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Translational Recognition of Messenger Ribonucleic Acid Caps as a Function of pH†

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ABSTRACT: The degree to which cell-free translation of eukaryotic mRNA is stimulated by the presence of a 5′-terminal 7-methylguanosine-containing cap is affected by a variety of factors including ionic strength, temperature, mRNA concentration, and the type of mRNA. In this report, we show that pH also affects cap dependence. Translation of globin mRNA from which the cap had been enzymatically removed was relatively insensitive to pH compared with capped mRNA. Also, at low pH (6.6–7.2), the cap analogue m^7GTP caused little inhibition of globin mRNA translation in a cell-free system whereas at higher pH the degree of inhibition increased. Finally, the overall extent to which globin mRNA translation could be inhibited at saturating concentrations of m^7GTP increased with increasing pH. It is also shown that the pK_a of the N-1 proton of m^7GTP is affected by mono- and divalent cations. At the K^+ and Mg^{2+} concentrations optimal for cell-free translation, the pK_a is approximately 7.4. Since the pH optimum for translation is near 7.6, both keto and enolate forms of m^7G are present in appreciable amounts. One interpretation for the observed change in cap dependence with pH is that only the enolate form of m^7G is recognized by the cap-binding protein.

Eukaryotic messenger RNA differs from its prokaryotic counterpart in that it contains a 7-methylguanosine moiety linked 5′ to 5′ to the first coded nucleoside (Rottman et al., 1974; Furuichi & Miura, 1975; Adams & Cory, 1975). This structure, referred to as a "cap", is not an obligatory requirement for translation but does accelerate the rate of initiation 5–10-fold [for reviews, see Skatkin (1976) and Banerjee (1980)]. This effect is mediated through interaction of the 5′-terminus of mRNA with a cap-binding protein, presumably the entry point for mRNA into the initiation process (Sonenberg & Shatkin, 1977; Trachsel et al., 1980; Hellmann et al., 1982). The dependence of translation on the cap is strongly affected by ionic strength: at 50 mM potassium acetate (KOAc),[1] cap analogues such as m^7GTP fail to inhibit translation, while at 200 mM KOAc, they exert a maximal inhibition (Weber et al., 1977; Kemper & Stolarsky, 1978; Chu & Rhoads, 1978). The dependence of translation on the cap is also affected by temperature (Weber et al., 1978) and by mRNA concentration (Chu & Rhoads, 1980). While information of this type is useful for interpreting cell-free translational data, its greater importance is in gaining insight into the mechanism by which cap-binding protein and other factors recognize the 5′-terminus of mRNA. We describe here another factor which affects cap dependence, the pH of the cell-free system.

Materials and Methods

Cell-Free Translation System. The messenger-dependent rabbit reticulocyte system of Pelham & Jackson (1976) was used. The sources and concentrations of all components have been described previously (Chu & Rhoads, 1980) with the exception that buffer was added as indicated in the figure legends. Several preparations of reticulocyte lysate were used in the course of this study. These were either prepared as previously described (Chu & Rhoads, 1978) or purchased from Clinical Convenience Inc. (Madison, WI), or Hazelton

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[1] Abbreviations: TAP, tobacco acid pyrophosphatase; m^7GpppA, 7-methylguanosine linked 5′ to 5′ by a triphosphate bridge to adenosine; KOAc, potassium acetate; HPLC, high-performance liquid chromatography; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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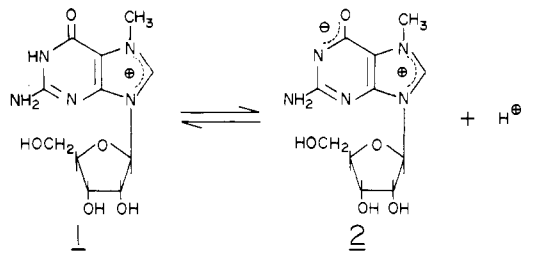

FIGURE 1: Ionization of m^7GTP: **1**, keto form; **2**, enolate form.

Dutchland Inc. (Denver, PA). Unless otherwise stated, the KOAc and $MgCl_2$ concentrations used in this study were 100 and 1.5 mM, respectively.

