β -globin mRNAs capped with m^7G , $m_2^{2,7}G$ or $m_3^{2,2,7}G$ differ in intrinsic translation efficiencyE.Darzynkiewicz¹, J.Stepinski², I.Ekiel³, Y.Jin⁺, D.Haber¹, T.Sijuwade and S.M.Tahara*

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Received April 29, 1988; Revised and Accepted August 22, 1988

ABSTRACT

We examined the intramolecular effect of altered cap structures on translation efficiency of artificial β -globin mRNAs. For these studies, synthetic dinucleotides of the form $X(5')ppp(5')G$ [$X=7$ -methyl guanosine (m^7G), 2,7-dimethyl guanosine ($m_2^{2,7}G$) or 2,2,7-trimethyl guanosine ($m_3^{2,2,7}G$)], were transcriptionally incorporated into mRNAs, containing rabbit β -globin coding sequences, using T7 RNA polymerase and a β -globin cDNA template. These synthetic mRNAs were assayed in reticulocyte lysate for activity relative to m^7G -capped mRNA. $m_2^{2,7}G$ -Capped mRNA was found to be 1.5-fold more active than m^7G -capped mRNA. Messenger RNA capped with $m_3^{2,2,7}G$ was less active with activity of 0.24 relative to its m^7G -capped counterpart (activity = 1.0). These data suggest that m^7G -capped mRNAs become more active as translation templates after addition of a single N^2 methyl moiety, which is especially pertinent to gene expression in togaviridae. The latter are observed to synthesize $m_2^{2,7}G$ and $m_3^{2,2,7}G$ -capped mRNAs in addition to m^7G -capped templates during the course of infection in animal cells.

INTRODUCTION

A role of the 5'-cap structure, found in all eukaryotic mRNAs, is to facilitate ribosome binding during the initiation phase of translation (1,2). In eukaryotic translation, several key interactions between the 5'-cap of mRNAs and cap binding proteins are required for optimum recognition/binding of 5'-caps to these ligand binding proteins (1,3-6; for review of initiation see ref. 7). Individually, these proteins have been identified as eIF-4A (8,9), eIF-4B (8), eIF-4E or CBP I (10,11), and eIF-4F (8,9,11,12). Of these only eIF-4F and eIF-4E have been shown to be true cap binding proteins; eIF-4A probably does not bind the cap directly but apparently binds at a site very near to the cap structure (13). The chemical features of the cap which are recognized include: 1) the N7 substituent of the capping guanine nucleotide (3,14); 2) the purine C2 amino and C6 keto substituents (3); and 3) the α -phosphate of the capping nucleotide (4,6). Other non-translation roles for the cap have been described, these include stabilization against 5'-exonucleolytic activities (15,16), transcription initiation (1), RNA splicing (17,18), 3'-processing of histone mRNA (18) and termination of transcription of influenza mRNAs (20).

Another naturally occurring type of cap structure is found at the 5'-end of snRNAs. Unlike mRNA m^7G caps, snRNAs are capped with $m_3^{2,2,7}G$ (21). These molecules do not appear to have a function in translation but instead participate in several partial reactions of RNA splicing. The $m_3^{2,2,7}G$ -cap structure has been found in viral mRNAs. Togaviruses such as Sindbis and Semliki Forest virus synthesize

mRNAs capped with $m_3^{2,2}7G$ and $m_2^{2,7}G$, in addition to m^7G , during the course of infection (22,23). We became interested in the biological activity of di- and trimethylated guanine caps through our studies pertaining to ligand binding properties of cap binding proteins as noted above.

Previous studies by us (4-6) and others (3,24) employed nucleotide cap analogs as competitive inhibitors in partial reaction and complete translation assays. However because several cap binding activities are present in partially fractionated cell extracts, competitive inhibition studies in reality characterize the combined cap binding requirements of the translation machinery. In an effort to analyze individual ligand binding requirements of the various cap binding proteins for the cap of a native mRNA molecule, we prepared mRNAs with different 5'-caps. Since m^7G , $m_2^{2,7}G$, and $m_3^{2,2}7G$ caps can be found *in vivo*, we decided to first compare the relative activities of these structures on a model synthetic mRNA. The experiments described here report a newly identified intramolecular influence on mRNA translation activity by biologically occurring hypermethylated caps.

