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Methylated Messenger RNA Synthesis *In Vitro* by Purified Reovirus

(methylation/RNA virus)

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ABSTRACT **Purified human reovirus contains RNA methylase activity in addition to an RNA polymerase. Virions incubated under appropriate conditions in the presence of *S*-adenosyl-L-methionine synthesize mRNA that is specifically methylated in the 5′-terminal guanosine. Alkaline digestion of the methylated RNA released a 5′-terminal dinucleotide, ppG′pCp, indicating that the guanosine contains 2′-*O*-methylribose. The possible roles of methylation in viral and cellular mRNA function is discussed.**

The genome of human reovirus consists of ten segments of double-stranded RNA (1), each containing the same two 5′-terminal sequences: GpApUp in the minus (−) strand and G′pCp in the plus (+) strand (2). G′ presumably is 2′-*O*-methylated guanosine, since the G′pCp sequence is resistant to hydrolysis by alkali or T1 RNase (2). A virion-associated RNA polymerase transcribes only the (−) strand of each reovirus duplex segment (3–6). The resulting ten (+) species of single-stranded RNA function as viral mRNA (7, 8). In addition, they serve as precursors to the double-stranded genome segments (9), suggesting that reovirus mRNA may also be methylated at the 5′-termini. A similar situation exists for another double-stranded RNA virus, cytoplasmic polyhedrosis virus (CPV) of insects (10, 11). Furuichi recently found that the single-stranded viral RNA synthesized *in vitro* by the cytoplasmic polyhedrosis virus-associated transcriptase in the presence of *S*-adenosyl-L-methionine contains 1.2 methyl groups per RNA molecule of average chain length 2300 nucleotides (12). In the view of the above results and the report that mouse L cell mRNA contains 2.2 methyl groups per 1000 nucleotides (13), it was of interest to test the possibility that reovirus mRNA is methylated.

MATERIALS AND METHODS

Reovirus type 3, Dearing strain, was grown in mouse L cells in suspension culture and purified as described (3).

RNA was synthesized by the virion-associated transcriptase by incubating 70 μg of purified reovirus with 30 μg of chymotrypsin for 30 min at 45° in a standard reaction mixture containing 7 μmoles of Tris·HCl (pH 8.0), 0.75 μmole of Mg^{+2}, 15 μmoles of KCl, 0.2 μmole each of GTP, CTP, and UTP, 0.1 μmole of ATP, and 0.25 μmole of cyclohexylammonium phospho*enol*-pyruvate in a volume of 0.065 ml. The volume was then adjusted to 0.1 ml by addition of 0.5 unit of pyruvate kinase, [α-^{32}P]ATP, *S*-adenosyl-L-[*methyl*-^{3}H]methionine, and the other, indicated constituents. The incubation was continued at 45°, and samples of 0.02 ml were removed to 5 ml of trichloroacetic acid–sodium pyrophosphate (10% each) at the indicated times. After 30 min at 0°, they were filtered through Millipore filters, washed, and counted in toluene-based scintillation fluid.

Analysis of RNA products by velocity sedimentation in sucrose gradients (3) and chromatography on DEAE–cellulose in the presence of 7 M urea has been described (14).

[α-^{32}P]ATP (specific activity = 116 Ci/mmole) and *S*-adenosyl-L-[*methyl*-^{3}H]methionine (8.5 Ci/mmole) were purchased from New England Nuclear Corp., Boston, Mass. [^{3}H]CTP (specific activity = 19 Ci/mmole) and [^{3}H]valine (16 Ci/mmole) and [β,γ-^{32}P]GTP (9.5 Ci/mmole) were from Schwarz/Mann, Orangeburg, N.Y. and ICN, Irvine, Calif., respectively.

