Translational Recognition of the 5′-Terminal 7-Methylguanosine of Globin Messenger RNA as a Function of Ionic Strength†

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ABSTRACT: The translation of rabbit globin mRNA in cell-free systems derived from either wheat germ or rabbit reticulocyte was studied in the presence of various analogues of the methylated 5′ terminus (cap) as a function of ionic strength. Inhibition by these analogues was strongly enhanced by increasing concentrations of KCl, K(OAc), Na(OAc), or NH_4(OAc). At appropriate concentrations of K(OAc), both cell-free systems were equally sensitive to inhibition by m^7GTP. At 50 mM K(OAc), the reticulocyte system was not sensitive to m^7GMP or m^7GTP, but at higher concentrations up to 200 mM K(OAc), both nucleotides caused strong inhibition. The compound $m^7G^{5'}ppp^{5'}Am$ was inhibitory at all concentrations of K(OAc) ranging from 50 to 200 mM, although more strongly so at the higher concentrations. Over the same range of nucleotide concentrations, the compounds GMP, GTP, and $G^{5'}ppp^{5'}Am$ were not inhibitors. The mobility on sodium dodecyl sulfate-polyacrylamide electrophoresis of the translation product was that of globin at all K(OAc) concentrations in the presence of m^7GTP. Globin mRNA from which the terminal m^7GTP group had been removed by chemical treatment (periodate-cyclohexylamine-alkaline phosphatase) or enzymatic treatment (tobacco acid pyrophosphatase-alkaline phosphatase) was translated less efficiently than untreated globin mRNA at higher K(OAc) concentrations, but retained appreciable activity at low K(OAc) concentrations.

Most cellular and viral messenger RNAs of eukaryotic cells have been found to contain 7-methylguanosine linked by a 5′,5′-triphosphate bridge to the 5′-terminal residue of the polynucleotide chain, a structure commonly referred to as a cap (Shatkin, 1976; Griffin, 1976). The role of this structure in mRNA function has not yet been unequivocally established. Furuichi et al. (1977) have suggested that capping confers stability against degradation of mRNA in vivo. Others have presented evidence that the cap is a recognition signal necessary for efficient translation of mRNA. One type of approach supporting the latter role has been to use analogues of the cap such as m^7GMP[1] (Hickey et al., 1976; Weber et al., 1976; Lodish & Rose, 1977) and $m^7G^{5'}ppp^{5'}Nm$ (Lodish & Rose, 1977) as competitive inhibitors of mRNA translation. Another approach has been to compare the translation of viral mRNAs synthesized in vitro in the presence or absence of a methyl donor (Both et al., 1975a,b; Furuichi & Shatkin, 1976; Rose & Lodish, 1976). This technique cannot currently be applied to cellular mRNAs and, consequently, one must resort to chemical and enzymatic means to remove caps. Rao et al. (1975) reported that periodate oxidation virtually eliminated the translational activity of liver mRNA. Similarly, Muthukrishnan et al. reported that globin (1975a) and *Artemia salina* (1975b) mRNAs retained less than 20% activity following oxidation and β elimination. Treatment of brome mosaic virus mRNA in a similar way resulted in a product which retained 40% of the original activity (Shih et al., 1976). Vesicular stomatitis virus mRNA (Lodish & Rose, 1977) and parathyroid hormone mRNA (Kemper, 1976) also lost activity to varying degrees depending on the severity of chemical treatment. Enzymatic methods have also been applied to remove mRNA caps. Abraham & Pihl (1977) treated globin and immunoglobulin mRNAs with polynucleotide kinase and reported that, even though m^7GDP was liberated, the mRNAs retained full activity. These authors argued that the reagents used for chemical decapping have deleterious side reactions on the polynucleotide chain. By contrast, Zan-Kowalczewka et al. (1977) treated several mRNAs with potato nucleotide pyrophosphatase and found only 20% of the activity remained.

One way to test the question of secondary damage to the polynucleotide chain of mRNA, caused by either chemical side reactions or contaminating enzymatic activities, would be to assay the mRNA in a cell-free system which did not recognize the cap structure. The possibility that some systems may be more cap dependent than others was suggested by the finding of Rose & Lodish (1976) that cap structures appear more important for translation in cell-free systems derived from wheat germ than from reticulocytes. More recently, Weber et al. (1977b) reported that increasing the K^+ concentration in both reticulocyte and wheat germ cell-free systems reduced the translation of unmethylated vaccinia mRNA relative to methylated controls.