EXPERIMENTAL METHODS

Construction of pT7rG4

The 489 bp *Hind* III/*Bgl* II fragment containing an intact rabbit β -globin coding region was excised from pSV2Ra β G (obtained from Paul Berg; ref. 25) and subcloned into *Hind* III/*Bam* HI cut pT7/T3-19 (Bethesda Research Laboratories) by standard methods (26). This recombinant plasmid was sequenced to verify the expected construction (27).

In vitro Transcription

5'-Capped RNA transcripts were synthesized in a 0.05 ml reaction volume containing: 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 0.5 mM NTPs (except GTP), 0.1 mM GTP, 0.5 mM m^7GpppG (New England Biolabs or Pharmacia-PL) or other capped dinucleotide (see below), 1000 U/ml RNasin (Promega), 100 μ g/ml *Ava* I linearized pT7rG4, 7.5 μ Ci [3H]ATP (ICN; 30 Ci/mmol) and 400 U/ml T7 RNA polymerase. Mixtures were incubated for 30 min at 37°C. A second aliquot of enzyme was added and the mixture was incubated 30 min longer. For synthesis of uncapped transcripts, capped dinucleotide was omitted from the incubation. Template DNA was digested with 5 μ g/ml DNase I (Cooper Biomedical) at 37°C for 10 min. After digestion, the mixture was extracted with phenol- $CHCl_3$ -isoamyl alcohol (25:24:1, v/v/v) and $CHCl_3$ -isoamyl alcohol (24:1, v/v). Transcripts were separated from unincorporated nucleotides and oligoDNA by precipitation from 2.5 M NH_4OAc with 2.5 volumes of ethanol at -20°C. RNA was collected by centrifugation (13,200 g), washed in 70% ethanol, dried, resuspended in sterile water and reprecipitated from 2.5 M NH_4OAc as above. Twice precipitated RNA was resuspended in 0.025 ml of sterile water and an aliquot was quantitated by liquid scintillation spectrometry in Safety Solve (RPI). Messenger RNA solutions were routinely stored at the vapor temperature of liquid nitrogen. Use of pT7rG4 for transcription by T7 RNA polymerase gives an RNA

transcript with the following 5'-untranslated sequence (the capped transcript is shown):

m⁷GpppGGGAGACCCAAGCUUGGACUGUGUUUACUUGCAAUCCCCCAAACAGACAGAAUG.

The β -globin transcripts contain 17 5'-proximal bases, corresponding to vector sequences, followed by 35 nucleotides of the native β -globin 5'-untranslated region, the entire β -globin coding region of 438 nts and 9 nts of the 3'-untranslated region.

In vitro Translation

Preparation of rabbit reticulocyte lysate and *in vitro* translation assays were performed as described earlier (6,11). Translation assays measured the incorporation of [³⁵S]Met (1347 Ci/mmol, Amersham) into hot trichloroacetic acid (TCA) precipitable material.

Synthesis of mono and dinucleotide cap analogs

m₃^{2,2} 7GMP and m₂^{2,7} GMP--These analogs were synthesized from a common precursor, 2-thio 6-keto purine riboside. The latter was oxidized with H₂O₂ and reacted with either dimethylamine or methylamine to yield m₂^{2,2} guanosine or m²guanosine, respectively (28). 5'-Phosphorylation of these nucleosides was performed as described earlier (5,6) followed by N7 methylation with methyl iodide (5,6) to give m₃^{2,2} 7GMP and m₂^{2,7} GMP, respectively. Products were homogenous as judged by chromatography in two solvent systems (5) and exhibited proton NMR spectra consistent with methyl substitutions at the N7 and N² positions, as expected.