RESULTS

Synthesis-Dependent Methylation of Reovirus mRNA. Purified reovirus incubated under standard conditions of RNA synthesis in the presence of [α-^{32}P]ATP and *S*-adenosyl-L-[*methyl*-^{3}H]methionine catalyzed the incorporation of both isotopes into acid-insoluble products at a linear rate for 60 min (Fig. 1). In the absence of GTP, no RNA synthesis occurred, and there was no incorporation of [^{3}H]methyl group. To test whether exogenous mRNA can be methylated in the absence of RNA synthesis, I added purified single-stranded RNA made *in vitro* in the absence of *S*-adenosylmethionine to the incubation mixture lacking GTP. No incorporation of [^{3}H]methyl group was found (Fig. 1). [*methyl*-^{3}H]Methionine did not replace *S*-adenosylmethionine as a methyl donor. As described for the reovirus RNA polymerase (3–5), methylase activity is also associated with purified cores prepared from chymotrypsin-digested virions (data not shown).

RNA synthesis was unaffected by the analogue of *S*-adenosylmethionine, *S*-adenosyl-L-homocysteine (Table 1). However, [^{3}H]methyl incorporation from adenosyl [methyl-^{3}H]-methionine was reduced by increasing levels of the analogue, and at an adenosylhomocysteine to adenosylmethionine ratio of 100, inhibition was 93%. In other experiments (data not shown) the rate of RNA synthesis was unchanged by addition of 0.003–1 mM adenosylmethionine, and a K_m of 1.1 μM was determined for methylation. The results indicate that purified reovirus contains, in addition to RNA polymerase, a methylase activity that synthesizes methylated mRNA *in vitro* when provided with the methyl donor, *S*-adenosyl-L-methionine.

Characterization of Products Formed In Vitro. The [^{3}H]-methyl-labeled products formed *in vitro* were degraded by treatment with pancreatic RNase or KOH but not by pancreatic DNase or chymotrypsin, indicating that the methyl group is incorporated into RNA (Table 2).

Sedimentation analysis of the products synthesized in the presence of [α-^{32}P]ATP and *S*-adenosyl[*methyl*-^{3}H]methionine is shown in Fig. 2. The two radioactivity profiles coincided and included the three size classes of reovirus mRNA previously

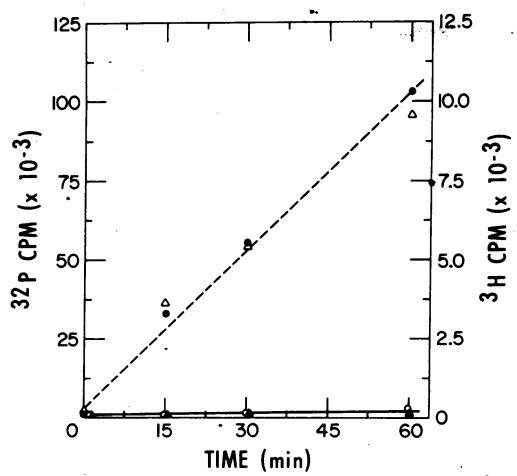

FIG. 1. *Kinetics of methylated RNA synthesis.* RNA synthesis by purified reovirus under standard conditions in the presence of *S*-adenosyl-[*methyl*-^{3}H]methionine (2.6 μM, specific activity = 8.5 Ci/mmole) and [α-^{32}P]ATP (47 mCi/mmole) was assayed as described in *Materials and Methods*. [^{32}P]AMP (△) and [^{3}H]-methyl group (●) incorporation into acid-insoluble products; incorporation of [^{32}P]AMP and [^{3}H]methyl group in the absence of GTP with (○) and without (▲) 7 μg of reovirus mRNA that was synthesized *in vitro* in the absence of adenosylmethionine and purified by phenol extraction, gel filtration, and ethanol precipitation (3, 14).