We have examined the translation of globin mRNA as a function of ionic strength and have used various cap analogues as a probe to distinguish the degree to which translation depends on the cap structure. We report here conditions of low ionic strength in which the reticulocyte cell-free system retained high activity and was capable of synthesizing full-length globin, but was almost completely insensitive to m^7GMP and m^7GTP. At high ionic strength, on the other hand, both reticulocyte and wheat germ systems were equally sensitive to those inhibitors. Considerably less dependence on ionic strength was observed with the inhibitor $m^7G^{5'}ppp^{5'}Am$. Decapped globin mRNA assayed under the cap-independent conditions exhibited appreciable activity while it was virtually inactive under cap-dependent conditions.

Materials and Methods

Materials. m^7GMP, m^7GTP, $m^7G^{5'}ppp^{5'}Am$, and $G^{5'}$-

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[1] Abbreviations used: m^7GMP, 7-methylguanosine 5′-monophosphate; Am, 2′-*O*-methyladenosine; m^6Am, 2′-*O*-methyl-N^6-methyladenosine; Gm, 2′-*O*-methylguanosine; VSV, vesicular stomatitis virus; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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ppp$^{5'}$Am were purchased from P-L Biochemicals, and GMP and GTP from Sigma Chemical Co. The following extinction coefficients were used: m^{7}GMP, 11.0 A_{256}/μmol at pH 2.0; m^{7}GTP, 10.5 A_{257}/μmol at pH 2.0; m^{7}G$^{5'}$ppp$^{5'}$Am, 25 A_{260}/μmol at pH 7.0; and G$^{5'}$ppp$^{5'}$Am, 27 A_{260}/μmol at pH 7.0. [^{3}H]Leucine (62 Ci/mmol) was obtained from Schwarz/Mann. NCS is a product of the Amersham Corp.

Reticulocyte Cell-Free System. Reticulocyte lysate was prepared from anemic rabbits as described previously (Evans & Lingrel, 1969) except that lysis of cells was performed using water instead of 5 mM $MgCl_2$. The lysate was then made dependent on exogenous mRNA for protein synthesis using the procedure of Pelham & Jackson (1976). Reaction (25 μL final volume) contained 15 μL of lysate, 10 mM creatine phosphate, 40 μM each of 19 amino acids except leucine, 0.25 mM $MgCl_2$, and 3 μCi of [^{3}H]leucine. K(OAc), NH_4(OAc), Na(OAc), or KCl was added at the concentrations indicated. The contribution of monovalent ions by lysate (Bernstein, 1954) was not taken into account. Incubations were carried out for 60 min at 30 °C. Duplicate 5-μL samples were assayed for radioactivity as previously described (Schimke et al., 1974) except $\frac{1}{2}$-in. filter discs, 0.2 mL of NCS, and 4-mL shell vial were used.

Wheat Germ Cell-Free System. Wheat germ extract, a generous gift from Dr. C. Bancroft, Memorial Sloan-Kettering Cancer Center, was prepared by the procedure of Roberts & Paterson (1973) without preincubation. Reactions contained, in a final volume of 25 μL, 7.5 μL of wheat germ extract, 2.0 mM Mg(OAc)$_2$, 16 mM Hepes, pH 7.6, 2.4 mM dithiothreitol, 1.4 mM ATP, 0.28 mM GTP, 21 mM creatine phosphate, 20 units/mL creatine kinase, 60 μM each of 19 amino acids except leucine, 25 μM spermine, and 3 μCi of [^{3}H]leucine. All reactions contained 36 mM KCl, contributed by the wheat germ extract, plus additional K(OAc) to arrive at the indicated K^+ concentrations. The incubation and assay of radioactivity incorporated were the same as for the reticulocyte lysate system.

Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out in Plexiglas tubes (6 mm i.d.) using the gel system of Laemmli & Favre (1973). Aliquots from cell-free protein synthesis assays were dialyzed against sample buffer and separated on a 6% spacer gel (1.5 cm) and 15% separating gel (8 cm). After electrophoresis, the separating gel was cut into 2-mm slices, and the radioactivity was solubilized in NCS at 37 °C overnight and determined by liquid scintillation spectrometry.

