Inhibition of Cell-Free Messenger Ribonucleic Acid Translation by 7-Methylguanosine 5′-Triphosphate: Effect of Messenger Ribonucleic Acid Concentration†

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ABSTRACT: The translation of rabbit globin and hen ovalbumin messenger ribonucleic acid (mRNA) was studied in protein-synthesizing systems prepared from whole (untreated) and depleted (micrococcal nuclease treated) rabbit reticulocyte lysate. Translation of both messengers was 32-fold more sensitive to inhibition by 7-methylguanosine 5′-triphosphate ($pppm^7G$) in the depleted lysate than in the whole lysate system, as previously observed in several laboratories. To understand this phenomenon, we added purified globin mRNA to the depleted lysate system to restore its original mRNA content. In this "restored" lysate system the translation of both messengers again became insensitive to inhibition. Inhibition of both globin and ovalbumin mRNA translation by $pppm^7G$ in the depleted lysate system was reversed by increasing concentrations of globin mRNA, with higher concentrations of inhibitor requiring higher concentrations of mRNA for equivalent reversal. These results suggested that $pppm^7G$ and the caps of globin and ovalbumin mRNA compete reversibly for a common binding site. As direct evidence for competition, globin mRNA was shown to be an inhibitor of ovalbumin mRNA translation, being approximately 1000-fold more potent than $pppm^7G$ on a molar basis. A kinetic model was developed in which cap analogues are treated as competitive inhibitors of mRNA binding to the 40S initiation complex. Theoretical curves generated with this model exhibit the same property of resistance to inhibition at high mRNA concentrations. In addition, it can be shown using this model that two mRNAs which differ in either their rate constant of initiation or their rate constant of elongation will be inhibited to different degrees by cap analogues. From both the experimental data and the theoretical model, it is concluded that the total mRNA concentration is a strong determinant in the sensitivity of either endogenous or exogenous mRNA translation to inhibition by cap analogues and is responsible for the difference between whole and depleted lysate systems.

Analogues of the 7-methylguanosine-containing 5′ terminus (cap) of eucaryotic mRNA have been used to study the mechanism of initiation of protein synthesis. The interpretation of results obtained with these cap analogues, however, has been complicated by the finding that their degree of inhibition of cell-free translation is affected by a number of factors. These factors include the source of the cell-free system (Lodish & Rose, 1977; Canaani et al., 1976; Sharma et al., 1976), the type of mRNA translated (Hickey et al., 1976; Suzuki, 1976; Sharma et al., 1976; Asselbergs et al., 1978; Chu et al., 1978; Willems et al., 1979), and the ionic strength of the cell-free system (Kemper & Stolarsky, 1977; Weber et al., 1977; Chu & Rhoads, 1978). Another observation is that the rabbit reticulocyte cell-free system is relatively insensitive to cap analogues (Shafritz et al., 1976; Suzuki, 1976; Lodish & Rose, 1977) whereas a fractionated reticulocyte system (Shafritz et al., 1976) or one treated with micrococcal nuclease (Wu et al., 1977; Asselbergs et al., 1978) is sensitive to inhibition by cap analogues. Wu et al. (1977) have proposed that exogenous mRNA translated in the depleted (nuclease-treated) lysate lacks some tightly bound protein which is present on endogenous mRNA and that cap analogues prevent exogenous mRNA from binding this protein. Asselbergs et al. (1978) have also advanced this explanation for the difference between depleted and whole lysate and in addition have presented evidence that exogenous mRNA acquires this protein during the course of the incubation.

In this report we present experimental evidence supporting a different explanation, namely, that the total concentration of mRNA in a cell-free system determines the degree of sensitivity to cap analogues. According to this explanation, the only difference between the whole and depleted lysate systems is the endogenous mRNA concentration. It is this variable that is solely responsible for the difference in sensitivity of translation to cap analogues. We also present a kinetic model of translation, an extension of the model of MacDonald et al. (1968) and Lodish (1974), which mimics these changes in sensitivity as a function of mRNA concentration. In this model, the loss of sensitivity to inhibition in the presence of high concentrations of mRNA is due to the depletion of 40S ribosomes available for initiation.

Materials and Methods

Materials. $pppm^7G$[1] was purchased from P-L Biochemicals, and [^{3}H]leucine (51.6 Ci/mmol) was from New England Nuclear.