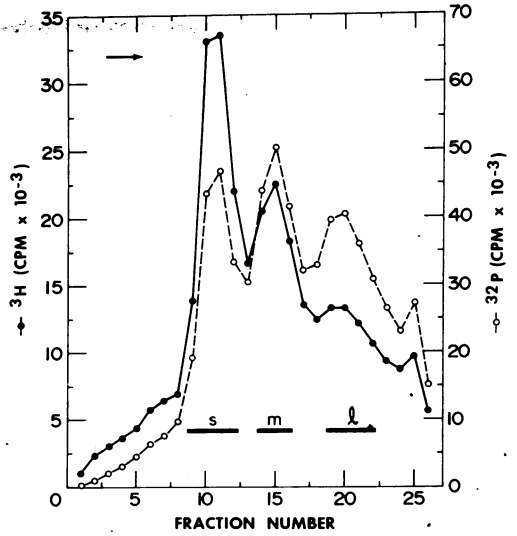

FIG. 2. *Sedimentation analysis of RNA products.* The products of a 2-hr standard incubation reaction (0.5 ml; 0.018 mM *S*-adenosyl-[methyl-^{3}H]methionine, specific activity = 8.5 Ci/mmole; 1 mM [α-^{32}P]ATP, specific activity = 26 mCi/mmole) were purified by phenol extraction, gel filtration on Sephadex G-100, and ethanol precipitation (3, 14). The RNA was dissolved in 0.15 ml of H_2O and layered onto a 15–30% sucrose gradient containing 0.5% sodium dodecyl sulfate, 0.1 M NaCl, 1 mM EDTA, 10 mM Tris buffer, pH 7.4. After centrifugation (SW-41, 18 hr, 27,500 rpm, 23°), samples of 0.5 ml were collected from the top, diluted with 0.5 ml of H_2O and 10 ml of aquasol (New England Nuclear Corp.), and counted. ●, [^{3}H]Methyl; ○, [^{32}P]AMP.

designated *l*, *m*, and *s* (3–5). The [^{3}H]methyl content of each RNA class was inversely proportional to its size. Chain lengths of 3500, 2000, and 1000 nucleotides have been estimated for the *l*, *m*, and *s* RNA classes, respectively (14). On the basis of the specific radioactivities of the RNA precursors, and an AMP content of 25%, molar ratios of [^{3}H]methyl/total nucleotides for the *l*, *m*, and *s* RNA classes were 1/1940, 1/1250, and 1/720, respectively, consistent with an average of 1–2 methyl groups per chain.

5′-Terminal Position of Methyl Groups. Reovirus genome RNA is hydrolyzed by KOH treatment to mononucleotides and an alkali-resistant dinucleotide, ppG′pCp (ref. 2; Chow and Shatkin, unpublished results). The dinucleotide results from a specific modification, presumably 2′-*O*-methylation, of the 5′-terminal guanosine in the (+) strands of all ten double-stranded RNA segments. The same 5′-dinucleotide was found in reovirus mRNA synthesized *in vitro* in the presence of adenosylmethionine. mRNA was synthesized with the radioactive precursors, [β,γ-^{32}P]GTP to label the 5′-termini (14) and *S*-adenosyl[*methyl*-^{3}H]methionine to methylate residues in the RNA products. Since reovirus contains a phosphohydrolase activity that cleaves the γ-phosphate from nascent RNA products, the 5′-termini of the resulting ten species of mRNA are ^{32}ppG (14). As shown in Fig. 3*A*, after alkaline digestion of the radioactive mRNA, most of the ^{3}H and all of the ^{32}P eluted from a DEAE–cellulose–urea column with a net negative charge between −5 and −6 (about 0.18 M NaCl),

TABLE 1. *Effect of S-adenosyl-L-homocysteine on RNA synthesis and methylation*

Adenosylhomocysteine (mM)	cpm incorporated	
	methyl[^{3}H]	[^{32}P]AMP
0	4,262	27,098
0.0025	3,005	29,396
0.025	1,125	26,983
0.25	302	26,757
1.0	202	26,368

RNA was synthesized under standard conditions in the presence of 0.0026 mM *S*-adenosyl-[*methyl*-^{3}H]methionine and 1 mM [α-^{32}P]ATP (specific activities = 8.5 Ci/mmole and 25 mCi/mmole, respectively). Incorporation of radioactivity into acid-insoluble products after 30 min was assayed as described in *Materials and Methods*.

TABLE 2. *Properties of* [^{3}*H*]*methyl-labeled products*

Treatment	Acid-insoluble cpm
None	3,064
Pancreatic RNase	119
KOH	45
Pancreatic DNase	2,916
Chymotrypsin	2,759

Products synthesized under standard conditions in the presence of *S*-adenosyl-[*methyl*-^{3}H]methionine were isolated by phenol extraction and ethanol precipitation (3, 14) and dissolved in 10 mM Tris buffer, pH 8. Samples were digested with pancreatic RNase (10 μg/ml), chymotrypsin (10 μg/ml), or pancreatic DNase (5 μg/ml in 5 mM Mg^{+2}) for 30 min or 0.3 M KOH for 18 hr at 37°. Acid-insoluble radioactivity was counted on Millipore filters.