Treatment of mRNA with Tobacco Acid Pyrophosphatase (TAP). Tobacco cells (*Nicotiana tabacum L.*, cultivar KY 17) were grown in suspension culture in PG.5 medium and kindly donated by Dr. Glen Collins, University of Kentucky. TAP was purified from cells by the method of Efstratiadis et al. (1977) and assayed by the method of Shinshi et al. (1976). One unit is defined as 1 nmol of *p*-nitrophenol liberated per min. Globin mRNA (Chu & Rhoads, 1980) was incubated at a concentration of 120 μg/mL with TAP (final concentration 6.3×10^{-6} unit/μL) for 30 min at 37 °C in 50 mM NaOAc buffer, pH 6.0, containing 10 mM β-mercaptoethanol.

Removal of the mRNA cap by TAP was demonstrated two ways. First, control mRNA and TAP-treated mRNA were translated in the cell-free system at KOAc concentrations ranging from 0 to 200 mM. The activity of the TAP-treated mRNA compared to the control decreased from 100% to 15% over this range. These results were identical with those obtained previously (Chu et al., 1978) and demonstrate that at low ionic strength, where the cap is not "recognized", the TAP-treated mRNA appeared to be intact (i.e., the TAP preparation did not contain nuclease). At high ionic strength, where the cap is recognized, the treated mRNA was functionally uncapped. A second demonstration that the TAP preparation removed the cap but did not degrade the mRNA was carried out by using methods described by Breter et al. (1979). Globin mRNA was labeled at the 5′- and 3′-termini with $NaIO_4$ and NaB^3H_4 and treated with TAP. The presence of a m^7GpppN structure was assayed by digestion with nuclease P_1 and by HPLC. Untreated globin mRNA contained primarily m^7Gpppm^6Am with a small amount of $m^7GpppAm$, but the TAP-treated sample contained none. Gel electrophoresis (Rhoads & Hellmann, 1978) indicated that the mRNA was otherwise intact (data not shown).

Measurement of pH. The pH of translation reaction mixtures containing various buffers was determined by diluting a 15-μL aliquot to 100 μL with water and using a microelectrode (Vanlab).

Determination of the pK_a of m^7GTP. The absorption maximum for m^7GTP is 258 nm in the acidic range (pH 3–7) and 282 nm in the alkaline range (pH 8–11). Thus, the A_{258}:A_{282} ratio is the most sensitive measure of the relative amounts of compounds **1** and **2** (Figure 1). It is not necessary, however, to measure the A_{258}:A_{282} ratio over the complete range of pH values. Knowing the molar extinction coefficients of compounds **1** and **2** at 258 and 282 nm, one can determine the pK_a from a single measurement of the A_{258}:A_{282} ratio (R) at a given pH by using the equation

$$pK_a = \text{pH} - \log \frac{Rc_1 - c_2}{c_3 - c_4R}$$

where c_1 is the ϵ_M^{282} of compound **1** ($=7.34 \times 10^3$), c_2 is the ϵ_M^{258} of compound **1** ($=11.4 \times 10^3$), c_3 is the ϵ_M^{258} of compound **2** ($=6.05 \times 10^3$), and c_4 is the ϵ_M^{282} of compound **2** ($=8.78 \times 10^3$). These extinction coefficients were determined from absorption spectra of m^7GTP at pH 6 (10 mM Mes buffer) or pH 9 (10 mM Tris buffer).

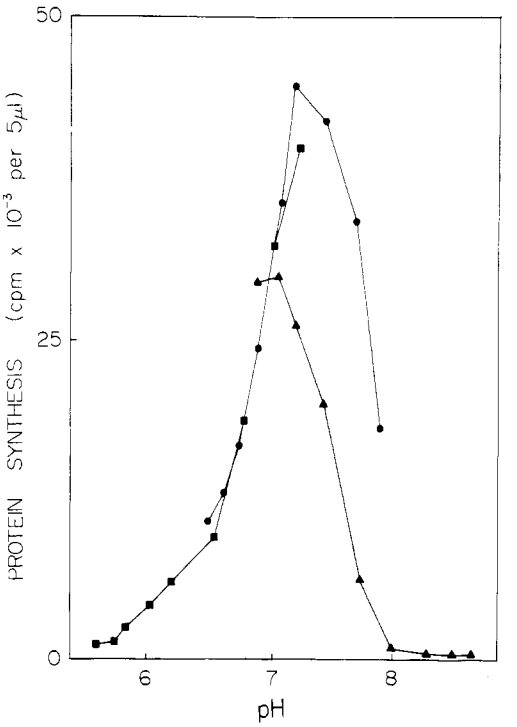

FIGURE 2: Cell-free translation of globin mRNA at various pH values. A reticulocyte messenger-dependent cell-free system was used to translate globin mRNA as described under Materials and Methods. Various buffers were used at 50 mM to adjust the pH: Mes (■); Hepes (●); Tris (▲). The pH of each reaction mixture was measured following incubation as described under Materials and Methods.