Reticulocyte Cell-Free System. Reticulocyte lysate was prepared from anemic rabbits as described previously (Chu & Rhoads, 1978). Whole lysate refers to lysate with 25 μM hemin and 50 μg/mL creatine kinase added. Depleted lysate was made from the whole lysate by treatment with micrococcal nuclease as described by Pelham & Jackson (1976). Reaction mixtures of 25 μL contained 10 μL of either whole or depleted lysate and the following components: 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 40 μM each of the 20 amino acids, 1.5 mM $MgCl_2$, 150 mM K(OAc), 3 μCi of [^{3}H]leucine. Incubations were at 30 °C. Radioactivity in total protein was

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[1] Abbreviations used: pm^7G, 7-methylguanosine 5′-phosphate; $pppm^7G$, 7-methylguanosine 5′-triphosphate; $m^7G^5ppp^5N(m)$, 7-methylguanosine linked by 5′,5′-triphosphate to a nucleoside which may or may not be 2′-*O*-methylated; VSV, vesicular stomatitis virus; TMV, tobacco mosaic virus; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; STNV, satellite tobacco necrosis virus; TVMV, tobacco vein-mottling virus.

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assayed by trichloroacetic acid precipitation as described previously (Chu & Rhoads, 1978). Ovalbumin synthesis was assayed by immunoprecipitation as described previously (Rhoads et al., 1973).

mRNA Preparations. Rabbit globin mRNA was prepared from reticulocyte lysate as follows. Polyribosomes were extracted with 0.5 M KCl (Schreier & Staehelin, 1973), and the ribosomal fraction was extracted twice with 1:1 phenol–chloroform (Rhoads, 1975). The resulting RNA was precipitated with ethanol and purified by chromatography on oligo(dT)–cellulose (Desrosiers et al., 1974) and sucrose gradient centrifugation (Rhoads et al., 1971). The 9S region of the gradient was pooled and used as the globin mRNA preparation. Ovalbumin mRNA was prepared from hen oviduct as described previously (Chu et al., 1978).

Kinetic Model. Q_i is defined as the number of new polypeptide chains initiated per minute (picomole per milliliter per minute), k_1 as the rate constant of initiation (milliliter per picomole per minute), m_f as the concentration of mRNA which contains a free 5′ terminus, and R_f as the concentration of 40S initiation complexes. Then

$$Q_i = k_1 m_f R_f \quad (1)$$

An alternative formulation is that mRNA binds to a soluble cap-binding protein which in turn binds to the 40S initiation complex. Our reasons for choosing eq 1 rather than the alternative formulation are discussed below (see Discussion).

An expression for m_f is given by Lodish (1974) as

$$m_f = m_0(1 - nL) \quad (2)$$

where m_0 is the total concentration of mRNA, n is the probability that a codon is covered by a ribosome [see MacDonald et al. (1968) for the precise definition], and L is the number of codons covered by one ribosome.

If the total concentration of competent 40S ribosomes is R_0, then

$$R_0 = R_f + R_b + R_i \quad (3)$$

where R_b and R_i are the concentrations of ribosomes bound to mRNA in polysomes and to a competitive inhibitor of initiation, respectively.[2] We now express R_b as

$$R_b = m_0 n N \quad (4)$$

where N is the total number of codons in the mRNA. If I is the concentration of a competitive inhibitor of mRNA binding to ribosomes which participates in the reaction

$$R_f + I \rightleftharpoons R_i \quad (5)$$

and K_I is the dissociation constant of the R–I complex, then

$$R_i = I R_f / K_I \quad (6)$$

(Here we make the normal assumption that $I = I_{total} = I_{free}$; i.e., the concentration of inhibitor is vastly greater than the concentration of ribosomes.)

Combining eq 1–4 and 6, we obtain

$$Q_i = k_1 m_0 (1 - nL)\left(\frac{R_0 - m_0 n N}{1 + I/K_I}\right) \quad (7)$$

If Q_e is defined as the flux of ribosomes across a particular codon and one assumes (1) that ribosomes are uniformly distributed along the mRNA and (2) that the release of chains does not limit the overall rate of protein synthesis, then Q_e is given by the expression (Lodish, 1974)

$$Q_e = \frac{m_0 k_e n (1 - nL)}{1 - n(L - 1)} \quad (8)$$

in which k_e is the rate constant of elongation (codons per minute).

If one assumes that the polysome size does not change over the course of the experiment (typically 60 min), then the rate of attachment of ribosomes to mRNA equals the rate of release and $Q_i = Q_e$. Thus, n can be evaluated by setting eq 7 equal to eq 8 and using the quadratic formula:

$$n = [m_0 N + R_0(L - 1) + (k_e/k_1)(1 + I/K_I) - [[m_0 N + R_0(L - 1) + (k_e/k_1)(1 + I/K_I)]^2 - 4(L - 1)m_0 N R_0]^{1/2}]/[2(L - 1)m_0 N] \quad (9)$$

This expression for n is substituted into eq 7.