Globin mRNA Preparations. Rabbit globin mRNA was prepared as described previously (Lockard & RajBhandary, 1976). For one preparation, the cap was removed by treatment with periodate, cyclohexylamine, and alkaline phosphatase as described previously (Lockard & Rajbhandary, 1976; Lockard et al., 1978). For a second preparation, the cap was removed by treatment with tobacco acid pyrophosphatase (Shinshi et al., 1976) and alkaline phosphatase. The detailed procedure will be published elsewhere (Chu, L.-Y., Lockard, R. E., RajBhandary, U. L., & Rhoads, R. E., manuscript in preparation). The extinction coefficient used for globin mRNA was 20 A_{260}/mg. These preparations were a generous gift from Drs. R. Lockard and U. RajBhandary, Massachusetts Institute of Technology.

Results

Comparison of Cell-Free Systems. Lodish & Rose (1977) presented data indicating that the translation of VSV mRNA in a reticulocyte cell-free system was insensitive to the cap analogues m^{7}GMP and m^{7}G$^{5'}$ppp$^{5'}$N(m), whereas translation in a wheat system was inhibited. More recently, Weber et al. (1977b) showed that the inhibition of vaccinia mRNA translation in a wheat germ system by m^{7}GMP was enhanced at high K^+ concentrations. We set out to determine whether these systems were equally sensitive to cap analogues when the appropriate K^+ concentration for each system was used. It was desirable to use inhibitors which were analogues of various parts of the cap as probes of cap recognition by the translation machinery. We therefore chose to study the inhibition of rabbit globin mRNA, one of the few cellular mRNAs for which the complete covalent structure of the cap has been determined.

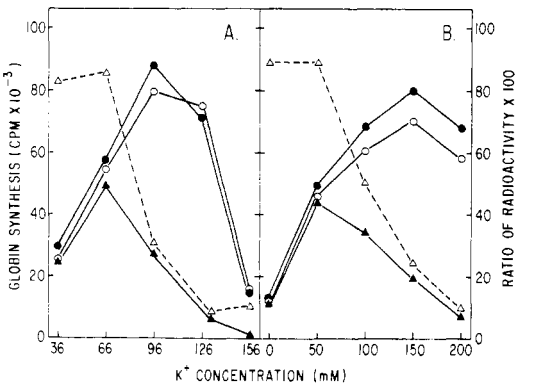

FIGURE 1: Translation of globin mRNA at different K^+ concentrations with or without m^{7}GTP. K(OAc) was added to give the final concentrations indicated. (A) Translation in the wheat germ cell-free system. The globin mRNA concentration was 2.69 μg/mL. (B) Translation in reticulocyte lysate system. The globin mRNA concentration was 2.23 μg/mL. (●—●) Control; (○—○) reactions contained 0.2 mM GTP; (▲—▲) reactions contained 0.2 mM m^{7}GTP; (△---△) synthesis of globin in the presence of 0.2 mM m^{7}GTP expressed as a percentage of the synthesis in the control reaction.

The inhibition of globin mRNA by m^{7}GTP in wheat germ and reticulocyte cell-free systems is presented in Figure 1. The K^+ optimum of translation in the absence of m^{7}GTP was considerably broader for reticulocyte than for wheat germ, a finding observed by Weber et al. (1977a) as well. For example, in reticulocyte, the activity of the system was 85% of the maximal activity over a 100 mM range of K^+ concentrations, whereas, in wheat germ, the range was only 37 mM. The sensitivity of translation to 0.2 mM m^{7}GTP, however, was remarkably similar in both systems. At low K^+ concentrations, globin translation was only slightly inhibited (less than 15%). At the K^+ optimum (96 mM for wheat germ, 150 mM for reticulocyte), the inhibition was 70–75% of control in both systems. At high K^+ concentrations, sufficient to reduce translation to 75% of maximal, inhibition by m^{7}GTP was 90% of control in both cases. Substituting GTP for m^{7}GTP had no effect on translation (Figure 1). We conclude that, by the criterion of sensitivity to this particular inhibitor, these two systems exhibited equal dependence on the cap structure for efficient translation of mRNA, provided the appropriate K^+ concentration was used.

Comparison of Different Inhibitors. The cap structure of rabbit globin mRNA has been determined to consist of m^{7}G$^{5'}$ppp$^{5'}$m^{6}Am, m^{7}G$^{5'}$ppp$^{5'}$Am, and m^{7}G$^{5'}$ppp$^{5'}$A (Hunt & Oakes, 1976; Lockard & RajBhandary, 1976). It therefore was possible to use analogues corresponding to an increasingly larger portion of the cap to further investigate the phenomenon of K^+-dependent inhibition. These comparisons were performed both to determine the portions of the cap structure which are important for binding to the translational machinery and also to rule out the possibility that inhibition was due to competition with GTP at some other step in protein synthesis.