Extended dinucleotide caps were synthesized by the general method described by Nakagawa et al. (29) and Yamaguchi et al. (30). A full description concerning the synthesis and physical characterization of the extended cap series of compounds and di- and trimethylated guanine nucleotides will be reported elsewhere.

Cap analysis

Analyses of 5'-ends of T7 transcribed RNAs were performed as described by Konarska et al. (17). ³²P-labeled, 5'-capped transcripts were prepared as above except that [α -³²P]GTP (3000 Ci/mmol; NEN) was used as radiolabel. ³²P-labeled transcripts (50,000 cpm) were digested with ribonuclease T2 (40 U/ml) for 15 min at 37°C. A portion of the digest (10,000 cpm) was analyzed by two-dimensional chromatography on cellulose thin layers. pppGp and XpppG markers were routinely co-chromatographed with digests as migration markers and visualized under UV light. Positions of ³²P-labeled, 5'-cap nucleotides were visualized by autoradiography at -70°C for 24 hr.

RESULTS

As part of a systematic examination of the structural features of the cap which are recognized by cap binding proteins, we examined the inhibitory properties of m₃^{2,2} 7GMP and the related compound m₂^{2,7} GMP in translation. A titration of both analogs in a reticulocyte translation assay indicated that m₂^{2,7} GMP was an effective inhibitor of translation (50% inhibition at 0.02 mM), whereas m₃^{2,2} 7GMP was

Figure 1--Inhibition of globin synthesis in reticulocyte lysate by added cap analogs.

Translation was performed in micrococcal-treated rabbit reticulocyte lysate containing cap analogs at different concentrations. Native rabbit β -globin mRNA (100 ng) was incubated in a 0.025 ml reaction at 37°C for 15 min. Aliquots (0.015 ml) were removed from the reaction mixture after incubation and measured for incorporation of [³⁵S]Met into hot TCA precipitable material by liquid scintillation spectrometry. Cap analogs were assayed for effect on translation at increasing concentrations as indicated in the figure. Relative translation is ratio of activity observed in the presence of analog relative to activity in the absence of analog, expressed as a percentage. m⁷GMP (○); m^{2,7}GMP (◐); m^{3,2,7}GMP (●).

inactive as an inhibitor (Fig. 1). In comparison to the inhibitory activity of m⁷GMP (50% inhibition at 0.06 mM), m^{2,7}GMP was a much more effective inhibitor. This result suggested that it possessed a higher affinity for cap binding initiation factors than did m⁷GMP. Other N7 substituted cap analogs have been synthesized by us and tested for inhibitory activity in reticulocyte lysate. Substituents larger than ethyl were essentially inactive as inhibitors (Darzynkiewicz et al., manuscript in preparation). Based on the results of cap analog inhibition shown in Fig. 1 we were initially interested in examining the effect of replacing the base of the 5'-capping nucleotide of mRNA with other guanine derivatives. We expected that m^{2,7}G would increase the activity of the cap moiety as suggested by the competitive inhibition results.

For the translation studies, extended caps of the form XpppG (X=m^{2,7}G, and m^{3,2,7}G) were synthesized. Our intent was to incorporate these dinucleotides into the 5'-ends of synthetic transcripts using T7 RNA polymerase. Several groups, including our own, have observed that SP6 and T7 RNA polymerase can initiate transcription using the capped dinucleotide m⁷GpppG (17,18). However it was not known for sure if modified capped dinucleotides other than m⁷GpppG could also initiate transcription by phage RNA polymerases. In an effort to confirm this, RNAs were synthesized using T7 RNA polymerase from a cloned rabbit β -globin transcription template. These mRNAs were initiated with different extended cap dinucleotides and labeled with [α -³²P]GTP. Presumptively capped transcripts were subjected to T2 RNase digestion and analyzed for transfer of radiolabel into the 3'-position of the extended cap (XpppGp*). As shown in Fig. 2, mRNA transcripts were observed to have quantitatively

Figure 2--5'-End analyses of artificial mRNAs initiated with different extended cap analogs.

