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Influence of 5′-Terminal m^7G and 2′-O-Methylated Residues on Messenger Ribonucleic Acid Binding to Ribosomes†

S. Muthukrishnan,*,‡ Maureen Morgan, Amiya K. Banerjee, and Aaron J. Shatkin

ABSTRACT: Removal of 80% of the 5′-terminal 7-methylguanosine (m^7G) from methylated reovirus mRNA by β elimination results in a concomitant loss of the ability to bind to wheat germ ribosomes. The mRNA molecules that retain the m^7G account for most of the residual binding. Removal of the m^7G from all molecules in preparations of methylated reovirus and vesicular stomatitis virus mRNA reduces the extent of binding to wheat germ ribosomes from 80% to 5–7%. In the reticulocyte lysate, however, significant binding (17–34%) of the β-eliminated viral RNAs occurs. This m^7G-independent binding appears to be due to recognition by ribosomes of other structural features of the 5′-proximal sequences. Initiation complexes involving β-eliminated animal virus mRNAs and rabbit reticulocyte ribosomes appear to be more stable than the more heterologous combination of the same viral mRNAs and wheat germ ribosomes. In addition, evidence is presented to show that the 2′-O-methylated nucleoside of the 5′-terminal cap has a positive influence on the ribosome binding of viral mRNA and of capped synthetic ribopolymers. A model involving recognition of multiple structural features of the 5′-terminal region of mRNA by ribosomes during initiation of protein synthesis is presented.

The 5′ termini of many eukaryotic mRNAs are blocked with the methylated "cap" structure, $m^7G(5')ppp(5')N$ (Shatkin, 1976). Several lines of evidence have suggested a functional role for the m^7G[1] at the initiation step of capped mRNA translation. In cell-free protein synthesizing extracts derived from wheat germ or *Artemia salina* embryos, translational efficiencies were considerably greater for the methylated forms of reovirus and vesicular stomatitis virus (VSV) mRNAs than their unmethylated counterparts (Both et al., 1975a; Muthukrishnan et al., 1975b). Removal of the m^7G from globin

† From the Department of Cell Biology, Roche Institute of Molecular Biology, Nutley, New Jersey 07110. *Received July 26, 1976.*

‡ Present address: National Institutes of Health, Bethesda, Maryland 20014.

[1] Abbreviations used: VSV, vesicular stomatitis virus; m^7pG, 7-methylguanosine 5′-phosphate; m^7G, 7-methylguanosine; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

mRNA or reovirus mRNA by β elimination markedly reduced their translational capacity (Muthukrishnan et al., 1975a). The low efficiency of translation of unmethylated viral mRNAs was probably due to a decrease in their ability to bind to wheat germ 40S ribosomal subunits (Both et al., 1975b). Furthermore, binding of reovirus mRNA to 40S ribosomes conferred resistance of the cap and the adjacent sequence to RNase digestion, indicating that the m^7G comprises part of the ribosome binding site in this system (Kozak and Shatkin, 1976). Additional support for the concept that m^7G recognition occurs during initiation was obtained by Hickey et al. (1976) and, subsequently, by Roman et al. (1976) and Canaani et al. (1976) who reported that 7-methylguanosine 5′-monophosphate (m^7pG) inhibited in vitro translation of several viral and cellular capped mRNAs in wheat germ, rabbit reticulocyte, and mouse L-cell-free systems by blocking the formation of mRNA–ribosome initiation complexes. Presumably, m^7pG competes for some cellular component that recognizes the 5′ end of mRNA during initiation of protein synthesis. More recently, a protein fraction that specifically recognizes the 5′-terminal m^7G in mRNA has been detected in high salt washes of *A. salina* ribosomes (Filipowicz et al., 1976), and Shafritz et al. (1976) have reported that the binding of eukaryotic initiation factor IF-M_3 to capped mRNAs is inhibited by m^7pG. These results taken together strongly implicate the cap structure as an important functional constituent of most eukaryotic mRNAs.

However, some viral RNAs, e.g., those from satellite tobacco necrosis virus (Lesnaw and Reichmann, 1970) and the picornaviruses, polio (Nomoto et al., 1976; Fernandez-Munoz and Darnell, 1976; Hewlett et al., 1976), and encephalomyocarditis virus Nuss et al., 1975), are uncapped, contain no m^7G, but direct the synthesis of virus-specific polypeptides in vitro and in vivo (Shatkin et al., 1977). Bacteriophage T3 and T7 mRNAs containing 5′-terminal triphosphates also are translated in eukaryotic cell-free systems, although less effectively than capped eukaryotic mRNAs (Anderson et al., 1976). In addition, short 5′-terminal oligonucleotides derived from reovirus mRNA by T1 RNase digestion and certain synthetic ribopolymers that contain caps fail to form stable complexes with ribosomes (Both et al., 1975b, 1976). Thus, the absence of 5′-terminal m^7G does not preclude translation of some mRNAs and the presence of cap by itself does not ensure binding to ribosomes. Apparently, other structural features of mRNA including 5′-terminal sequence (Both et al., 1976; Muthukrishnan et al., 1976) and possibly the location of initiator AUG codon relative to cap also influence initiation complex formation. The resultant of these different components may determine an intrinsic translational efficiency for each mRNA (Muthukrishnan et al., 1976).