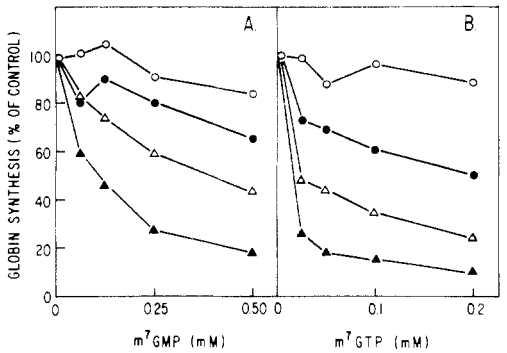

FIGURE 2: (A) Effect of m^7GMP on the translation of globin mRNA in the reticulocyte lysate system at different K(OAc) concentrations. Incubations contained 2.23 μg/mL globin mRNA and the concentrations of m^7GMP indicated. K(OAc) was added to give the following final K^+ concentrations: (○—○) 50 mM K^+; (●—●) 100 mM K^+; (△—△) 150 mM K^+; (▲—▲) 200 mM K^+. (B) Effect of m^7GTP on the translation of globin mRNA in the reticulocyte lysate system at different K(OAc) concentrations. Details are as in A except that m^7GTP was used instead of m^7GMP.

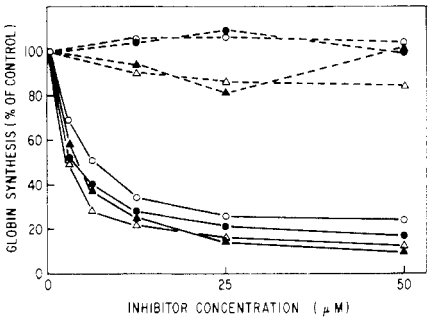

FIGURE 3: Effect of $m^7G^{5'}ppp^{5'}Am$ and $G^{5'}ppp^{5'}Am$ on the translation of globin mRNA in the reticulocyte lysate system at different K(OAc) concentrations. The effect of $m^7G^{5'}ppp^{5'}Am$ is indicated by solid lines and $G^{5'}ppp^{5'}Am$ by broken lines. Otherwise, details are as in Figure 2.

The reticulocyte system was used for subsequent studies because of its lower background.

The effect of m^7GMP on globin translation as a function of both nucleotide and K^+ concentration is shown in Figure 2A. At 50 mM K^+, the system was relatively insensitive to the analogue, translation being inhibited less than 20% at the highest concentration of m^7GMP tested. However, at 200 mM K^+, translation was inhibited 82% at the highest concentration of inhibitor used. A larger cap analogue, m^7GTP, was also tested as a function of both inhibitor and K^+ concentrations (Figure 2B). The same dependence on K^+ was observed as with m^7GMP. Very little inhibition occurred at 50 mM K^+ (approximately 10% at the highest concentration of nucleotide tested), but inhibition was nearly total (90%) at 200 mM K^+. GMP and GTP tested over the same range of nucleotide and K^+ concentrations exhibited less than 20% inhibition (data not shown).

Finally, a dinucleotide analogue of the 5′ terminus of globin mRNA, $m^7G^{5'}ppp^{5'}Am$, was tested at various K^+ and nucleotide concentrations (Figure 3). Unexpectedly, this compound inhibited globin translation at both high and low K^+ concentrations. Although the concentration of K^+ had some influence on inhibition, the magnitude of the effect was much less than with either m^7GMP or m^7GTP. For example, if one chooses a nucleotide concentration that produced 50% inhibition at 200 mM K^+, the same concentration of nucleotide at 50 mM K^+ produced no detectable inhibition, within experimental error, in the case of either m^7GMP (Figure 2A) or m^7GTP (Figure 2B). However, with $m^7G^{5'}ppp^{5'}Am$, such a concentration produced 30% inhibition at 50 mM K^+ (Figure 3). The compound used as a control in this experiment, $G^{5'}ppp^{5'}Am$, exhibited essentially no inhibition over the same concentration ranges of K^+ and nucleotide (Figure 3).

TABLE I: Comparison of Inhibitors at Various K(OAc) Concentrations.[a]

K(OAc) concn (mM)	Concn of nucleotide to achieve 50% inhibition (μM)		
	m^7GMP	m^7GTP	$m^7G^{5'}ppp^{5'}Am$
50	>500	>200	6.7
100	>500	200	3.8
150	390	34	3.1
200	106	17	4.4

[a] The concentration of each nucleotide required to achieve 50% inhibition of globin synthesis was determined as described in Figure 2.