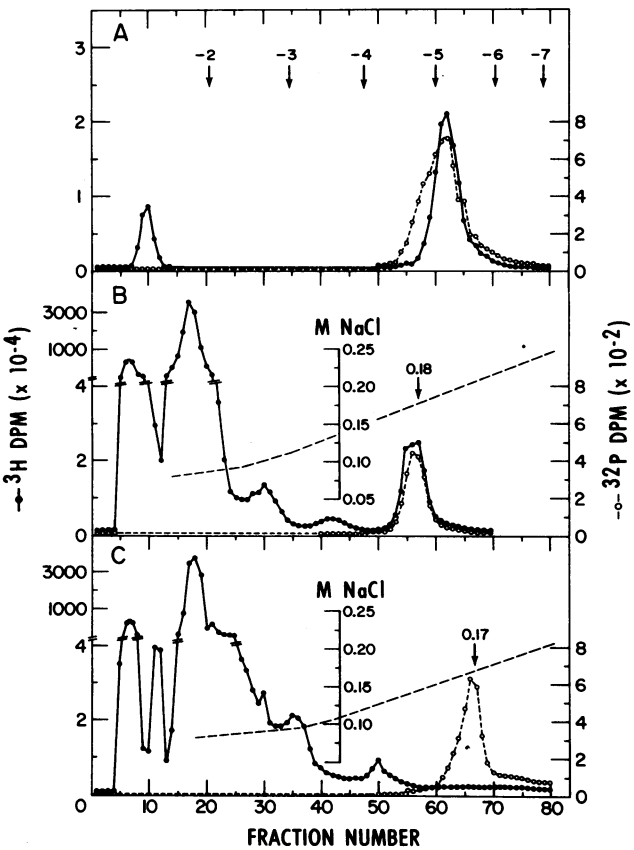

FIG. 3. *Chromatography of alkaline digests of RNA products.* RNA was synthesized under standard conditions with the following changes in the reaction mixtures: (*A*) 0.07 mM *S*-adenosyl-[*methyl*-^{3}H]methionine (specific activity = 1.3 Ci/mmole) and 0.5 mM [β,γ-^{32}P]GTP (specific activity = 0.17 Ci/mmole); (*B*) 0.07 mM unlabeled adenosylmethionine, 0.1 mM [^{3}H]CTP (specific activity = 4 Ci/mmole) and 0.5 mM [β,γ-^{32}P]GTP (specific activity = 0.17 Ci/mmole); (*C*) as in *B* but no adenosylmethionine was added. RNA products, purified as in the *legend* to Fig. 2, were digested with 0.3 M KOH for 18 hr at 37°, neutralized, and analyzed by chromatography on a 1 × 20-cm column of DEAE-cellulose (14). Samples of 2 ml were eluted with a linear NaCl gradient [0.05–0.3 M in 50 mM Tris buffer (pH 8.0), 7 M urea], and aliquots were counted in a mixture of 2-methoxyethanol, 2,5-diphenyloxazole (PPO), 1,4-bis[2-4-(methyl-5-phenyloxazolyl)]-benzene (POPOP), and toluene (14). The elution positions of oligonucleotides of net negative charge −2 to −6 (*arrows*) were determined by mixing with the sample a digest of yeast tRNA (1 mg treated with 0.1 mg of pancreatic RNase for 7 hr at 37° in 0.5 ml of 0.1 M Tris buffer, pH 7.2) and recording the $A_{260\ nm}$ profile.

the position of a dinucleotide with the structure ppG'pCp. The same peak of radioactivity was obtained from T1 plus pancreatic RNase-digested mRNA (data not shown). The ^{3}H that did not bind to the column at 0.05 M NaCl (18% of the total) was not further characterized. The ratio in pmoles of [^{3}H]methyl/^{32}ppG in the alkaline-resistant material estimated from the specific activities of the precursors in two separate experiments was 32/38 and 38/30, or an average of 1 methyl group per 5'-terminal dinucleotide.