The relative contribution of m^7G to ribosome binding affinity and translational efficiency may vary among several mRNAs and possibly differs in protein synthesizing systems prepared from different sources. For example, unmethylated VSV mRNA is translated poorly, if at all, in wheat germ extract (Both et al., 1975a). In contrast, Rose and Lodish (1976) found that chemical removal of m^7G by β elimination from viral mRNA, isolated from VSV-infected cells, reduced by only about threefold its rate of ribosome binding and efficiency of translation in reticulocyte lysate. Because there was only a small decrease (20–30%) in the extent of mRNA binding to ribosomes after β elimination, it was suggested that these effects may be nonspecific ones due to periodate oxidation (Rose and Lodish, 1976). Rao et al. (1975) also reported that periodate oxidation of liver mRNA abolished its translation in wheat germ extract, while for brome mosaic virus RNA the inhibitory effect of β elimination on translation was incomplete and partially overcome in wheat germ extract at saturating levels of mRNA (Shih et al., 1976). In an effort to determine if these differences are the result of mRNA structural features other than m^7G that are recognized in homologous systems (i.e., animal virus mRNA in reticulocyte lysate or plant virus mRNA in wheat germ extract) but not in heterologous systems (i.e., animal viral or cellular mRNA in wheat germ extract), we have compared the efficiency of initiation complex formation of mock-treated, periodate oxidized, and β-eliminated VSV and reovirus mRNAs in wheat germ extract and reticulocyte lysate.

Experimental Procedures

Preparation of Viral mRNAs. Reovirus mRNA labeled with [^{3}H] in the 5′-terminal methyl groups was prepared by incubating chymotrypsin-treated reovirus for 2 h at 45 °C in a standard transcription reaction mixture containing 10 μM [^{3}H]-*S*-adenosylmethionine (specific activity 11.6 Ci/mmol, Amersham/Searle). The virion cores were pelleted by high-speed centrifugation, and the mRNA in the supernatant fraction was phenol-extracted and purified by gel filtration in Sephadex G-50 as described previously (Both et al., 1975c; Shatkin, 1974). Unmethylated reovirus mRNA was prepared in the same way except that *S*-adenosylmethionine was replaced by 200 μM adenosylhomocysteine and was labeled internally with [^{3}H]GTP.

Methylated and unmethylated VSV mRNAs were synthesized in vitro in the presence and absence of *S*-adenosylmethionine (Rhodes and Banerjee, 1976); *S*-adenosylhomocysteine was not used in order to avoid any possible effects of the analogue on VSV mRNA synthesis (Rose and Lodish, 1976). The mRNA's were labeled with ^{3}H in the 5′-terminal methyl groups and in internal U residues, respectively. Both mRNA preparations were purified by oligo(dT)cellulose column chromatography followed by glycerol gradient centrifugation. They include the three resolved peaks of radioactivity in the 12–18S region previously shown to code for the structural proteins G, N, and M, plus NS (Both et al., 1975d).

Methylated VSV mRNA was isolated from infected BHK cells that had been labeled with [^{3}H]methionine (Schwarz/Mann, Orangeburg, N.Y., specific activity 5 Ci/mmol) as described by Moyer et al. (1975).

Periodate Oxidation and β Elimination. mRNAs were dissolved in 0.25 ml of 0.1 M sodium acetate buffer (pH 5.3) containing 10 mM EDTA at 0 °C. A freshly prepared solution of 10 mM potassium periodate was added to give a final concentration of 0.2 mM and incubated for 2 h in the dark. Sterile glycerol (10 μl; 50% v/v) was than added to destroy the excess periodate, and the RNA was purified by gel filtration in Sephadex G-100 and ethanol precipitation.

β elimination of the periodate-treated mRNA was performed in 0.25 ml of 0.33 M steam-distilled aniline, adjusted to pH 5.0 with HCl, containing 0.1% sodium dodecyl sulfate. For incomplete removal of m^7G as in the case of mRNA described in Figures 1 and 2, the reaction was stopped after 2 h in the dark at room temperature. For complete β elimination, the incubation time was increased to 6 h. The treated mRNAs were then collected by precipitating two times with ethanol.

Mock-treated mRNA was subjected to all steps of the β elimination except that periodate was omitted during the oxidation step.

Analysis of 5′ Termini. mRNA (0.5–1 μg) was digested

with P_1 nuclease followed by alkaline phosphatase and analyzed by paper electrophoresis at pH 3.5 as described previously (Furuichi et al., 1975).

Ribosome Binding of mRNA. Mock-treated, periodate-oxidized or β-eliminated mRNA (0.5 pmol/50 μl assay) was incubated in wheat germ extract S_{23} at 25 °C or in rabbit reticulocyte lysate at 30 °C in the presence of 200 μM sparsomycin to prevent polypeptide chain elongation and thus obtain 80S monosomes (Darnbrough et al., 1973). *S*-Adenosylhomocysteine (200 μM) was included to prevent methylation by enzymes present in the extracts. The standard binding conditions were as described previously for wheat germ extracts (Both et al., 1976). For reticulocyte lysate, binding reactions were carried out in 50 μl consisting of 30 μl of lysate (kindly provided by Dr. W. F. Anderson, National Institutes of Health; Crystal et al., 1974), 12 mM Hepes buffer, pH 7.5, 75 mM KCl, 0.6 mM magnesium acetate, 0.8 mM ATP, 0.2 mM GTP, 0.1 mg/ml of creatine phosphokinase, 10 mM creatine phosphate, 5 mM dithiothreitol, 20 μM of each of 20 amino acids, and 20 μg/ml of hemin. [^{3}H]Methyl-labeled mRNA or synthetic ribopolymers were added at a level of 0.5 pmol/50 μl assay, a concentration of six- to tenfold below the saturation value. At the indicated times, samples were diluted fourfold with cold Tris buffer (20 mM Tris, pH 7.5, mM KCl, 3 mM magnesium acetate and the mixtures loaded onto 5 ml, 10 to 30% v/v glycerol gradients. After centrifugation for 90 min at 48 000 rpm in an SW-50.1 rotor at 4 °C, fractions were collected and counted directly in aqueous counting scintillant (Amersham/Searle).

