Another RNA virus, vesicular stomatitis virus, synthesizes GppA RNA caps by a different mechanism that involves addition of pG from GTP to 5′-terminal pA (50). The 5′-terminal monophosphate involved in this type of blocking reaction could arise by cleavage of a larger RNA. Recent studies (51–53) suggest that the 5′ vesicular stomatitis virus mRNAs may be formed by specific cleavage of a large transcription product as observed for “early” mRNA of T7 bacteriophage (54). The vesicular stomatitis virus cleaved products presumably are then capped by the reaction: ppG + pA → GpppA + P. Phosphate rather than pyrophos-
phate is released, and mRNAs with ppA . . . ends are not obtained. The vesicular stomatitis virus-capping mechanism represents a model for the type of processing that would be expected in eukaryotic cells if mRNA is formed from heterogeneous nuclear (hn) RNA by nucleolytic hydrolysis and modification. Caps are present in HeLa and L-cell hnRNA (25, 26), in the fast-sedimenting RNA as well as in nuclear RNA molecules that are similar in size to cytoplasmic mRNA. However, it is not known if the large capped hn RNA which has poly(A) at the 3’-terminal end consists of molecules that have already been cleaved from precursors or if they are primary transcripts. Our studies with reovirus cores demonstrate that, at least for some eukaryotic mRNAs, 5’-terminal caps are formed during initiation of transcription.

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