Extended caps were incorporated into RNA molecules using T7 RNA polymerase and *Ava* I linearized pT7rG4. Reaction conditions were as described earlier except that 13 μ Ci of [α - 32 P]GTP (3000 Ci/mmol) was used in each transcription mixture. RNA transcripts were subjected to ribonuclease T2 digestion (16) and analyzed by two-dimensional TLC on cellulose sheets (8 X 8 cm; Baker). Chromatography in the first dimension was in isobutyric acid/ $\text{NH}_4\text{OH}/\text{H}_2\text{O}$, 577/38/385, pH4.3 (system I); the second dimension solvent was saturated (NH_4) $_2\text{SO}_4$ /1 M NaOAc/iPrOH, 80/18/2 (system II). Positions of XpppG and pppGp markers on each chromatogram are shown as dotted circles (compare panel A with other panels for position of pppGp). Autoradiograms are shown. PI = inorganic phosphate.

- A. Uncapped mRNA: **1** = pppGp*
 B. GpppG capped mRNA: **2** = GpppG; **2a** = GpppGp*
 C. m⁷GpppG capped mRNA: **3** = m⁷GpppG; **3a** = m⁷GpppGp*
 D. m₂^{2,7}GpppG capped mRNA: **4** = m₂^{2,7}GpppG; **4a** = m₂^{2,7}GpppGp*
 E. m₃^{2,2,7}GpppG capped mRNA: **5** = m₃^{2,2,7}GpppG; **5a** = m₃^{2,2,7}GpppGp*
 F. Summary of relative migration of 3'-nucleoside monophosphates and mRNA 5'-end dinucleotides.

incorporated a 5'-cap corresponding to the synthetic extended cap which was used for transcription initiation. An undetectable fraction of the 5'-ends corresponded to uncapped mRNAs as judged by the absence of pppGp*. We observed no conversion of pppGp* to the bis or triphosphate forms, even after longer exposure of the autoradiograms thus indicating the absence of any degradation of the mRNA 5'-nucleotide. Removal of the labeled 3'-phosphate of the digestion products was not significant due to the very low levels of 5'-capped nucleotides as compared to the 3'-labeled Np cleavage products. Any Pi produced during T2-treatment probably was derived from the latter digestion products. Furthermore, transcripts initiated with different extended caps were homogeneous and equal in expected size to globin transcripts initiated with m⁷GpppG (not shown). Therefore, from these studies

Figure 3--Translation activity of globin mRNAs with nonstandard caps.

Translation assays were performed as described in the legend to Fig. 1 except that the reaction volume was 0.05 ml and incubation was at 30°C. Synthetic mRNAs (200 ng) terminated with m^7GpppG or nonstandard 5'-caps were added to separate reactions and incubated. Aliquots (0.005 ml) were removed at the indicated times and hot-TCA precipitable radioactivity was measured. m^7GpppG -mRNA (○); $m_{3,2,7}GpppG$ -mRNA (●); $m_{2,7}GpppG$ -mRNA (●). Relative initial rates of translation for the various capped mRNAs were: m^7GpppG (1.0); $m_{2,7}GpppG$ (1.5); $m_{3,2,7}GpppG$ (0.24).

transcription by T7 RNA polymerase can initiate with extended cap dinucleotides to make full length and appropriately 5'-capped β -globin mRNAs.