Equation 7 was used to compute the theoretical rate of protein synthesis, Q ($= Q_i = Q_e$), as a function of different variables. Numerical values were assigned to each of the constants (see Results), and Q was computed and automatically plotted with the aid of a Wang 2200 programmable calculator.

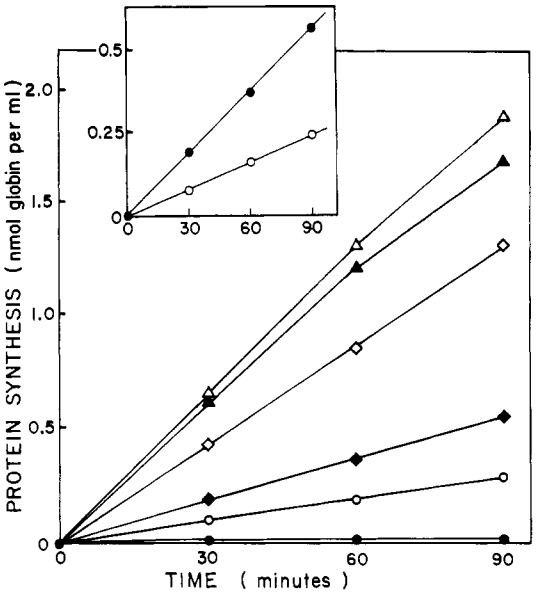

FIGURE 1: Kinetics of protein synthesis in the depleted lysate system at different globin mRNA concentrations. The globin mRNA concentrations were (●) none, (○) 1, (♦) 2, (◇) 5, (▲) 10, and (△) 15 μg/mL. One picomole of globin corresponded to 37 000 cpm. Inset: effect of pppm^{7}G on the kinetics of protein synthesis in the depleted lysate system. Globin mRNA concentrations were 2 μg/mL. (●) No pppm^{7}G; (○) 0.025 mM pppm^{7}G.

Results

A. *In Vitro Assays*

Properties of the Cell-Free System. With the conditions we used previously for the depleted lysate system (Chu & Rhoads, 1978), protein synthesis ceased in less than 60 min when high concentrations of mRNA were used. Others have also reported nonlinear kinetics with this cell-free system (Weber et al., 1978; Suzuki, 1978). To improve the kinetics of translation, the concentration of added leucine was increased to 40 μM. Under these new conditions, protein synthesis remained linear for almost 90 min at both low and high mRNA concentrations (Figure 1). Synthesis was also linear to 90 min in the presence of pppm^{7}G (Figure 1, inset), unlike

[2] Some of the 40S ribosomes are undoubtedly present in inactive 80S monosomes. We estimate these to constitute at most 30% of the 40S ribosomes. If we assume that these remain inactive at all mRNA concentrations, they will only affect the value assigned to R_0 (see Results). The actual value of R_0 has a profound affect on the absolute rate of protein synthesis (Q) but has a very small effect on the shape of mRNA saturation curves (Figure 7) or inhibition curves (Figures 6 and 8). Therefore, a term for inactive 40S ribosomes is omitted in the subsequent derivation for simplicity.

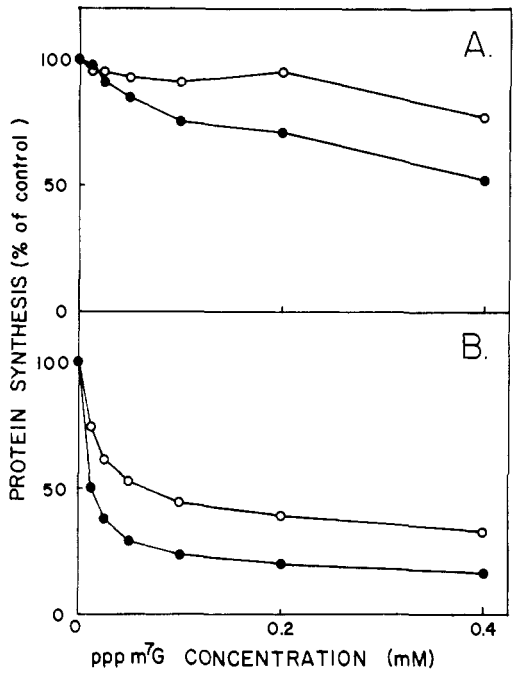

FIGURE 2: Effect of pppm^{7}G on the translation of mRNAs in the whole and depleted lysate systems. (A) Translation in the whole lysate system. (●) Total protein synthesis (primarily globin) with no exogenous mRNA added; 100% corresponded to 222 000 cpm/5 μL; (○) ovalbumin synthesis with 3.0 μg/mL ovalbumin mRNA added (detected by antibody precipitation); 100% corresponded to 15 000 cpm/20 μL. (B) Translation in the depleted lysate system. (●) 1.0 μg/mL globin mRNA; 100% corresponded to 46 299 cpm/5 μL; (○) 3.0 μg/mL ovalbumin mRNA (detected by antibody precipitation); 100% corresponded to 46 500 cpm/20 μL.