*Preparation of [^{3}H]Methyl-Labeled m^7GpppG_pmC and $m^7GpppGpC$ and ^{32}P-Labeled G*pppGpC.* [^{3}H]Methyl-labeled m^7GpppG_pmC was prepared as described previously (Both et al., 1976) by digesting reovirus methylated mRNA with T_2 ribonuclease followed by alkaline phosphatase and subsequent purification by paper electrophoresis. $m^7GpppGpC$ was synthesized by incubating washed reovirus cores (2 mg/ml) with 0.05 mM ppGpC (kindly provided by Dr. J. Tomasz, Szeged, Hungary; Simoncsits et al., 1975), 2 mM GTP, 10 mM phosphoenolpyruvate, 1.0 unit of pyruvate kinase, 4 mM $MgCl_2$, and 10 μM [^{3}H]-*S*-adenosylmethionine for 16 h at 45 °C (Furuichi et al., 1976). The reaction mixture was extracted with phenol, applied to Whatman 3 MM paper, and electrophoresed at pH 3.5 for 60 min at 2600 V. The paper was dried, cut into 1-cm strips, and counted. A broad peak of ^{3}H in the region of 5′-AMP consisted of a mixture of $m^7GpppGpC$ and smaller amounts of m^7GpppG_pmC. It was eluted and the two components were resolved by paper chromatography in isobutyric acid–0.5 M NH_4OH (10:6 v/v). They were used as primers for ribopolymer synthesis by polynucleotide phosphorylase. [^{32}P]G*pppGpC was prepared as described previously (Both et al., 1976) by incubating reovirus cores with ppGpC and [α-^{32}P]GTP in the presence of *S*-adenosylhomocysteine.

*Synthesis of Ribopolymers Containing 5′-Terminal $m^7GpppGpC$, m^7GpppG_pmC, and G*pppGpC.* Appropriate amounts of trinucleotide primer (usually 40–50 pmol) were incubated in 100 μl of 0.05 M Tris buffer, pH 8, 0.01 M $MgCl_2$ with 1.0 μg of polynucleotide phosphorylase (kindly provided by Drs. C. Klee and M. Singer, National Institutes of Health, Bethesda, Md.) and the indicated ribonucleoside diphosphates under primer-dependent conditions (0.2 M NaCl) for 2 h at 37 °C, and the resulting polymers were purified by phenol extraction and gel filtration in Sephadex G-100 (Both et al., 1976). The specific activities were 5000 cpm/pmol for $m^7GpppGpC$, 10 000 cpm/pmol for m^7GpppG_pmC, and 1200 cpm/pmol for G*pppGpC.

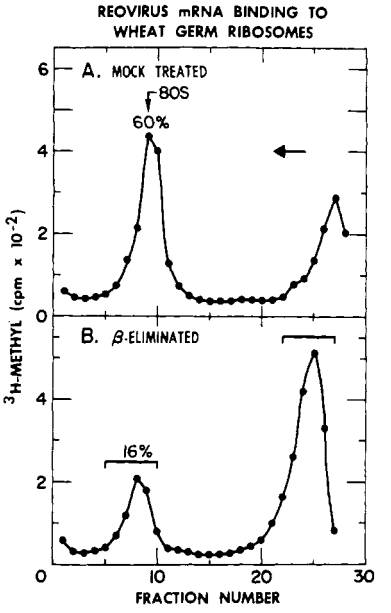

FIGURE 1: Reovirus mRNA binding to wheat germ ribosomes. Mock-treated or β-eliminated reovirus [^{3}H]methyl-labeled mRNA from which 80% of the 5′-terminal m^7G had been removed was incubated (0.5 pmol per 50-μl assay) for 10 min at 25 °C in wheat germ S_{23} extract in the presence of 200 μM sparsomycin and 200 μM *S*-adenosylhomocysteine as described in the Experimental Procedures section. The incubation mixtures were diluted with 3 volumes of cold Tris buffer (20 mM Tris, 70 mM KCl, 3 mM magnesium acetate), layered onto 5 ml glycerol gradients (10–30%), and centrifuged for 90 min at 48 000 rpm in an SW 50.1 rotor at 4 °C. Fractions were collected and counted in aqueous counting scintillant (Amersham/Searle).

Results

Preferential Binding to Ribosomes of mRNA with 5′-Terminal m^7G. Previously, it was reported that removal of the 5′-terminal m^7G from reovirus mRNA by β elimination resulted in a parallel decrease in translation of this mRNA in wheat germ extract, consistent with an important role for m^7G in protein synthesis (Muthukrishnan et al., 1975a). Because the introduction of internal scissions in mRNA during β elimination would also interfere with polypeptide chain elongation, we have reexamined the effect of this chemical treatment on translation specifically at the level of initiation. [^{3}H]methyl-labeled reovirus mRNA was synthesized by the virion-associated RNA polymerase in the presence of [^{3}H]methyl-*S*-adenosylmethionine. The mRNA contained radioactivity only in the 5′-terminal structure, m^7GpppG^m. Since ribosomes bind to reovirus mRNA at the 5′ end (Kozak and Shatkin, 1976), use of mRNA labeled specifically at this site permits a direct estimate of the efficiency of ribosome binding by assaying for 80S ribosome-associated radioactivity.

Ribosome binding of reovirus β-eliminated mRNA was compared with that of mock-treated mRNA, i.e., exposed to aniline but not periodate oxidized (Figure 1). Sixty percent of the mock-treated mRNA radioactivity was recovered in 80S complexes as compared with 16% for β-eliminated mRNA. A fourfold reduction in ribosome binding is consistent with the observed extent of removal of the 5′-terminal m^7G as determined by electrophoretic analysis of mRNA digested with P_1 nuclease and alkaline phosphatase (Figure 2A). Two ^{3}H-labeled components were resolved in the positions of G^m (68%) and m^7GpppG^m (32%), and their identities were confirmed by

