Inhibition of initiation of protein synthesis by 7-methylguanosine-5' -monophosphate
(protein synthesis/methylated nucleotides/modified 5'-termini)

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ABSTRACT Translation of rabbit globin mRNA in a wheat germ protein-synthesizing system is inhibited by the nucleotide 7'-methylguanosine-5'-monophosphate (m7G5'p) but not by other guanosine nucleotides without the 7-methyl group or with the phosphate in a different position. Translation of RNA of tobacco mosaic virus and poly(A) + HeLa RNA is also inhibited by m7G5'p. We show that m7G5'p prevents the association of mRNA with ribosomal subunits to form initiation complexes. We propose that m7G5'p interacts with a site on initiation factor(s) or ribosomes which is involved in mRNA recognition, presumably by binding to the 5'-terminal sequence m7G5'ppp. m7G5'p does not inhibit translation of poly(U) and RNA of satellite tobacco necrosis virus, which do not have the 5'-terminal sequence m7G5'ppp. In the case of RNA of satellite tobacco necrosis virus, some stimulation of its translation is consistently observed in the presence of m7G5'p; possible interpretations of this finding are discussed.

Rottman et al. (1) have described a dinucleotide at the 5'-terminus of a variety of mRNAs that consists of 7-methylguanosine linked through its 5'-hydroxyl group via a triphosphate to the 5'-hydroxyl group of a ribosemethylated nucleotide. The 5'-terminal sequence m7G5'ppp . . . has been reported for reovirus (2), vesicular stomatitis (3), vaccinia (4), and silkworm polyhedrosis virus RNAs (5) and for rabbit globin (6). HeLa (7), myeloma (8), and L cell mRNAs (6). These reports suggest that methylated blocked 5'-termini may be a rather general feature of eukaryotic mRNA.

Both et al. (9) have demonstrated a specific role for the 7-methyl group of the 5'-terminal guanosine of reovirus RNA. Viral RNA can be synthesized in vitro in the presence of associated RNA polymerase. This RNA is methylated when Sadenosylmethionine is added to the incubation mixture, but is unmethylated when S-adenosylhomocysteine, an inhibitor of methylation, is added. The methylated RNA is translated effectively and with fidelity by a wheat germ protein-synthesizing system; whereas the unmethylated RNA is translated only after being methylated during the incubation in the wheat germ extract (9). Addition of S-adenosylhomocysteine to the wheat germ extract prevents methylation and abolishes translation of unmethylated reovirus RNA. Similarly, the removal of the 5'-terminal m7G5'p from rabbit globin mRNA causes a loss of the template activity of this mRNA (6). The presence of the methylated 5'-terminal nucleotide seems to be specifically required for the interaction of reovirus RNA with ribosomal subunits in the formation of initiation complexes (10). A functional role for the m7G5'p . . . nucleotide sequence in mRNA translation has not yet been directly shown. It seems possible, however, that this group acts as a recognition signal for mRNA. We report here that this is the most likely function of the 5'-end of mRNA by showing that the nucleotide m7G5'p specifically inhibits translation of mRNAs with the sequence m7G5'ppp . . . , but not of other RNAs that do not have this group at the 5'-terminus. Other analogs of guanosine do not show inhibitory activity.

MATERIALS AND METHODS

7-Methylguanosine and 7-methylguanosine monophosphates were purchased from Terra-Marine Bioresearch (La Jolla, Calif.). Globin mRNA was isolated by repeated sucrose gradient centrifugation as described (11). Tobacco mosaic virus (TMV) was given to us by Dr. Bryan E. Roberts, Massachusetts Institute of Technology. TMV RNA was prepared by phenol extraction (12). Poly(A) + RNA was prepared from HeLa cell polysomes as described (13). Reovirus RNA synthesized by virions and labeled with S-adenosyl[CH3-3H]methionine (10) was a gift of Gerald W. Both and Aaron J. Shatkin of the Roche Institute of Molecular Biology, Nutley, N.J. Satellite tobacco necrosis virus RNA was given to us by Dr. Abraham Marcus, Institute for Cancer Research, Fox Chase, Philadelphia. Sparsomycin was a gift of Upjohn Co., Kalamazoo, Mich. Radioactive amino acids were purchased from New England Nuclear.