the findings of Asselbergs et al. (1978). In subsequent experiments it was therefore possible to measure initial rates by using a single time point of 60 min. We have previously shown that the only polypeptide made in response to globin mRNA migrates on electrophoresis with the mobility of full-length globin, both in the presence and absence of pppm^{7}G (Chu et al., 1978).

Comparison of Cap Analogue Inhibition in Whole and Depleted Reticulocyte Lysate Systems. The translation of globin and ovalbumin mRNA was studied in cell-free systems containing either whole reticulocyte lysate or lysate depleted of endogenous mRNA by nuclease treatment. Protein synthesis was inhibited by the cap analogue pppm^{7}G in both systems, but the inhibition was greater in the depleted lysate system (Figure 2). For globin mRNA, the concentration of pppm^{7}G which produced 50% inhibition of translation was 0.4 mM in the whole lysate system (Figure 2A, filled circles) but only 0.0125 mM in the depleted lysate system (Figure 2B, filled circles). For ovalbumin mRNA, the concentration of pppm^{7}G which produced 22% inhibition was 0.4 mM in the whole lysate system (Figure 2A, open circles) but only 0.0125 mM in the depleted lysate system. For both messengers, this represented a 32-fold reduction in inhibitor concentration required to achieve the same degree of inhibition. The difference in sensitivity of ovalbumin and globin mRNA translation to pppm^{7}G has been observed previously (Chu et al., 1978). Although the explanation is not known, several possibilities are discussed below (see Discussion).

The 32-fold increase in sensitivity to inhibition by pppm^{7}G cannot be explained by the additional presence of micrococcal nuclease, Ca^{2+}, and EGTA in the nuclease-treated lysate. To show this, we prepared control lysates in which micrococcal nuclease and Ca^{2+} were added as usual, but in addition, EGTA was included to prevent the action of the nuclease. Lysates prepared in this way, either with or without the incubation at 20 °C which is normally performed when making depleted lysate, exhibited the same resistance to inhibition by pppm^{7}G as whole lysate (data not shown).

Restoration of mRNA Concentration in Nuclease-Treated Lysate with Exogenous Globin mRNA. To test the possibility that the concentration of endogenous mRNA accounted for these differences between whole and depleted lysate systems, purified globin mRNA was added back to the depleted lysate. It was first necessary to determine the concentration of active globin mRNA in the whole lysate. Whole lysate was used as a source of mRNA for translation in the depleted lysate system and compared to purified globin mRNA in programming protein synthesis. One microliter of whole lysate in 50 μL of reaction mixture gave the same activity as 0.6 μg/mL purified globin mRNA. From this we calculated that the concentration of active globin mRNA was 30 μg/mL in whole lysate or 12 μg/mL in the reaction mixture since 10 μL of whole lysate is used per 25 μL of reaction mixture.

Using the value of 12 μg/mL as a guide, the effect of adding increasing amounts of exogenous purified globin mRNA to the depleted lysate system was tested. As shown in Figure 1, the rate of protein synthesis increased with added mRNA until the concentration reached 10–15 μg/mL, after which the rate of synthesis remained constant. The linear kinetics of incorporation shown in Figure 1 demonstrates that this upper limit of protein synthesis is not due to exhaustion of some component such as amino acids, ATP, etc. with time. It is more likely that, at these concentrations of mRNA, a large fraction of the ribosomes are present in polysomes, and consequently the rate of initiation cannot increase due to a scarcity of ribosomes. Quantitatively, the rate of protein synthesis in the depleted lysate system containing 15 μg/mL or more exogenous globin mRNA was the same as that in the untreated whole lysate system. Thus, the protein synthetic activity of the depleted lysate system can be restored to that of the whole lysate system by the addition of equivalent "activity units" of exogenous mRNA, and no other factors appear to be required.

Cap Analogue Inhibition vs. mRNA Concentration. We next tested the sensitivity of translation in the "restored" whole lysate system to inhibition by pppm^{7}G. Inhibition over a range of pppm^{7}G concentrations was tested with increasing amounts of globin mRNA (Figure 3A). Inhibition was greatest at low globin mRNA concentrations and became less severe at higher concentrations. The concentration of pppm^{7}G which produced 50% inhibition of translation was 0.0125 mM for globin mRNA at 1 μg/mL but increased to 0.3 mM for globin mRNA at 20 μg/mL, a difference of 24-fold. These results mimic the difference between depleted and whole lysate systems (Figure 2).