The relative potencies of these three inhibitors are shown in Table I. At the K^+ optimum (150 mM), the concentration of m^7GTP required for 50% inhibition was 11-fold lower than that of m^7GMP. The dinucleotide was even more effective; the concentration required for 50% inhibition was 126-fold lower than that of m^7GMP.

Effect of Different Electrolytes. To further understand the mechanism by which K(OAc) enhanced inhibition by cap analogues, we tested other electrolytes. Varying amounts of $NH_4(OAc)$ were added to the cell-free system already containing 50 mM K(OAc). The total activity of the system was highest with no $NH_4(OAc)$ added, decreased to 90% of this activity at 100 mM added, and to 46% at 150 mM added (data not shown). In spite of these differences, the inhibition of translation of m^7GTP became more pronounced with increasing $NH_4(OAc)$ (Figure 4A). Thus, substitution of a cation which resembles K^+ in structure (Cotton & Wilkinson, 1966) did not alter the basic phenomenon.

The effect of Na(OAc), in which the hydrated radius of the cation is larger than that of K^+ (Na^+, 2.76 Å; K^+, 2.32 Å), was tested. In a system containing 50 mM K(OAc), optimal total activity was observed at 50 mM added Na(OAc), and 95% of that activity, at 100 mM Na(OAc) (data not shown). The effect on the potency of m^7GTP as an inhibitor was, nevertheless, the same as with K(OAc) or $NH_4(OAc)$ (Figure 4B).

When KCl was added to the cell-free system, the total activity of the system peaked at 75 mM, the shape of the curve being similar to that reported by Weber et al. (1977a). Again, we found that inhibition by m^7GTP increased with increasing KCl (Figure 4C). However, the concentrations of KCl needed to cause equivalent effects were approximately half those of K(OAc).

Size of Translational Product. To verify that the product synthesized in response to added globin mRNA was in fact full-length globin under all conditions of ionic strength and inhibitor concentrations, we examined the electrophoretic mobility of the translation products on sodium dodecyl sulfate–polyacrylamide gels. With no inhibitor added and at 150 mM K(OAc), 85% of the radioactivity migrated as a single symmetric peak with the mobility of globin. In the presence of 50 mM m^7GTP, the radioactivity decreased as the K(OAc) concentration was increased from 50 to 200 mM, but the distribution was identical with that observed with no inhibitor. In the absence of added mRNA there were no detectable products.

Translation of Decapped Globin mRNA. In the foregoing

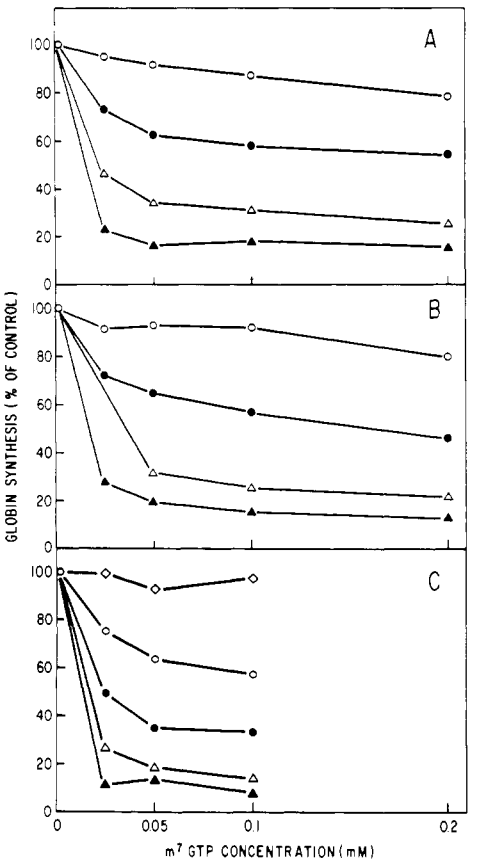

FIGURE 4: Effect of m^7GTP on the translation of globin mRNA in the reticulocyte lysate system at different $NH_4(OAc)$, Na(OAc), and KCl concentrations. Incubations contained 2.23 μg/mL globin mRNA and the concentrations of m^7GTP indicated. (A) Translation at different NH_4^+ concentrations. Reactions contained 50 mM K(OAc), and $NH_4(OAc)$ was added to give the following final NH_4^+ concentrations: (○—○) 0 mM NH_4^+; (●—●) 50 mM NH_4^+; (△—△) 100 mM NH_4^+; (▲—▲) 150 mM NH_4^+. (B) Translation at different Na^+ concentrations. Reactions contained 50 mM K(OAc), and Na(OAc) was added to give the following final Na^+ concentrations: (○—○) 0 mM Na^+; (●—●) 50 mM Na^+; (△—△) 100 mM Na^+; (▲—▲) 150 mM Na^+. (C) Translation at different KCl concentrations: (◇—◇) 25 mM KCl; (○—○) 50 mM KCl; (●—●) 75 mM KCl; (△—△) 100 mM KCl; (▲—▲) 125 mM KCl.