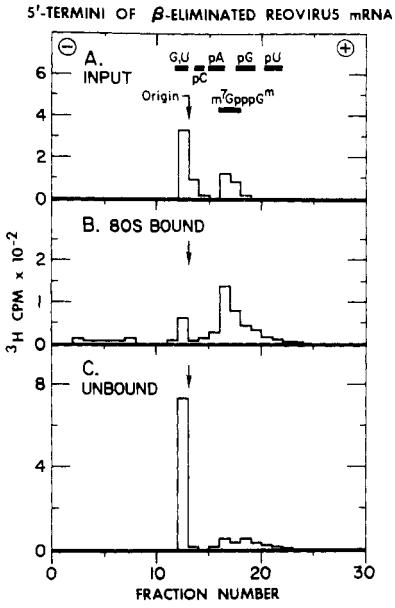

FIGURE 2: Analysis of 5′ termini of incompletely β-eliminated reovirus mRNA. (A) An aliquot of incompletely β-eliminated reovirus mRNA that was used for ribosome binding in the experiment described in Figure 1B was digested with P_1 nuclease and alkaline phosphatase. The digest was analyzed with the indicated marker compounds by paper electrophoresis at pH 3.5 for 60 min at 2600 V. The paper was dried, cut into 1-cm strips, and counted in toluene-based scintillant (Furuichi et al., 1975). (B) The 80S ribosome-bound mRNA was pooled (fractions 6–10 of Figure 1B), phenol-extracted, and ethanol-precipitated. The RNA pellet was digested with P_1 nuclease and alkaline phosphatase and analyzed as in A. (C) The radioactive mRNA not bound to ribosomes was pooled (fractions 23–27 of Figure 1B) and analyzed as in B.

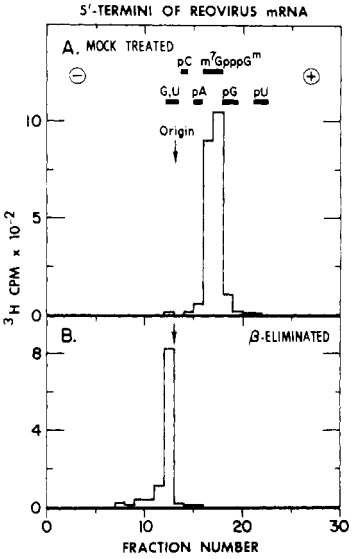

FIGURE 3: Analysis of 5′ termini of mock-treated and completely β-eliminated reovirus mRNA. [^{3}H]Methyl-labeled reovirus mRNA was β-eliminated by incubation for 6 h in aniline after periodate oxidation or mock-treated without addition of periodate as described in Experimental Procedures. The mRNAs were digested with P_1 nuclease plus alkaline phosphatase and analyzed by high-voltage paper electrophoresis.

paper chromatography in 2-propanol–HCl and isobutyric acid–NH_4OH solvents, respectively (Furuichi et al., 1975; Muthukrishnan et al., 1975a). On the basis of the presence of 1 vs. 2 methyl groups in these two structures, it was calculated that the β-eliminated [^{3}H]methyl-labeled mRNA consisted of a 4:1 molar ratio of molecules with 5′-terminal $pppG^m$ vs. m^7GpppG^m. The latter structure presumably contained the dialdehyde form of m^7G since all the 5′ termini were converted to $pppG^m$ when the incubation time in aniline was increased from 2 to 6 h (see below). After incubation in wheat germ extract, the 80S ribosome-bound and unbound mRNAs were recovered and similarly analyzed by enzymatic digestion and electrophoresis. m^7GpppG^m comprised 78% of the [^{3}H]methyl radioactivity in the bound mRNA (Figure 2B). This value is consistent with a 2:1 molar ratio of molecules with 5′-terminal m^7GpppG^m vs. $pppG^m$. Thus, although the residual capped molecules constituted only about 20% of the input β-eliminated mRNA, the ribosome-bound fraction was predominantly of the capped type, resulting in an enrichment of molecules with m^7GpppG^m associated with 80S ribosomes. The unbound mRNA yielded mostly G^m after treatment with P_1 nuclease and alkaline phosphatase (Figure 2C), indicating that it was depleted of molecules with 5′-terminal m^7GpppG^m. The results demonstrate that, in a mixture of β-eliminated reovirus mRNA, molecules retaining the 5′-terminal m^7G were preferentially bound to ribosomes in wheat germ extract. Since the mixture of molecules with 5′-terminal m^7GpppG^m and $pppG^m$ had been subjected to the same chemical treatment and chains with m^7G were selectively bound, it is likely that the decrease in ribosome binding and translation of β-eliminated reovirus mRNA was due to removal of m^7G rather than to nonspecific effects of the treatment.

Comparison of Reovirus mRNA Binding to Wheat Germ and Reticulocyte Ribosomes. The dependence on 5′-terminal m^7G for initiation complex formation may be characteristic of reovirus mRNA or the result of translation of an animal virus mRNA in a plant cell extract. We, therefore, tested the ribosome binding properties of reovirus mRNA in wheat germ extract and rabbit reticulocyte lysate. For comparison of the influence of m^7G on mRNA binding, [^{3}H]methyl-labeled mRNA was periodate oxidized and β-eliminated under conditions that modified the 5′ termini quantitatively. Digestion of the treated mRNA with P_1 nuclease and alkaline phosphatase yielded G^m as the only radioactive constituent (Figure 3B), while m^7GpppG^m was obtained from the mock-treated mRNA (Figure 3A).