Cell-Free Protein Synthesis. Previously published methods were used to prepare the wheat germ extract (14). The wheat germ protein-synthesis assay contains in 50 μl: 15 μl of wheat germ extract, 1.2 mM ATP, 0.25 mM GTP, 15 mM creatine phosphate, 0.67 unit of creatine phosphokinase (Sigma), 22 mM N-2-hydroxyethylphosphazene-N'-ethanesulfonic acid (Hepes)/KOH pH 7.1, 3.0 mM Mg-acetate, 0.1 M KCl, 2.6 mM dithiothreitol, 1.8 mM mercaptoethanol, 50 μM unlabeled amino acids minus lysine or phenylalanine, and 5 μCi of [3H]lysine (38 Ci/mmol) or [3H]phenylalanine (59 Ci/mmol). RNA, guanosine nucleotides, and inhibitors were added at the concentrations indicated in the figure legends. The incubations were carried out at 24°C, and 5-μl samples were taken in duplicate before starting the incubation and at the specified time intervals. The samples were processed for determination of radioactivity as described (15).

Binding of Reovirus [3H]RNA to Initiation Complexes. The binding reactions were carried out in 50 μl of the wheat germ cell-free system containing 50 μM unlabeled amino acids, 0.1 mM sparsomycin, 2500 cpm of reovirus...
Methods.

The concentrations of the guanosine nucleotides added in the presence of incorporation at sampled were calculated. Translation in the presence of the nucleotides is expressed as % of the untreated control; the endogenous incorporation of the control was 1160 cpm. The control incorporation with globin mRNA added was 37,000 cpm.

\[^{3}H\text{mRNA}\text{ (specific activity 8000 cpm/µg), and the indicated concentration of m}^{7}\text{G}\text{5'p. After 10 min at 24°, the incubations were stopped by addition of 0.45 ml of 1 mM cycloheximide in 0.1 M KCl, 20 mM Tris-HCl (pH 7.5), and 5 mM Mg-aceate. The samples were applied to 15–30% sucrose gradients in the same buffer and centrifuged at 22,000 rpm in a Beckman SW-27 rotor. The }A_{260}\text{ of the gradients was monitored in a Gilford recording spectrophotometer, and the radioactivity of the fractions collected was determined after addition of 12 volumes of 1 part Triton X-100, 2 parts 0.8% butyl-PBD (C.I.B.A.) in toluene, and 10% water.}

RESULTS

The presence of a 5'-terminal m\(^{7}\text{G}\text{5'p in rabbit globin mRNA affects its translation by wheat germ extracts (6). When the 5'-terminal m}\(^{7}\text{G}\text{5'p is removed by periodate oxidation and β-elimination, the mRNA loses most of its ability to stimulate protein synthesis (6). In experiments directed at establishing a specific role for m}\(^{7}\text{G}\text{5'p in protein synthesis, we have translated rabbit globin mRNA in the presence of mononucleotide analogs of the 5'-terminal end of this mRNA. Only m}\(^{7}\text{G}\text{5'p inhibits protein synthesis, whereas the other compounds tested (m}\(^{7}\text{G}, \text{m}\(^{7}\text{G}\text{5'p, and G}\text{5'p) show negligible inhibition and only at very high concentrations (Fig. 1). This indicates that the chemical groups essential for inhibition of globin mRNA translation by m}\(^{7}\text{G}\text{5'p are the methyl group in position 7 and the phosphate in 5'.' Compounds with the phosphate group in a different position or without the methyl group show no inhibition. Endogenous protein synthesis of the wheat germ extract is also inhibited by m}\(^{7}\text{G}\text{5'p (Fig. 1).}

We have observed inhibition of translation of other mRNAs by m\(^{7}\text{G}\text{5'p in wheat germ extracts and also in a different cell free system. Translation of TMV RNA and of HeLa cell poly(A) + RNA in the wheat germ extract is inhibited, though incompletely or at slightly higher concentrations of the inhibitor (Fig. 2). Poly(A) + RNA of HeLa cells has been shown to have 5'-terminal m\(^{7}\text{G}\text{5'p (7). Protein synthesis programmed by endogenous mRNA in a HeLa cell extract is also inhibited by m}\(^{7}\text{G}\text{5'p (to be published elsewhere)).}