Results

Cell-Free Translation at Various pH Values. In order to investigate the dependence of cap recognition on pH, the range over which protein synthesis is active was determined. Three buffers, Mes, Hepes, and Tris, were used to alter pH. Due to the buffering capacity of hemoglobin in the reticulocyte lysate, it was necessary to use fairly high concentrations (50 mM) of buffer. As shown in Figure 2, the pH optimum of translation was 7.6. Incorporation of amino acids was approximately the same for Hepes and Mes at the same pH, indicating that the presence of the buffers themselves did not alter the activity of the system. Tris, on the other hand, appeared to inhibit translation. Hepes was chosen for subsequent experiments since its buffering range spanned the pH optimum for translation.

pH Dependence of Capped and Uncapped mRNA. Tobacco acid pyrophosphatase was used to specifically remove the m^7G moiety from globin mRNA, producing a single phosphate group at the 5′-terminus. The mRNA was translated over a range of pH values (Figure 3, open circles) and compared with untreated mRNA (closed circles). Translation of uncapped mRNA was relatively insensitive to pH, increasing approximately 3-fold from pH 6.5 to the optimum at pH 7.5. Capped mRNA translation was more dependent on pH, rising approximately 5-fold over the same pH range. In addition to the difference in overall dependence on pH, the ratio of uncapped to capped mRNA translation decreased as the pH was increased (triangles). These results suggest that some step during cap recognition is affected by pH changes over the range 6.5–7.5.

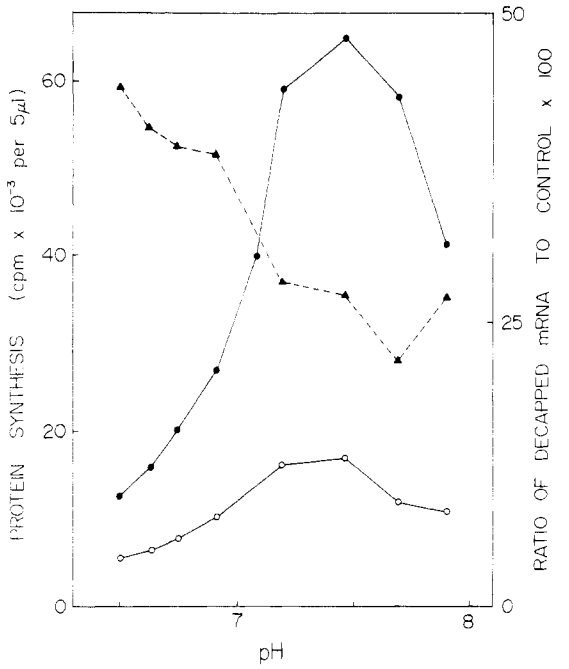

FIGURE 3: Translation of decapped mRNA as a function of pH. Tobacco acid pyrophosphatase was used to remove the cap of globin mRNA as described under Materials and Methods. Translation was as in Figure 3, with Hepes buffer used to adjust the pH: (●) translation of untreated globin mRNA; (○) translation of decapped mRNA; (▲) ratio × 100 of decapped mRNA translation to control translation.

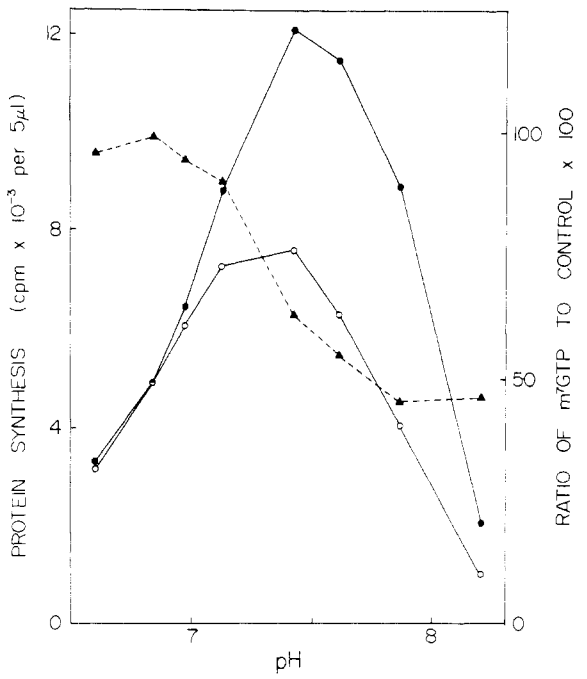

FIGURE 4: Translation of globin mRNA in the presence and absence of m^7GTP as a function of pH. Hepes buffer was used to adjust the pH of the translation system as in Figure 3: (○) translation in the presence of 80 μM m^7GTP; (●) translation in the absence of m^7GTP; (▲) ratio × 100 of translation in the presence of m^7GTP to that in its absence.