In order to determine the composition of the alkali-resistant material, synthesis was carried out with [β,γ-^{32}P]GTP and [^{3}H]CTP in the presence and absence of unlabeled adenosylmethionine, and alkaline digests of the methylated and unmethylated RNA were compared. The elution profile of the digest of methylated RNA, i.e., synthesized with adenosylmethionine in the incubation mixture, is shown in Fig. 3*B*. The ^{32}P eluted quantitatively in one peak at 0.18 M NaCl, as in Fig. 3*A*. There was also a coincident peak of ^{3}H, comprising 0.1% of the total ^{3}H-labeled material, although most of the ^{3}H eluted as Cp at 0.08 M NaCl. The radioactivity in combined fractions 52–62 corresponds to a total of 15 pmoles of ^{3}H and 13 pmoles of ^{32}P, i.e., a molar ratio close to the value of 1.0 expected for ppG'pCp.

The elution profile of the digested unmethylated RNA is shown in Fig. 3*C*. A single ^{32}P-labeled component was present again, but it eluted at a NaCl concentration of 0.17 M, consistent with a structure having a net charge of −4 to −5. Furthermore, in contrast to the profile of digested methylated RNA shown in Fig. 3*B*, no ^{3}H-labeled material eluted with the ^{32}P-labeled nucleotides. It is concluded that alkali released ^{32}ppGp from the 5'-ends of unmethylated mRNA.

The results indicate that reovirus mRNA synthesized in the presence of adenosylmethionine, like the (+) strand of the genome double-stranded RNA, contains methylated guanosine at the 5'-termini. Since a 5'-terminal dinucleotide, ppG'pCp, is released from methylated RNA by alkali (or T1 plus pancreatic RNase) digestion, it is likely that methylation is at the 2'-position of the ribose moiety, but an average value of between 1 and 2 methyl groups per RNA chains before alkaline hydrolysis (Fig. 2) may indicate that a ring position of the 5'-terminal guanosine can also be substituted.

TABLE 3. *Stimulation of protein synthesis by reovirus mRNA*

mRNA	[^{3}H]Valine incorporated (cpm/60 min)
None	278
Unmethylated	8,129
Methylated	9,838

Cell-free extracts of wheat germ were prepared and incubated as described (15) in a reaction mixture containing 0.7 μM [^{3}H]-valine and 50 μg/ml of reovirus mRNA. After 60 min at 25°, samples were adjusted to 10% trichloroacetic acid–casamino acids, heated for 15 min at 100°, filtered, washed, and counted. Reovirus mRNA was synthesized in the presence and absence of 0.1 mM adenosylmethionine under standard conditions. After synthesis at 45° for 2 hr, viral cores were removed by centrifugation (20 min, 27,000 × *g*), and the mRNA was purified by phenol extraction, gel filtration through Sephadex G-50, and ethanol precipitation (3, 14).

Messenger Activity of Methylated RNA. Unmethylated, single-stranded reovirus RNA synthesized *in vitro* has been translated with fidelity in cell-free systems from several sources (7, 8), including wheat germ extracts (Both, Lavi, and Shatkin, unpublished results), which are notable for their low endogenous activity (15). Methylation of the 5'-position of a viral mRNA may be expected to alter its messenger function in infected cells, for example by changing its ribosome-binding capacity. However, protein synthesis in wheat germ cell-free extracts was stimulated to the same extent by methylated and unmethylated RNA (Table 3), and the rates of synthesis were similar. It remains to be determined if the mRNA remains methylated during protein synthesis *in vitro*. Further studies, including analysis of the polypeptide products and the use of mammalian cell-free systems, may reveal functional differences between modified and unmodified mRNA.

DISCUSSION

Reoviruses contain an RNA polymerase that transcribes the double-stranded genome RNA (3–5, 16, 17). They apparently also possess a methylase, since the RNA products synthesized *in vitro* in the presence of *S*-adenosylmethionine are methylated. Both enzyme activities are located in the viral cores that are formed when RNA synthesis is activated by chymotrypsin digestion of the virion outer protein shell (3–5, 17). In addition to the genome RNA, reovirus cores consist of 1 minor and 3 major polypeptides (3, 18), and they may correspond to the RNA polymerase, methylase, and phosphohydrolase activities.