Messenger RNAs with different capping nucleotides were assayed in rabbit reticulocyte lysates for relative rates of translation. It was observed that mRNA capped with a $m_{2,7}G$ was the most active. Messenger RNAs capped with $m_{2,7}G$ was 1.5 fold more active than m^7G -capped mRNA based on initial rates. Replacement of m^7G by $m_{3,2,7}G$ led to a major decrease in activity to only 24% of the control translation rate (Fig. 3). Under these conditions, GpppG and pppG ended mRNAs were tested as translation templates and had the same activity as $m_{3,2,7}G$ -capped mRNA. The latter result was of interest since it has been reported that reticulocyte lysates are less cap dependent than wheat germ extracts (1,31,32). On the contrary, we observe that cap dependent translation is the rule for our translation studies under the reaction conditions we employed. At low K^+ concentrations, cap dependence is lost for translation of mRNAs (32). The translation assays described in this report were performed at a KOAc concentration of 150 mM. We routinely observe that inhibition of translation by cap analog and, by inference, cap dependent mRNA translation is also enhanced at higher ionic strength. An analysis of polypeptides synthesized from the different 5'-capped templates showed that, as expected, only full length β -globin polypeptides were made. Therefore the increased activity observed with $m_{2,7}G$ -capped mRNA was not a consequence of increased accumulation of truncated polypeptides with a corresponding increase in reinitiation. In control experiments the stability of differently capped globin transcripts was analyzed in reticulocyte lysate translation mixtures. All transcripts were observed to undergo slight degradation during the standard incubation period. At the end of a 15 min incubation, under mock translation conditions, less than 10% of the input mRNA was found to be smaller than full length in size. There was no difference in degradation rates of the variously capped mRNAs. Thus the observed differences in translation rates could not be attributed to any differences in mRNA stability due to a change in the capping nucleotide.

Figure 4--Inhibition of mRNAs with non-standard caps by nucleotide cap analogs.

Translation assays were performed as described in the legend to Fig. 1. Cap analogs were assayed over a concentration range of 0.025 to 0.1 or 0.01 to 0.05 mM as indicated. m⁷GpppG-mRNA (○), m₂⁷GpppG-mRNA (●). Relative translation is the same as defined in legend to Fig. 1. A. Effect of increasing m⁷GMP; B. Effect of increasing m₂⁷GMP.

The increased translation activity of m₂⁷G capped mRNA probably occurred via a more facile recruitment of translation factors; however it was not clear if this type of cap obviated a requirement for cap binding proteins in initiation. In an effort to resolve this question we assayed mRNAs containing m₂⁷G and m⁷G caps for inhibition of translation by the corresponding nucleotides: m⁷GMP and m₂⁷GMP. A similarity in the inhibition kinetics for the two differently capped mRNAs by each mononucleotide cap homolog would indicate that the increase in translation activity conferred on the mRNA by m₂⁷G caps was due to an increase in relative cap affinity and not by action through another translation component. The inhibition data indicated that this was indeed the case (see Fig. 4). m₂⁷GMP (50% inhibition at 0.005 to 0.007 mM) was found to be more active than m⁷GMP (50% inhibition at 0.018 mM) as translation inhibitor in translation assays regardless of the mRNA used. Both capped mRNAs had essentially the same inhibition kinetics when assayed against m⁷GMP, although it appeared that m⁷G-capped mRNA was slightly more sensitive to inhibition by m₂⁷GMP (Fig. 4B). The lower 50% inhibition concentrations of cap analog observed in Fig. 4 vs. Fig. 1 probably were due to sequence differences between native and synthetic translation templates. For example, native globin mRNAs were used to obtain the experimental results shown in Fig. 1 whereas T7 globin transcripts were used in the experiments shown in Fig. 4. Differences in the 5'-untranslated region could conceivably have an effect on other mRNA-factor interactions (e.g., binding of eIF-4A/eIF-4B to mRNA). Inhibition of translation initiation by these analogs might be more effective if other determinants of mRNA initiation activity, perhaps contributions from the 5'-untranslated region, are weakened or absent.

DISCUSSION

By the approach we have described it should be possible to cap synthetic mRNAs with virtually any guanine nucleotide by transcription initiation with dinucleotides of the form XpppG and T7 RNA

polymerase. Previous studies which examined the effect of exchanging the native m⁷G of the cap with another base were limited to replacement by either G (33), 7-methyl inosine (m⁷I; ref. 33) or e⁷G (14). It was concluded that m⁷I caps were recognized by cap binding proteins and could be appropriately bound to ribosomes (34). A similar observation was made for e⁷G capped mRNAs (14). In both cases, the non m⁷G caps were observed to result in decreased mRNA efficiency consistent with a decreased affinity of cap binding proteins for these cap structures.