The kinetics of 80S–mRNA initiation complex formation of methylated reovirus mRNA that was mock-treated, oxidized or β-eliminated are shown in Figure 4. The mock-treated mRNA bound rapidly with a high efficiency (>80%) in both wheat germ extract (Figure 4A) and reticulocyte lysate (Figure 4B). Periodate oxidation of the mRNA resulted in a 25% decrease in binding to wheat germ ribosomes and a 35% reduction in reticulocyte lysate. These results suggest that alteration of the m^7G in mRNA affects initiation, a suggestion also supported by the finding that 7-methylxanthosine 5′-diphosphate and 7-methylinosine 5′-diphosphate were several-fold less effective than 7-methylguanosine 5′-diphosphate as inhibitors of reovirus mRNA binding to wheat germ ribosomes (Muthukrishnan, Morgan, Hecht, and Shatkin, unpublished results). After removal of all of the 5′-terminal m^7G by β elimination, the binding efficiency of reovirus mRNA to wheat germ ribosomes decreased from 80 to about 5%, in agreement with the inhibitory effect of β elimination or of addition of m^7pG on translation of reovirus mRNA in wheat germ extract (Muthukrishnan et al., 1975a; Hickey et al., 1976). However, the same β-eliminated mRNA bound to reticulocyte ribosomes to the extent of 17% of the input mRNA (Figure 4B). This residual binding appears to be due to m^7G-independent interaction(s) of mRNA with ribosomes

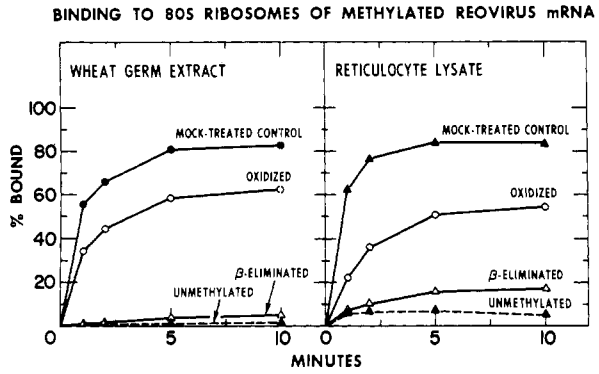

FIGURE 4: Binding of reovirus mRNA to 80S ribosomes. Reovirus [^{3}H]methyl-labeled mRNA was oxidized and a portion was β-eliminated to remove all of the 5′-terminal m^{7}G or mock-treated as described in the legend of Figure 3. The treated mRNAs were incubated in wheat germ extract or reticulocyte extract under standard conditions. At the indicated times, the reactions were stopped by addition of cold Tris buffer, and the proportion of radioactivity associated with 80S ribosomes was determined by glycerol gradient centrifugation as in Figure 1.

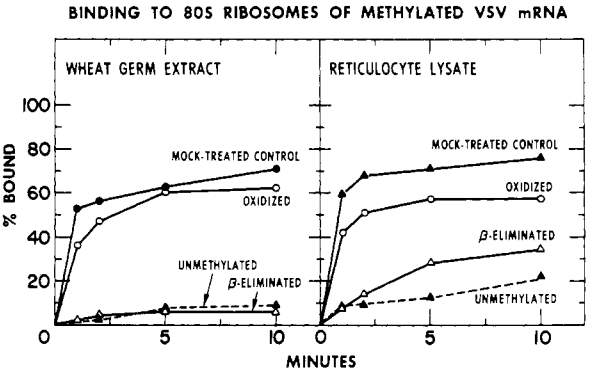

FIGURE 5: Binding of VSV mRNA to 80S ribosomes. VSV [^{3}H]methyl-labeled mRNA synthesized in vitro was oxidized and β-eliminated or mock-treated. Binding of the mRNAs (0.5 pmol/50 μl) was determined as in Figure 1.

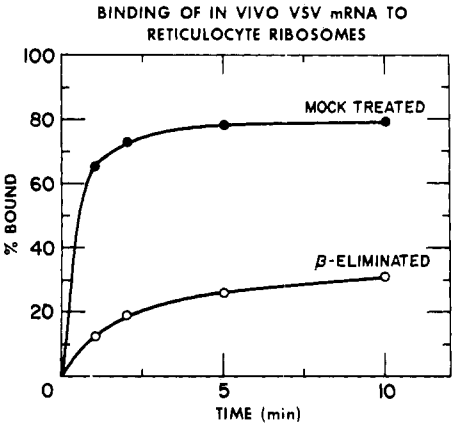

FIGURE 6: Ribosome binding of VSV mRNA isolated from infected BHK cells. BHK cells infected with VSV were labeled with [^{3}H]methionine (specific activity 5 Ci/mmol) from 2.5 to 7 h after infection. The mRNA was isolated by phenol extraction, oligo(dT)cellulose chromatography, and glycerol gradient centrifugation as described by Moyer et al. (1975). Aliquots of the purified mRNA were β-eliminated for 6 h or mock-treated. Ribosome binding of the mRNA (700 or 1400 cpm/μg; 2.2 μg) in reticulocyte lysate (50 μl) was determined as in Figures 4 and 5.

since the presence of 1 mM m^{7}pG did not further reduce the binding of the β-eliminated mRNA, whereas the binding of untreated, methylated mRNA was decreased from 80 to 18%, the level observed for β-eliminated mRNA (data not shown). Thus, in contrast to the 15-fold decrease in reovirus mRNA binding observed in the plant cell extract after β elimination, there was a four- to fivefold reduction in the animal cell lysate. The results demonstrate that the presence of an intact 5′-terminal m^{7}G promotes the association of reovirus mRNA to ribosomes, and the dependence on m^{7}G is greater in the wheat germ extract than in reticulocyte lysate.

Ribosome Binding of VSV mRNA. To begin to assess whether m^{7}G recognition is also a property of other animal virus capped mRNAs, similar experiments were done with methylated VSV mRNA synthesized in vitro by the virion-associated polymerase. Mock-treated mRNA bound to wheat germ and reticulocyte ribosomes to the extent of 70–80% (Figure 5). After periodate oxidation, binding was decreased, but the effect was less than with periodate oxidized reovirus mRNA. However, ribosome binding in wheat germ extract was almost completely inhibited after removal of 5′-terminal m^{7}G by β elimination (Figure 5A). In reticulocyte lysate, the same β-eliminated VSV mRNA retained the ability to form 80S complexes to the extent of 34% of the input mRNA as compared with 59 and 76%, respectively, for periodate- and mock-treated mRNAs (Figure 5B). The residual binding in reticulocyte lysate was not due to incomplete β elimination of the mRNA since digestion with P_1 nuclease plus phosphatase followed by electrophoretic analysis, as in Figure 3, yielded A^m but no 5′-terminal cap structure, m^{7}GpppA^m (data not shown) (Abraham et al., 1975a).