Effect of Cap Analogue. Another way to test the dependence of translation on the mRNA cap is to use analogues as inhibitors of initiation (Canaani et al., 1976; Hickey et al., 1976). A concentration of m^7GTP was chosen which caused partial inhibition, and its effect was tested as a function of pH (Figure 4). At low pH, there was little effect of cap analogue (open circles) compared with the control reactions containing

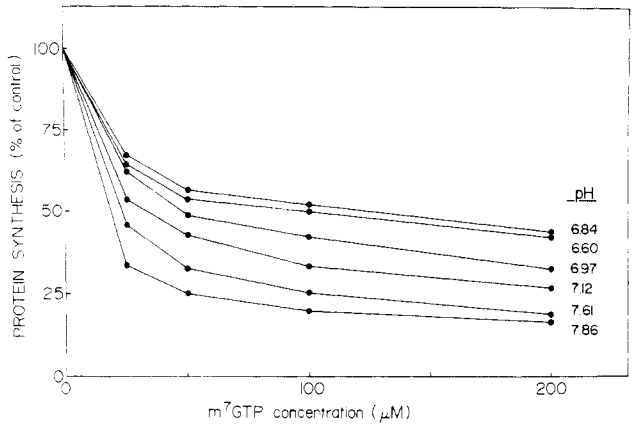

FIGURE 5: Inhibition of translation by m^7GTP at various pH values. The indicated concentrations of m^7GTP were used to inhibit the translation system as described in Figure 3. Hepes buffer was used to adjust the pH of the reaction mixture to the values shown in the figure.

no cap analogue (closed circles). As the pH increased from approximately 6.8 to 7.8, synthesis in the presence of analogue progressively decreased until a level of 50% inhibition was reached (triangles).

The experiment in Figure 4 was performed with a submaximal concentration of cap analogue, and the final degree of inhibition was only 50%. It was also of interest to determine the maximal extent to which translation could be inhibited at various pHs. In the experiment shown in Figure 5, the concentration of cap analogue was increased until a limiting degree of inhibition was reached. As can be seen, the level of inhibition increased with increasing pH, from 48% inhibition at pH 6.8 to 82% at pH 7.8. These results further suggest that some step in cap recognition becomes increasingly more important for translation as the pH is increased from approximately 6.8 to 7.8.

Ionization of m^7GTP. One interpretation of the foregoing results is that a protein which is involved in the cap recognition process contains an ionizable group with a pK_a in the 6.8–7.8 range and that this group must be in the alkaline form for the protein to be functionally active. Another possibility is that the m^7G moiety itself ionizes. 7-Methylguanosine is unique among the nucleoside residues in mRNA and among most other modified nucleosides in that it undergoes ionization in the physiological range (Figure 1); the N-1 proton dissociates with a pK_a of 6.7 (Shapiro, 1968). Adding one, two, or three phosphate residues to the 5′-position causes the pK_a to increase progressively (Hendler et al., 1970). The triphosphate derivative has a pK_a of 7.8. If the pK_a of the m^7G moiety in mRNA is similar to that of m^7GTP, then a mixture of the keto (**1**) and enolate (**2**) forms must exist at physiological pH. Considering the strict structural requirements of the guanine ring for active cap analogues (Adams et al., 1978), it is unlikely that both forms are recognized equally by the cap-binding protein.

The presence of mono- and divalent cations in the translation mixture might be expected to influence the pK_a of the N-1 position of m^7GTP, since it is a highly charged compound. Accordingly, the pK_a of m^7GTP was determined over a range of KCl and $MgCl_2$ concentrations. In Figure 6A, the A_{258}:A_{282} ratio of m^7GTP was measured over a complete pH range, both in the absence (closed circles) and in the presence (open circles) of 100 mM KCl. It is apparent that the pK_a was shifted to lower pH by approximately 0.3 unit. In Figure 6B, it can be seen that $MgCl_2$ at 1.5 mM (open circles) also lowered the pK_a compared to no salt (closed circles). The combined effects