studies, cap analogues were used as a probe to determine conditions under which the cell-free system either recognized or did not recognize cap structure. Using these conditions we next examined the translation of globin mRNA from which the terminal m^7GTP had been removed by two independent methods. In the first, the mRNA was treated with sodium periodate, cyclohexylamine, and alkaline phosphatase (see Materials and Methods) and its translation compared with untreated mRNA as a function of K^+ concentration (Figure 5). At 200 mM K^+, the activity was reduced 4% of control, indicating a strict dependence on the m^7GTP structure. With no added K^+, however, the activity of the treated sample was 48% that of control. Thus, the dependence on m^7GTP for translation was reduced tenfold in going from one extreme of K^+ concentration to the other.

Similar results were obtained with globin mRNA from which the terminal m^7GTP had been removed in an entirely different way. Globin mRNA was treated sequentially with tobacco acid pyrophosphatase and bacterial alkaline phosphatase and assayed over the same K^+ concentration range as the periodate-treated sample (Figure 5). At the highest K^+ concentration, the activity was reduced to 7.5% of control, while at the lowest K^+ concentration, the activity was 61%, an

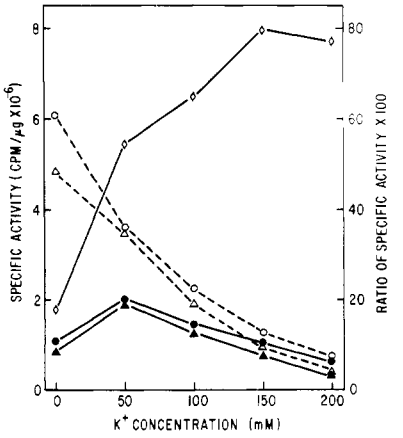

FIGURE 5: Translation of globin mRNA and decapped globin mRNA in the reticulocyte lysate system at different K^+ concentrations. Activity is expressed as cpm per μg of mRNA added (—) and as a percentage of untreated mRNA (- - -). K(OAc) was used to give the final K^+ concentrations indicated. (◇) Unmodified mRNA; (△, ▲) chemically decapped mRNA; (○, ●) enzymatically decapped mRNA.

eightfold reduction in the dependence of translation on the m^7GTP terminus. These results are completely different from those of Abraham & Pihl (1977) who observed no decrease in the translational activity of globin mRNA after removing more than 60% of the 5′-terminal m^7GDP with polynucleotide kinase. It is not clear at present whether this discrepancy is due to their use of a different enzyme to remove the cap, their use of a wheat germ system, or the fact that their mRNA preparation contained a 5′-phosphate moiety.

Discussion

The report by Lodish & Rose (1977) that m^7GMP, $m^7G^{5'}ppp^{5'}Am$, $m^7G^{5'}ppp^{5'}Gm$, and $m^7G^{5'}ppp^{5'}G$ inhibited the translation of VSV messengers in a wheat germ system but had no effect in a reticulocyte system made it seem likely that these systems differed markedly in their ability to recognize cap structures. Weber et al. (1977b) observed that altering the concentration of K(OAc) changed the ability of both systems to recognize cap structures. We therefore compared these systems in the hope of finding conditions of cell-free translation under which cap structures were not required. Our results indicate that, at the appropriate ionic strength, the two systems are quantitatively quite similar, both in terms of total incorporation of labeled leucine and in sensitivity to m^7GTP inhibition (Figure 1). Several considerations make the reticulocyte system preferable. First, amino acid incorporation in the absence of exogenous mRNA is fourfold lower in the reticulocyte system. Second, several laboratories have reported that large polypeptides are terminated prematurely in the wheat germ system at low K^+ concentrations (Harwood et al., 1975; Benveniste et al., 1976; Tse & Taylor, 1977), whereas there have been no comparable reports for the reticulocyte system to date. Our results with globin mRNA, admittedly a relatively small mRNA, indicate no change in the size of the translation product at low ionic strength. Finally, there is less source-dependent variability of reticulocyte preparations compared with wheat germ.