VSV mRNA isolated from infected BHK cells contains 5′ termini that are more extensively methylated than mRNA synthesized in vitro (Rose, 1975; Moyer and Banerjee, 1976). It was of interest to examine the effect of β elimination of this mRNA on ribosome binding in reticulocyte lysate. As shown in Figure 6, the results were similar to those obtained with methylated mRNA synthesized in vitro. The rates of binding of β-eliminated in vivo and in vitro VSV mRNA in reticulocyte lysate were markedly reduced as compared with mock-treated mRNA, and the extent of binding in each case was decreased by about 2.5-fold. In contrast, Rose and Lodish (1976) observed a smaller decrease (20–30%) of in vivo VSV mRNA binding to reticulocyte ribosomes after β elimination. This apparent discrepancy may be due to the lower binding (42–54%) observed for control mRNA by Rose and Lodish (1976) as compared with the value of 79% in the present experiments. In both studies, the residual binding of β-eliminated mRNA was similar: 28–37 vs. 31–34%.

While mock-treated methylated VSV and reovirus mRNAs bound to a similar extent (70–80%) in both cell-free systems, there were notable differences with the β-eliminated mRNAs. The reticulocyte ribosomes bound VSV mRNA more efficiently than reovirus mRNA after removal of the 5′-terminal m^{7}G. However, in the wheat germ system both reovirus and VSV mRNAs bound only inefficiently after β elimination (5–7%). The results suggest that in addition to 5′-terminal m^{7}G, reticulocyte ribosomes and/or soluble factors recognize other structural features in these viral mRNAs, e.g., nucleotide sequence (Both et al., 1976), and on this basis are capable of forming stable initiation complexes in the absence of 5′-terminal m^{7}G.

Binding of Unmethylated mRNAs. The contribution of nucleotide sequence to the stability of initiation complexes in the absence of methyl groups was estimated by comparing the ability of unmethylated reovirus and VSV mRNAs to bind to reticulocyte and wheat germ ribosomes. In the presence of

TABLE I: Ribosome Binding of Capped Synthetic Ribopolymers.[a]

Polymer	Wheat Germ Extract			Reticulocyte Lysate		
	G ppGpC	$m^7GpppGpC$	m^7GpppG^mpC	GpppGpC	$m^7GpppGpC$	m^7GpppG^mpC
A, U	12	46	65	3	5	28
U	5[b]	2	22	*d*	4	11
U, C	*d*	5	42	*d*	*d*	<1
A, C	*d*	2	11	*d*	*d*	<1
A	*d*	*d*	3[b]	31	46	77
A_2, U_2, G^c	20[b]	64	70	*d*	69	81

[a] Values expressed as percent of input counts per minute bound to 40S ribosomal subunits except for the A, U and A_2, U_2G polymers which formed 80S complexes. The 5′ end is designated. [b] From Table II (Both et al., 1976). [c] Subscript indicates input ratio of nucleoside diphosphates. [d] Not done.

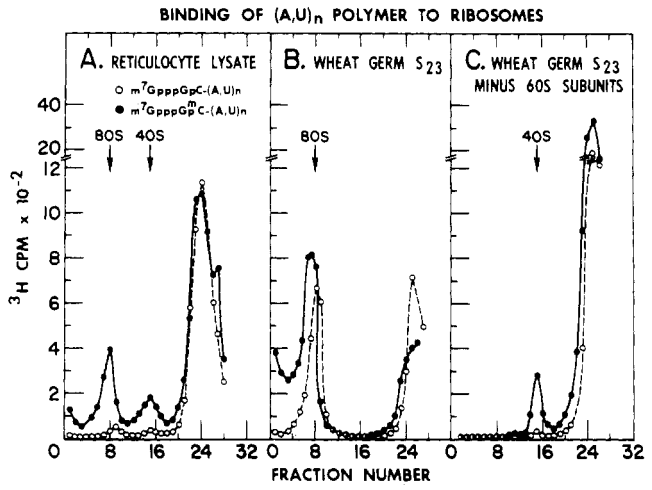

FIGURE 7: Ribosome binding of poly(A,U) containing 5′-terminal $m^7GpppGpC$ or m^7GpppG^mpC. Poly(A,U) with mono- or dimethylated 5′-terminal structures of size >5 S was incubated for 10 min under standard conditions with reticulocyte lysate, wheat germ S_{23} extract, or S_{23} depleted of 60S ribosomal subunits by centrifugation for 2 h at 60 000 rpm in the SW 65 rotor. The mixtures were diluted, sedimented in glycerol gradients, and analyzed as in Figure 1.

S-adenosylhomocysteine to prevent methylation by enzymes in the extract, reovirus and VSV unmethylated mRNA bound to reticulocyte ribosomes to the extent of about 7 and 20%, respectively (Figures 4 and 5). In wheat germ extracts, the binding efficiencies were less: 1 and 10% for reovirus and VSV unmethylated mRNA, reflecting again a more efficient interaction with reticulocyte ribosomes for these mRNAs lacking 5′-terminal methyl groups.