Mechanism of inhibition of protein synthesis by m\(^{7}\text{G}\text{5'p}

A direct demonstration that m\(^{7}\text{G}\text{5'p acts at the level of initiation of protein synthesis has been obtained by studying the formation of initiation complexes between reovirus RNA labeled in the methyl group of the 5'-terminal guanosine (2) and wheat germ ribosomes (Fig. 3). The binding reaction is carried out in the presence of sparsomycin, an inhibitor of elongation, as suggested by Darnbrough et al. (16). In the control experiment (Fig. 3A) 80% of the labeled RNA becomes associated with ribosomes. However, the association of reovirus RNA with ribosomes is partially inhibited by the presence of 0.125 mM m\(^{7}\text{G}\text{5'p (Fig. 3B) and is inhibited by

\begin{figure*}[h]
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\caption{Binding of reovirus \(^{[3]H}\text{RNA}} to the 80S initiation complex. The binding assay is carried out as indicated under Materials and Methods. The incubation mixtures are analyzed by sucrose gradient centrifugation. (A) Control; (B) 0.125 mM; and (C) 0.5 mM m\(^{7}\text{G}\text{5'p.}
\end{figure*}
over 70% by 0.5 mM m^7G^5p (Fig. 3C). No binding of reovirus RNA to 80S initiation complexes occurs in an incubation mixture kept at 0°C (not shown). The RNA that does not bind to the initiation complexes sediments at the top of the gradients because of degradation during the incubation. Several other labeled mRNAs have been shown to be degraded in a 10-min incubation (our unpublished observations). This degradation does not affect our results since the label in the reovirus RNA that we have used is in the 5′-terminal nucleotide, which is protected once the RNA is bound to ribosomes (10).

m^7G^5p has no effect on the binding of initiator Met-tRNA\textsubscript{i} to the 40S ribosomal subunit (to be published elsewhere). It seems possible that m^7G^5p interferes with the binding of mRNA in the formation of the 80S initiation complex by occupying one site with which the 5′-terminal nucleotide of mRNA interacts. This implies that the 5′-terminal nucleotide acts as a specific recognition signal during initiation. This suggestion is supported by the experiments of Both et al. (9), who have shown that unmethylated reovirus RNA is not translated by a wheat germ extract, whereas methylated reovirus RNA is efficiently translated. Messenger RNA and m^7G^5p may thus compete for binding to the same site with different affinity. We have calculated that the concentration of m^7G^5p at which we observe 50% inhibition of globin mRNA translation (0.063 mM; Fig. 1) is at least 1000-fold higher than the molar concentration of mRNA in the assay. We have used 220,000 as the molecular weight for globin mRNA (17) and have made the assumption that all the RNA added to the wheat germ extract is pure and active globin mRNA. In so doing, we have presumably overestimated the concentration of mRNA.

We postulate that m^7G^5p inhibits mRNA translation by binding to a site that interacts with the 5′-terminus of mRNA. We have attempted to learn more about this interaction by translating increasing concentrations of mRNA in the presence of a constant inhibitory concentration of m^7G^5p (Fig. 4). In the case of globin mRNA we have observed that the inhibition is constant over a 10-fold range of mRNA concentration (Fig. 4A). This can be explained by assuming that m^7G^5p inhibits an initiation factor in a dose-dependent fashion. Even in the presence of relatively high concentrations of the inhibitor there is some active factor that can participate in the initiation process. Translation of globin mRNA is linearly related to the amount of mRNA added under these conditions (Fig. 4A).

In the case of TMV RNA we find that the inhibition by m^7G^5p is overcome by increasing the RNA concentration (Fig. 4B). We propose a possible explanation for the discrepancy between the result obtained with globin mRNA and that with TMV RNA. We have seen previously that m^7G^5p does not completely inhibit translation of TMV RNA even at the highest concentrations tested (Fig. 2). This raises the possibility that TMV RNA is heterogeneous with respect to inhibition of translation by m^7G^5p. The result of the experiment shown in Fig. 4B may be explained by the addition of increasing amounts of RNA moieties that are not inhibited by m^7G^5p. Since these molecules may not have this methylated nucleotide at the 5′-terminus (see Discussion), it becomes extremely relevant to establish whether the translation of this type of RNA is inhibited by m^7G^5p.

Satellite tobacco necrosis virus (STNV) RNA is unique among a number of mRNAs in that it has a 5′-terminal se-
concentration of either ppApGpUp... (18, 19) or ppApGpUp... (20). Furthermore, accurate translation of STNV RNA occurs without the attachment of a 5′-terminal m7G5′p moiety (J. Brooker, S. N. Seal, and A. Marcus, in preparation). It is therefore of interest to ascertain whether or not m7G5′p has any effect on the translation of this mRNA. As shown in Fig. 5, in contrast to the results obtained with other mRNAs, STNV RNA translation is not inhibited, but is instead stimulated about 50% by relatively low concentrations of m7G5′p.