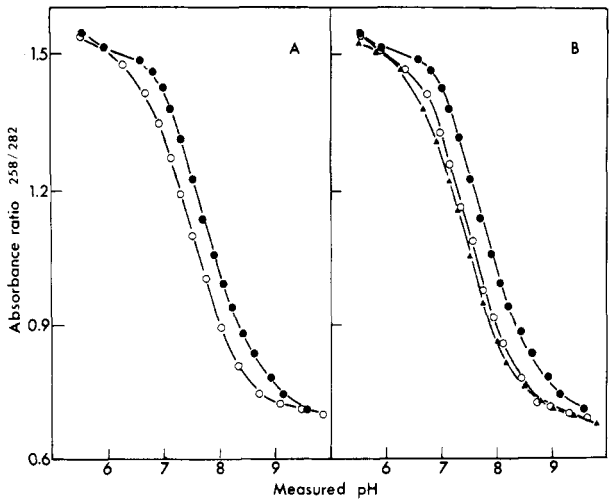

FIGURE 6: Titration of m^7GTP in the presence of various salts. The absorbance m^7GTP (50 μM) at 258 and 282 nm was measured at various pH values. The following buffers were used at a concentration of 10 mM: Mes, pH 5.6–6.8; Hepes, pH 7.1–7.9; Tris, pH 7.9–8.7; glycine, pH 8.9–10.0. The pH of each sample was measured with a pH meter. (A) (●) No added salt, pK_a = 7.76; (○) plus 100 mM KCl, pK_a = 7.52. (B) (●) No added salt, pK_a = 7.76; (○) plus 1.5 mM $MgCl_2$, pK_a = 7.53; (▲) plus 100 mM KCl and 1.5 mM $MgCl_2$, pK_a = 7.39.

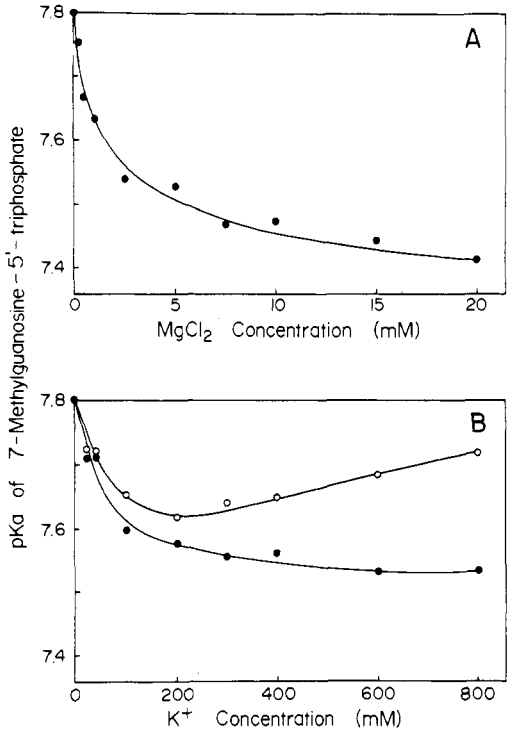

FIGURE 7: Variation of the pK_a of m^7GTP with salt concentration. The pK_a of m^7GTP was determined from single measurements of A_{258}, A_{282}, and pH as described under Materials and Methods. All measurements were made in 10 mM NaHepes buffer, pH 7.5. (A) Variation of pK_a with $MgCl_2$ concentration; (B) variation of pK_a with KCl (●) and KOAc (○).

of 1.5 mM $MgCl_2$ and 100 mM KCl caused a further decrease (triangles).

A more complete description of the dependence of this pK_a on ionic strength was determined by measuring the A_{258}:A_{282} ratio at a single pH value (near the pK_a) and calculating the pK_a from the known extinction coefficients of m^7GTP (see Materials and Methods). $MgCl_2$ decreased the pK_a from 7.8 to 7.4 (Figure 7A), and KCl decreased it from 7.8 to 7.5 (Figure 7B, closed circles). KOAc produced somewhat different results: the pK_a decreased to a minimum of 7.62 at 200

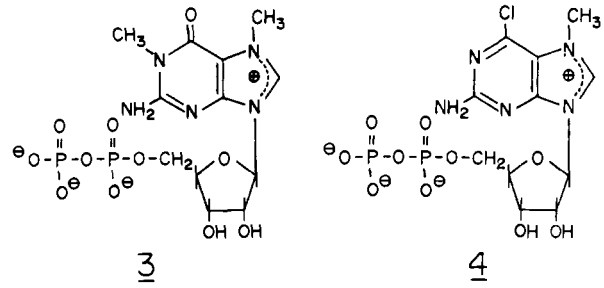

FIGURE 8: Cap analogues which are inactive as inhibitors of protein synthesis (Adams et al., 1978): **3**, 1,7-dimethylguanosine 5′-diphosphate; **4**, 6-chloro-7-methylguanosine 5′-diphosphate.

mM and thereafter increased to 7.7 (Figure 7B, open circles).