The use of m^7GMP, m^7GTP, and $m^7G^{5'}ppp^{5'}Am$ (Figures 2 and 3) made it possible to determine the quantitative effects on mRNA translation of increasing the size of the analogue. The addition of the β- and γ-phosphate groups increased the potency of the inhibitor 11-fold (Table I), suggesting that there are binding sites for this portion of the molecule in the putative

cap-recognizing initiation factor. Similarly, addition of a second nucleoside enhanced inhibition an additional 11-fold, a finding which suggests that this portion of the cap is recognized as well. Increasing the ionic strength caused more severe inhibition by all of the analogues tested. The dinucleotide inhibitor was considerably less sensitive to ionic strength than the mononucleotide inhibitors, perhaps indicating that the additional binding affinity contributed by the second nucleoside residue is independent of ionic strength in the range tested. The fact that $m^7G^{5'}ppp^{5'}Am$ was found to be a potent inhibitor even at 50 mM K(OAc) (Table I) is at variance with the results of Lodish & Rose (1977) who observed no effect with nucleotide concentrations as high as 130 μM. Whether this discrepancy is due to differences in the mRNAs being translated (globin vs. VSV structural proteins) or the translational systems (micrococcal nuclease-treated vs. whole reticulocyte lysate) remains to be determined. It is probably not due, however, to a difference in ionic strength since the reticulocyte system of Lodish & Rose (1977) contained 90 mM KCl.

With the mononucleotide inhibitors, the dependence on ionic strength was much more pronounced. The compound m^7GTP represents the exact portion of the mRNA cap that was lost in either of the decapping procedures employed in this study and can be used to define the range of ionic strengths useful for testing the activity of decapped mRNA. At the lower extreme, 50 mM K(OAc) was approximately the same as no added K^+ with respect to inhibition (Figure 1). This defines the ionic strength for minimal inhibition. At the other extreme, maximal inhibition for any combination of inhibitor or electrolyte was 88 to 92%. This degree of inhibition could be achieved at 200 mM K(OAc). The activity of the reticulocyte system for total incorporation of labeled leucine remained high (greater than 60% of the activity at the K^+ optimum) at both of these extremes. Two preparations of 5'-OH-terminated globin mRNA gave quite similar results when they were assayed over this range. The sample prepared by enzymatic treatment was slightly more active than the periodate-treated sample, possibly due to damage to the coding portion by the periodate treatment or to incomplete removal of m^7GMP by the enzyme. Regardless of these differences, it is clear that the modified mRNA exhibited markedly different behavior under this experimental protocol. This type of assay, then, provides an independent means, which does not depend on the use of cap analogue inhibitors, to distinguish whether an mRNA is terminated by a normal cap.

The fact that we observe enhanced recognition of the cap structure with increasing concentration of K(OAc), NH_4(OAc), Na(OAc), or KCl indicates that the effect is not due to a particular ion, but is a function of ionic strength. One explanation is that the secondary structure or conformation of mRNA is altered by ionic strength causing a change in mRNA-protein interactions. Another explanation for this phenomenon draws upon the use that Douzou & Maurel (1977) have made of polyelectrolyte theory in explaining the interdependence of pH and ionic strength in protein–nucleic acid interactions. One might suppose that the cap is recognized by a protein which has in its binding site a negative charge corresponding to the positive charge of m^7G. This is plausible since none of the unmethylated analogues had an effect on globin translation. The effect of increasing ionic strength is to decrease the p*K*s of functional groups on proteins which are in close proximity to a polyanion such as RNA, due to a reduction of the electrostatic potential of the polyanion. Thus, decreasing the p*K* of the group at constant pH would cause it to lose a proton, thereby increasing its electrostatic attraction for the m^7G group of the cap.