Both et al. (9) have shown that unmethylated reovirus RNA can be methylated by an endogenous methylating enzyme of the wheat germ and is then translated as well as methylated reovirus RNA. In some incubations we have added 0.32 mM S-adenosylhomocysteine, a competitive inhibitor of this enzyme (9), to the wheat germ extract. At this concentration S-adenosylhomocysteine prevents translation of unmethylated reovirus RNA (9). We have observed no effect on the translation of STNV RNA, TMV RNA, globin mRNA, and sea urchin egg mRNA in the presence of S-adenosylhomocysteine (R. Enos, L. Weber, and C. Baglioni, unpublished observations).

The translation of poly(U) is not inhibited by m7G5′p (Fig. 5). We have translated poly(U) under the same ionic conditions used for translation of natural mRNAs. At the relatively low Mg2+ concentration used, stimulation of protein synthesis by poly(U) is relatively modest, about 12-fold over the endogenous incorporation at 20 µg/ml of poly(U). This result supports our hypothesis that m7G5′p inhibits translation of only those RNAs having this nucleotide at the 5′-terminus.

**DISCUSSION**

The experiments described above show that m7G5′p can block the translation of some mRNAs, whereas it does not inhibit the translation of RNAs that lack the m7G5′ppp... 5′-terminal sequence. Therefore, m7G5′p does not inhibit protein chain elongation. We have shown that m7G5′p inhibits the binding of labeled reovirus mRNA to 80S initiation complexes. The inhibitory activity of guanosine nucleotides requires both the presence of the methyl group in the 7 position and the 5′ phosphate. We have not yet tested the activity of m7G5′pp or m7G5′ppp. These compounds may prove to be even more effective inhibitors, since they more closely resemble the 5′-terminus of mRNA than m7G5′p.

Translation of rabbit globin and HeLa poly(A) + RNA is inhibited by m7G5′p. These RNAs are known to have 5′-terminal m7G5′ppp (6, 7). Translation of TMV RNA is also inhibited by m7G5′p. While this manuscript was being prepared for publication, we learned that Keith and Frankel-Conrat (21) and Zimmern (22) have examined the 5′-terminal sequence of TMV RNA by the methods introduced for the study of other methylated viral and mammalian RNAs, and have determined that the majority of the molecules have m7G5′p at the 5′-terminus. Translation of TMV RNA is inhibited by m7G5′p to a less degree than globin mRNA (Fig. 2). We believe that TMV RNA is a homogenous template of molecular weight about 2 x 10^8 (12), containing enough nucleotides to code for 10 proteins the size of rabbit globin. We find that about 10-15% of the translational activity of TMV RNA is resistant to m7G5′p inhibition. The RNA translated in the presence of m7G5′p may correspond to molecules that do not have this nucleotide at the 5′-terminus; hidden breaks in TMV RNA may result in the formation of these molecules (22). Another explanation for the translation of TMV RNA being resistant to m7G5′p is worth mentioning. TMV RNA is a polycistronic mRNA, and it is possible that initiation of polypeptide chains can take place at internal sites, though the preferred initiation site involves the m7G5′ppp... 5′-terminal sequence.

The observation that the translation of STNV RNA is not inhibited by m7G5′p demonstrates the specificity of this inhibitor and also raises the possibility of the existence of separate mechanisms for the initiation of protein synthesis for classes of mRNAs that contain different 5′-termini. This has intriguing possibilities as a potential translational control mechanism. The stimulation of STNV RNA translation by m7G5′p is rather surprising. A possible explanation for this observation is that m7G5′p inhibits translation of endogenous mRNA or mRNA fragments present in the wheat germ extract, and thus eliminates competition between this RNA and STNV RNA.

Translation of HeLa poly(A) + RNA in the wheat germ extract does not appear to be completely inhibited at high m7G5′p concentrations. This result is open to different interpretations. One possibility is that, in the heterogeneous populations of mRNAs present in the poly(A) + RNA, there are mRNAs that do not possess the m7G5′ppp... terminal sequence and are not inhibited by m7G5′p. Whether the fraction of HeLa cell mRNA resistant to m7G5′p inhibition codes for proteins different from those coded for by the HeLa mRNA that is inhibited is a matter of speculation.

We believe that m7G5′p will be a very useful tool in elucidating the role of methylated 5′-termini of mRNA in protein synthesis. Our preliminary results suggest that this compound might be used to determine whether a template for protein synthesis has the 5′-terminal sequence m7G5′ppp... . It seems possible, moreover, that labeled m7G5′p can be used to establish whether a specific initiation factor or a ribosomal protein interacts with the 5′-terminus of mRNA.

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