These results demonstrate that the pK_a of m^7GTP is a function of the ions present. The pK_a of m^7GTP in a complete translational mixture is likely to be near 7.4, since reactions are normally run at 100 mM KOAc and 1.5 mM $MgCl_2$. Other factors, such as the contribution of endogenous ions by the reticulocyte lysate, may shift this slightly. Also, the pK_a of m^7GTP within an mRNA cap may be influenced by the proximity of other groups. The essential finding, however, is that the pK_a of m^7GTP is within the range over which the pH dependence of cap recognition was observed (Figures 3–5).

Discussion

The results presented here indicate that the degree to which initiation is stimulated by the presence of a cap is a function of pH. This was demonstrated both with mRNA from which the cap had been removed (Figure 3) and with cap analogues (Figures 4 and 5). The explanation for this pH dependence, however, is not straightforward. There are over 100 polypeptides and 70 RNA species involved in the overall reaction of protein synthesis, and pH undoubtedly affects the structure and activity of many of them. Nonetheless, it is clear that the pK_a of the N-1 proton of m^7GTP is near 7.4 under the ionic conditions of the cell-free translational system (Figures 6 and 7). This is within the pH range (6.8–7.8) in which the changes in cap recognition occur (Figures 3–5). It seems unlikely that the two forms of m^7G in mRNA caps (Figure 1, compounds **1** and **2**) are equally recognized by the cap-binding protein, considering their differences in electronic structure and charge distribution. Thus, one interpretation which is consistent with the observed results is that the enolate form (**2**) of the cap preferentially complexes with cap-binding protein.

Support for this idea comes from the study of Adams et al. (1978), in which analogues of m^7GDP were tested as inhibitors of cell-free translation. One compound which failed to act as a cap analogue inhibitor was 1,7-dimethylguanosine 5′-diphosphate (Figure 8, compound **3**), a compound which is unable to lose a proton from the N-1 position. Also inactive was 6-chloro-7-methylguanosine 5′-diphosphate (**4**), which is unable to bear a negative charge at the 6-position due to substitution of chlorine for oxygen. Thus, an important structural feature for an active cap may be the ability to exist in a zwitterionic form. Another consideration with regard to cap recognition is whether the m^7G moiety is stacked with the first (coded) base in mRNA. Kim & Sarma (1977) presented NMR evidence that at pH 7, the analogue $m^7GpppAm$ existed in a stacked array. Nishimura et al. (1980) subsequently used fluorescence spectroscopy to show that the stacked conformation is less favored when the m^7G is in the enolate form. Thus, if the cap-binding protein does in fact prefer the enolate form, it may be due either to the charge distribution of the cap, as discussed above, or to the reduction of base stacking. Proof of this hypothesis will require a demonstration of which

form of the cap is present in a complex with cap-binding protein. Studies of this type are in progress in our laboratory.

The pK_a of m^7GTP was shown to be affected by ionic strength (Figures 6 and 7). Since this pK_a is near the pH optimum for protein synthesis (Figure 2), changes in the ionic strength of the translation system should alter the ratio of keto to enolate forms of the cap. This pK_a change may account for at least some of the observed effect of ionic strength on cap recognition (Weber et al., 1977; Kemper & Stolarsky, 1977; Chu & Rhoads, 1978). However, considering the profound effects of ionic strength on nucleic acid structure and protein–nucleic acid interactions, it is unlikely that this is the primary explanation. The increase in cap recognition at higher pH may also play a role in the activation of protein synthesis which occurs upon fertilization of oocytes. This activation has been shown to be correlated with an approximately 0.5 unit increase in pH (Winkler, 1982). Activation of lymphocytes by mitogens is also accompanied by an increase in intracellular pH from 7.2 to 7.4 (Gerson et al., 1982). Thus, pH-dependent recognition of caps may be part of a regulatory system controlling the overall rate of initiation of polypeptide synthesis.

Acknowledgments

We thank Dr. Mary Barkley for helpful advice and criticism.

Registry No. m^7G, 20244-86-4; m^7GTP, 26554-26-7; K, 7440-09-7; Mg, 7439-95-4.

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