Acknowledgments

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Role of Hypermodified Bases in Transfer RNA. Solution Properties of Dinucleoside Monophosphates†

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ABSTRACT: The hypermodified dinucleoside monophosphates, uridylyl(3′–5′)-*N*-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]threonine (Upt^{6}A), adenylyl(3′–5′)-N^6-(Δ^2-isopentenyl)adenosine (Api^{6}A), adenylyl(3′–5′)-N^6-(Δ^2-isopentenyl)-2-methylthioadenosine (Apms^{2}i^{6}A), and adenylyl(3′–5′)-1,N^6-ethenoadenosine (ApεA, a synthetic model for adenylyl(3′–5′)wybutosine, ApyW), which represent the most common sequences found as the third letter of the anticodon triplet and its adjacent 3′ neighbor, have been isolated. Their solution properties have been investigated using ultraviolet absorption, circular dichroism (CD), and high resolution proton magnetic resonance. The properties of these molecules have been compared with those of their unmodified counterparts, uridylyl(3′–5′)adenosine (UpA) and adenylyl(3′–5′)-adenosine (ApA). These properties measured as a function of temperature have been analyzed employing a two-state intramolecular stacking model. All of the properties show that the stacking of Upt^{6}A is stabilized relative to UpA, while Api^{6}A, Apms^{2}i^{6}A, and ApεA are slightly destabilized relative to ApA. Thus, Upt^{6}A, Api^{6}A, Apms^{2}i^{6}A, and ApεA have comparable stacking equilibria, indicating that the modifications remove the large difference in stacking between UpA and ApA. Furthermore, cytidylyl(3′–5′)adenosine (CpA), which is the most common unmodified sequence in this particular anticodon region, exhibits a stability similar to those of the hypermodified dinucleoside phosphates. Hypermodification therefore seems to keep the flexibility of this crucial part of the tRNA constant. It is proposed that this may result in a more smoothly regulated translation step. Also, it is proposed that the enhanced stacking of Upt^{6}A relative to UpA prevents the incorrect wobble base pairing of this U residue in the tRNA during translation.

We have attempted in this investigation to define and understand on a molecular level the functions of hypermodified bases in tRNAs. That these bases perform some function during protein synthesis has been supported by experiments with polymer directed polypeptide synthesis, and binding assays using ribosomes and synthetic polymer messages (Gefter & Russell, 1969; Odom et al., 1974; Kitchingman et al., 1976; Miller et al., 1976). tRNAs modified or deficient in their normal amount of hypermodification were generally found to be less efficient in messenger binding and translation. In most of these studies, the aminoacylations of the tRNAs which were changed in their content of hypermodification were affected little if any by the changes. Binding studies with tRNAs and complementary trimers, or with two tRNAs having complementary anticodon triplets have also shown a stabilization of the binding when hypermodified bases are present (Högenauer et al., 1972; Grosjean et al., 1976). Thus, with only a few exceptions (Litwack & Peterkofsky, 1971; Kimball & Söll, 1974), the presence of the hypermodified bases has been demonstrated to affect in vitro polypeptide synthesis. This effect, at least in part, appears to be due to a more efficient binding of the tRNA to the ribosome–mRNA complex.

Aside from these effects upon translation, some authors have suggested that the role of t^{6}A[1] is to prevent wobble on the 3′ side of the anticodon triplet (Ghosh et al., 1967; Takeishi et al., 1968; Dube et al., 1968; Stewart et al., 1971; Jukes, 1973; Elkins & Keller, 1974). Also, on the basis of crystal structures of the hypermodified bases, several workers have proposed that the role of hypermodification is to prevent the formation of a fourth base pair between the tRNA and mRNA (Bugg & Thewalt, 1972; Parthasarathy et al., 1974a,b).

Except for the hypothesis of the prevention of a fourth base pair, it is not known how these possible functions are related to the molecular properties of the hypermodified bases. Therefore, we have chosen to approach this problem through the study of the smallest unit of a hypermodified tRNA which still exhibits the characteristics of a polynucleotide—the dinucleoside monophosphate. In this study, the solution spectral properties of Upt^{6}A, Api^{6}A, Apms^{2}i^{6}A, ApεA, and their

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[1] Abbreviations used: t^{6}A, *N*-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]threonine; i^{6}A, N^6-(Δ^2-isopentenyl)adenosine; ms^{2}i^{6}A, N^6-(Δ^2-isopentenyl)-2-methylthioadenosine; εA, 1-N^6-ethenoadenosine; yW, wybutosine (Kasai et al., 1976); pt^{6}A, 5′-monophosphate of t^{6}A; Upt^{6}A, uridylyl(3′–5′)-*N*-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]-threonine; Api^{6}A, adenylyl(3′–5′)-N^6-(Δ^2-isopentenyl)adenosine; Apms^{2}i^{6}A, adenylyl(3′–5′)-N^6-(Δ^2-isopentenyl)-2-methylthioadenosine; ApεA, adenylyl(3′–5′)-1,N^6-ethenoadenosine; ApyW, adenylyl(3′–5′)-wybutosine.

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