Like the (+) strand of the double-stranded genome RNA of reovirus (2) and insect cytoplasmic polyhedrosis virus (10), only the 5′-terminal nucleotide is modified in the single-stranded RNA synthesized by the reovirion transcriptase. Unmethylated, single-stranded reovirus RNA is not a substrate for the reovirion methylase activity, suggesting that the enzyme modifies only nascent RNA chains, as reported for the virion-associated phosphohydrolase (14). Alternatively, the cores may be impermeable to exogenous mRNA. The single-stranded RNA synthesized *in vitro* by cytoplasmic polyhedrosis virus is also methylated during the initial stages of transcription, and it has been suggested that the two processes are coupled because the transcriptase of cytoplasmic polyhedrosis virus is stimulated by the presence of *S*-adenosylmethionine (12). The similarity between the 5′-termini of the reovirus transcription products and the (+) strands of the double-stranded genome segments lends further support to the evidence that the genome of double-stranded RNA-containing viruses are formed by way of single-stranded intermediates (9).

The RNA synthesized *in vitro* by cytoplasmic polyhedrosis virus contains an average of 1.2 methyl groups per chain (12). Reovirus mRNA molecules also contain an average of 1 methyl group in the 5′-terminal guanosine, probably as 2′-*O*-methylribose. Since nucleotide methylations are mediated by specific enzymes, reovirus and cytoplasmic polyhedrosis virus presumably contain different methylases. Vesicular stomatitis virus also contains methylase activity in addition to RNA polymerase, and the single-stranded viral mRNA synthesized *in vitro* by purified virions is methylated to the extent of about 3 methyl groups per RNA chain (A. K. Banerjee, personal communication). Base-specific tRNA methylases have been described in preparations of RNA tumor viruses. These include N^2-guanine methylase in avian myeloblastosis virus, Rous sarcoma virus and Rous-associated virus, and N^1-adenine methylase in Rous sarcoma virus (19, 20). Vaccinia virus mRNA synthesized *in vitro* by viral cores is also methylated to the extent of 2.3 methyl groups per 1000 nucleotides (C. W. Wei and B. Moss, personal communication), a number identical to that reported for mouse L cell mRNA (13). Thus, methylation occurs in mRNAs synthesized on both DNA and RNA templates.

The functional significance of methylation of viral and cellular mRNAs is unknown. It has been suggested that mRNA methylation in mouse L cells is a post-transcriptional modification related to processing of heterogeneous nuclear RNA (13). Processing of viral mRNAs from larger precursor molecules in infected or transformed cells may also occur by enzymatic cleavage at or near specific, methylated sites. Methylation at the 5′-termini of reovirus mRNA and possibly other mRNAs may provide protection against 5′-exonucleolytic attack, resulting in a longer half-life. Binding of ribosomes to monocistronic mRNA probably occurs at, or close to, the 5′-termini, and methylation may provide a nuclease-resistant binding site. In addition, methylation may interfere with hydrogen bond formation and prevent intramolecular secondary structures involving the 5′-termini of mRNA. A similar denaturing effect, localized at the methylated ends of viral double-stranded RNA, could facilitate the initiation of genome transcription. It should be possible to explore some of these possibilities with the *in vitro* systems available for studying transcription and translation.

Note Added in Proof. Reovirus mRNA synthesized in the presence of adenosylmethionine contains 5′-terminal phosphates that are 40–50% resistant to hydrolysis by alkaline phosphatase (Muthukrishnan and Shatkin, unpublished results). The 5′-phosphates of the plus strands of reovirus genome RNA are also phosphatase-resistant (Chow and Shatkin, unpublished results), and the 5′ ends can be labeled with [^{32}P]phosphate by polynucleotide kinase only after sequential oxidation, β-elimination, and phosphomonoesterase treatment (2). Thus, reovirus mRNA and genome RNA plus strands both contain blocked 5′ termini.

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