The binding efficiency of the unmethylated viral mRNAs in the reticulocyte lysate was less than that of the corresponding β-eliminated mRNAs. We, therefore, considered the possibility that the efficiency of ribosome binding is influenced by the 2′-O-methylation of the 5′-terminal sequence. One basis for stable initiation complex formation of unmethylated mRNA presumably is an interaction of the 5′-terminal sequence with ribosomes since for reovirus mRNA (Kozak and Shatkin, 1976) and brome mosaic virus RNA (Dasgupta et al., 1975) the 5′ terminus constitutes the ribosome binding site. This 5′-proximal sequence is the same in the unmethylated and β-eliminated, methylated mRNA preparations, but they contain different 5′-terminal structures. The unmethylated mRNA molecules contain either an unblocked diphosphate or a blocked 5′ terminus with the structure GpppN (Both et al., 1975b; Abraham et al., 1975b). By contrast, the β-eliminated, methylated mRNAs contain predominantly 5′-terminal $pppN^m$. To test if the presence of an unblocked 5′ end rather than a blocked terminus increases mRNA binding, the binding efficiencies of β-eliminated, unmethylated VSV mRNA (5′-terminal pppA) and mock-treated unmethylated mRNA containing 5′-GpppA in the reticulocyte lysate were compared. Values of 8 and 13% were obtained for the β-eliminated and mock-treated mRNAs, respectively. Thus, it appears that the presence or absence of blocked 5′ termini or unblocked phosphates at the 5′ end does not significantly alter ribosome binding efficiency in vitro. Similarly, phosphatase treatment of β-eliminated brome mosaic virus RNA-4 did not alter its translation in wheat germ extract (Shih et al., 1976). The greater binding to reticulocyte ribosomes of β-eliminated, methylated VSV and reovirus mRNA as compared with the corresponding unmethylated mRNA is consistent with 5′-proximal 2′-O-methylated residues influencing ribosome recognition of mRNA.

Effect of 2′-O-Methyl Group on Ribosome Binding of Capped Ribopolymers. In previous studies, it was shown that the ribosome binding efficiency of synthetic ribopolymers, i.e., poly(A,U) was increased by the presence of the 5′-terminal "cap", m^7GpppG_pmC (Both et al., 1976). The dimethylated structure, m^7GpppG_pmC, derived from reovirus mRNA by T_2 RNase digestion followed by alkaline phosphatase treatment, was used with polynucleotide phosphorylase and ribonucleoside diphosphates under primer-dependent conditions for the synthesis of capped ribopolymers. ^3H-labeled monomethylated cap, $m^7GpppGpC$, was prepared by incubation of ppGpC, GTP, and [^3H]methyl-*S*-adenosylmethionine with reovirus cores (Furuichi et al., 1976) as described in the Experimental Procedures section. It was similarly used as a primer for ribopolymer synthesis. The ribosome binding efficiencies of poly(A,U) containing 5′-terminal m^7GpppG_pmC vs. $m^7GpppGpC$, i.e., cap structure with or without the 2′-*O*-methyl group, were compared in reticulocyte lysate and wheat germ extract. Capped poly(A,U) formed 80S and lower amounts of 40S complexes in reticulocyte lysate (Muthukrishnan et al., 1976; Figure 7A). The presence of the 2′-*O*-methyl group in the 5′-penultimate residue increased binding fivefold. In wheat germ extract where capped A,U-rich polymers bind strongly to 80S ribosomes (Both et al., 1976), the presence of the 2′-*O*-methyl group had only a small effect and increased binding from 46 to 65% (Figure 7B, Table I). However, in wheat germ extract that was depleted of 60S ribosomal subunits by high-speed centrifugation, there was binding of m^7GpppG_pmC-poly(A,U) but not of poly(A,U)

containing 5′-terminal $m^7GpppGpC$, i.e., lacking the 2′-*O*-methyl group (Figure 7C). Presumably the 40S initiation complex of $m^7GpppGpC$-$(A,U)_n$ has low stability and the presence of the 60S subunits in the incubation mixture increases the extent of complex formation by converting them to 80S complexes which are more stable (unpublished data).

Table I summarizes the extent of binding of various polymers as percent of input cpm associated with 40S or 80S ribosomes. Ribopolymers that formed only 40S complexes, i.e., poly(U), poly(A,C), and poly(U,C) showed a strong dependence on the presence of both methyl groups for stable complex formation. By contrast, polymers that also formed 80S complexes with wheat germ ribosomes including poly(A,U) and poly(A_2,U_2,G) had high binding efficiencies and showed only small increases when their caps contained both methyl groups. Capped poly(A,U) bound weakly in reticulocyte lysate, and the addition to the cap of the 2′-*O*-methyl group increased the binding markedly. Thus, the stimulatory effect on stable initiation complex formation of the 2′-*O*-methyl group in the cap was most apparent when the ribosome binding efficiencies of mRNA or of synthetic ribopolymers were low.

Discussion

The results presented in this paper extend our previous observations on the loss of translational activity of eukaryotic viral and cellular mRNAs after β elimination. After removal of the 5′-terminal m^7G residue from capped reovirus and VSV mRNAs, the ability to form 80S ribosome complexes in wheat germ extract is markedly decreased in agreement with the previous finding that unmethylated reovirus mRNA forms few or no 40S or 80S initiation complexes in wheat germ extract. Furthermore, the decreased ability to form stable complexes with ribosomes is correlated specifically with the loss of the m^7G residue since, in a preparation of incompletely β-eliminated reovirus mRNA, molecules containing m^7G are enriched in the ribosome-associated mRNA fraction while the unbound mRNA fraction consists mostly of molecules lacking m^7G. The residual m^7G-containing mRNA molecules constitute an internal control in these experiments, and their binding to ribosomes argues against the possibility that nonspecific effects of the chemical treatment are responsible for the observed loss of ribosome binding. Furthermore, β elimination of encephalomyocarditis virus RNA, which does not contain 5′-terminal m^7G, did not decrease its ability to stimulate polypeptide synthesis in ascites cell-free extract (data not shown).