The inability of m₃^{2,2} 7G capped mRNAs to be translated is consistent with their cellular localization *in vivo*. Trimethylated caps of the form m₃^{2,2} 7G, to date, have been normally found only in nuclear snRNAs which participate in nuclear post-transcriptional processes (21). Methylation of snRNA caps occurs in two separate cellular compartments. The N7 modification is effected in the nucleus during transcription initiation whereas N² exocyclic dimethylation occurs in the cytoplasm by one or more enzymes (35,36). Thus the immature snRNA must translocate to the cytoplasm for further cap methylation prior to returning to its site of action in the nucleus (21,35). This type of cap might serve to functionally compartmentalize snRNAs from mRNA dependent processes and vice versa.

Low activity for m₃^{2,2} 7G capped mRNAs was reported earlier for Sindbis 26S mRNA (22). However van Duijn et al. also reported that a small but significant proportion of the 26S mRNA population of Semliki Forest virus had m₂^{2,7} G caps (23). The latter were reportedly not active in translation as inferred by their low abundance in ribosomal fractions. However, these mRNAs were not directly assayed for translation efficiency as in this study. The existence of m₂^{2,7} G caps on normal cellular mRNAs has not yet been demonstrated; nonetheless, this type of cap structure is almost surely to be an intermediate in the synthesis of m₃^{2,2} 7G caps found in snRNAs (21,35,36). Thus one might predict that if m₂^{2,7} G capped snRNA molecules were allowed to accumulate in the cytoplasm of a cell then they could conceivably compete with cytoplasmic mRNAs for translation components.

The observation that m₂^{2,7} G capped mRNA was more active than m⁷G capped mRNA was intriguing. It has not been shown if cellular mRNAs are N² methylated during normal growth. This might be a possible mechanism whereby cytoplasmic mRNAs can be made more active. As a corollary, dimethylation of the exocyclic N² amino group of an mRNA cap could lead to inactivation of the message by reducing affinity of the cap binding protein for this portion of the template mRNA. This type of mechanism conceivably could be used by a cell to selectively inactivate cytoplasmic mRNAs; conversely demethylation could serve to reactivate such hypermethylated capped mRNAs.

The increase in activity observed by substituting m₂^{2,7} G for m⁷G as the 5' cap is not large. However, this increase in initiation should be placed in perspective as one contribution to the intrinsic activity of a given mRNA. Other features of mRNA which might contribute to a change in initiation rates include differences in secondary structure in the 5' region of mRNAs (37), where ribosome scanning occurs (38), or the presence of *cis* regulatory elements (39), which may act to augment initiation and

hence translation rates. Nonetheless, relatively small changes in translation rates are significant. For example, the intrinsic translation rates of α - and β -globin differ such that ~20-50% more polypeptide is synthesized from β -globin mRNA than from an equimolar amount of α -globin mRNA (40). Excess synthesis of either α - or β -globin chain can be responsible for thalassemia (40) thus underscoring the importance of regulating the synthesis of these polypeptide chains. In effect, erythropoietic cells recognize this difference in mRNA activity and must compensate by synthesizing more α -globin mRNA (42). From the studies presented here it would be of considerable interest to examine the differences in translation behavior of m^7G , $m_2^2,7G$ and $m_3^2,2,7G$ capped togavirus mRNAs and whether these viral cap structures are important in the virus life cycle. The approach described in this report will make these types of studies feasible.

ACKNOWLEDGMENTS

We thank Aaron Shatkin for helpful comments. This work was supported by grants to S.M.T. from the American Cancer Society (MV-257), the Life and Health Insurance Medical Research Fund, and the NIH (GM38512). E. D. was supported in part by grants from the Polish Academy of Sciences (CPBR-3.13) and the Polish Ministry of Science, Technology and Higher Education (CPBP 01.06). Y.J. was supported in part by a grant from the USC-Norris Cancer Center. We also thank Ms. Laura Steel for preparing the manuscript.

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