Comparison of the binding efficiencies of β-eliminated viral mRNAs in the wheat germ and the reticulocyte lysate revealed that the degree of dependence on m^7G for initiation complex formation varies in the two different cell-free protein synthesizing systems. While removal of 5′-terminal m^7G has a strong influence on initiation complex formation by viral mRNAs in the wheat germ system, it has only a moderate effect in the reticulocyte lysate resulting in a decrease in the extent of binding ranging from 2.5- to 4-fold. This may be due to a more efficient recognition of some other structural feature of the animal virus mRNAs in the mammalian cell-free system as compared with the plant extract. In agreement with this interpretation is our finding that unmethylated reovirus and VSV mRNAs bind more efficiently to the reticulocyte ribosmes than to wheat germ ribosomes. The strong dependence on 5′-terminal m^7G for mRNA binding is apparently not a property unique to wheat germ but appears to reflect the more heterologous combination of an animal virus mRNA in plant cell extract. Ribosome binding and translation of brome mosaic virus mRNA in wheat germ, a normal host for this virus, are incompletely dependent upon the presence of 5′-terminal m^7G (Shih et al., 1976). The translation of β-eliminated BMV RNA at low concentrations of mRNA comparable to those used in our experiments (10 μg/ml or less) was reduced about eightfold, but at high concentrations (100 μg/ml) there was only a small decrease (~30%) in translation as compared with m^7G-containing mRNA. Under conditions that are not optimal for protein synthesis, the dependence on 5′-terminal m^7G may be greater. For example, Shih et al. (1976) showed that, at Mg^{2+} concentrations of 3 and 5.5 mM, the effect of β elimination on translation of brome mosaic virus RNA 4 was more pronounced than at 4 mM Mg^{2+} which is optimal for translation.

Ribosome binding of mRNA in a more homologous situation may involve recognition of a 5′-proximal sequence since our studies with capped synthetic ribopolymers suggest that, in addition to m^7G, an A,U-rich sequence near the 5′ end increases stable association with ribosomes (Both et al., 1976; Muthukrishnan et al., 1976). Ribosome binding to the 5′ ends of brome mosaic virus RNA (Dasgupta et al., 1975), and reovirus mRNA (Kozak and Shatkin, 1976) in wheat germ extracts has been demonstrated previously. 5′-Terminal sequences vary among different mRNAs and thus probably differ in their contribution to the overall stability of mRNA-initiation complexes. This may account for differences in intrinsic translational efficiencies among capped mRNAs (Nuss and Koch, 1976). Consistent with this suggestion, β-eliminated reovirus and VSV mRNAs have different binding efficiencies in reticulocyte lysate. These differences are not evident when both m^7G-dependent and internal sequence interactions occur, and the m^7G-containing reovirus and VSV mRNAs have almost the same high binding efficiency in reticulocyte lysate. In addition to affecting the extent of binding, removal of the m^7G also decreases the rate of association of mRNA with ribosomes in the reticulocyte lysate. We have previously reported similar effects of 5′-terminal m^7G on the binding of the capped polymer $m^7GpppG^mpC(A_2,U_2,G)_n$ to wheat germ and reticulocyte ribosomes (Both et al., 1976; Muthukrishnan et al., 1976).

β-eliminated VSV and reovirus mRNAs have significantly higher ribosome binding efficiencies than the corresponding unmethylated mRNAs in the reticulocyte lysate, suggesting a stabilizing effect of the 5′-terminal 2′-*O*-methyl group on initiation complex formation. In the case of β-eliminated, methylated vs. β-eliminated, unmethylated VSV mRNA, the 5′-terminal sequences differ only with respect to the presence or absence of the 2′-*O*-methyl group in the 5′terminal $pppA^{(m)}$, and its presence presumably accounts for the observed increase in binding. Studies with synthetic ribopolymers containing 5′-terminal $m^7GpppGpC$ vs. m^7GpppG^mpC also suggest that the 2′-*O*-methyl group may be part of the recognition mechanism for mRNA–ribosome interaction. The effect of the 2′-*O*-methyl group on synthetic polymer binding is most evident in those instances where the ribopolymer forms 40S complexes but not 80S ribosome complexes, possibly due to the absence of a sequence needed to form the 80S complex. The capped synthetic polymers, $(U)_n$, $(U,C)_n$, and $(A,C)_n$ bind to wheat germ 40S ribosomes only when they contain both methyl groups in the 5′-terminal structure. However, it is possible that these polymers do form complexes with 40S subunits even when they lack the 2′-*O*-methyl group. If these complexes have low stability and are dissociated during the centrifugation step, glutaraldehyde fixation may be needed to detect their formation. The $(A,U)_n$ polymer which binds weakly to reticulocyte

ribosomes (5%) shows a fivefold increase in binding when the 2′-*O*-methyl group is present in the cap. In wheat germ extract, however, where the binding of this polymer is strong (46%), the stimulatory effect of the 2′-*O*-methyl group is less and binding increases to 65%. These results suggest that the effect of the 2′-*O*-methyl group in the cap can be demonstrated only when other factors affecting mRNA–ribosome initiations are weak or absent. In agreement with this hypothesis, the presence of a 2′-*O*-methyl group has only a small effect on the binding of $(A_2,U_2,G)_n$, a polymer which binds strongly to wheat germ and reticulocyte ribosomes. The binding of this polymer with ribosomes may be stabilized by nucleotide sequence interactions that include base pairing between the AUG initiator codon and the Met-tRNA$_f$ anticodon.

In conclusion, our results indicate that multiple structural features of mRNA influence initiation complex formation. They include both the m^{7}G and 2′-O-methylated residue of the 5′-terminal cap, internal nucleotide sequence(s) which may be 5′-proximal as in brome mosaic virus RNA (Dasgupta et al., 1975), and reovirus mRNA (Kozak and Shatkin, 1976), and the initiator AUG codon. Each of these mRNA structural components may be recognized by ribosomes or soluble factors, serving to position the mRNA and stabilize initiation complexes. The relative importance of each of these interactions varies among different mRNAs or for the same mRNA in different protein synthesizing systems. The overall efficiency of initiation would be determined by a summation of these interactions. Some mRNAs may be translated even when one or more of these structural components are absent, as in the case of poliovirus or encephalomyocarditis virus RNAs which do not have methylated 5′ termini. These RNAs may have a sequence or structure that permits their efficient recognition by ribosomes. The translation of these viral messages would be favored if other mechanisms operate to inhibit translation of capped mRNAs of the host.

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