Our interpretation of this phenomenon is that globin mRNA and pppm^{7}G compete for the same site on a protein which binds to caps during the initiation process. A protein of molecular weight 24 000 having this property has been identified by Shatkin and co-workers (Sonenberg et al., 1978). If globin mRNA and pppm^{7}G compete for a common site on such a protein, then inhibition by the analogue should be overcome by increasing the globin mRNA concentration. Furthermore, at higher analogue concentrations, more globin mRNA should be required for reversal. This is shown to be the case in Figure 4, in which the data of Figure 3A were replotted by using actual incorporation data rather than percentages of controls. At low concentrations of analogue (0.025 mM), inhibition was reversed almost completely by

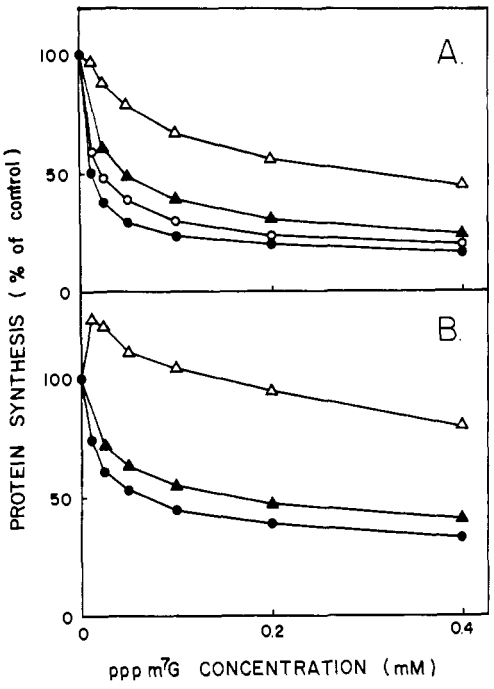

FIGURE 3: Effects of pppm^{7}G on the translation of globin mRNA and ovalbumin mRNA in the depleted lysate system at different globin mRNA concentrations. (A) Translation of globin mRNA. The globin mRNA concentrations were (●) 1, (○) 5, (▲) 10, and (△) 20 μg/mL. The 100% points for each curve were respectively 46 299, 204 428, 316 372, and 268 040 cpm/5 μL. (B) Translation of 3.0 μg/mL ovalbumin mRNA (detected by antibody precipitation) in the presence of the following concentrations of globin mRNA: (●) none; (▲) 10 μg/mL; (△) 20 μg/mL. The 100% points for each curve were respectively 46 500, 37 500, and 9800 cpm/20 μL.

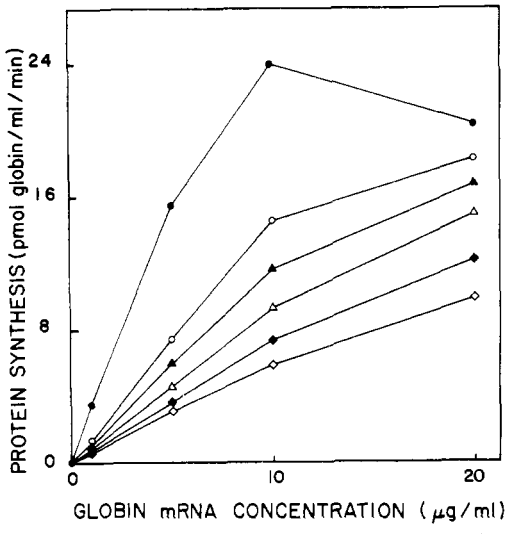

FIGURE 4: Translation of globin mRNA in the presence of various concentrations of pppm^{7}G in the depleted lysate system. One picomole of globin corresponded to 44 000 cpm. The inhibitor concentrations used were (●) none, (○) 0.025, (▲) 0.05, (△) 0.1, (♦) 0.2, and (◇) 0.4 mM.

increasing the globin mRNA concentration. At higher analogue concentrations, more globin mRNA was needed for reversal. However, it appears that at all analogue concentrations tested the same maximal rate of protein synthesis was approached.

Translation of Ovalbumin mRNA in Restored Lysate. The foregoing results are compatible with a competitive relationship between pppm^{7}G and globin mRNA. As the ratio of globin mRNA to pppm^{7}G is increased, the translation of globin mRNA increases. However, one can also demonstrate an

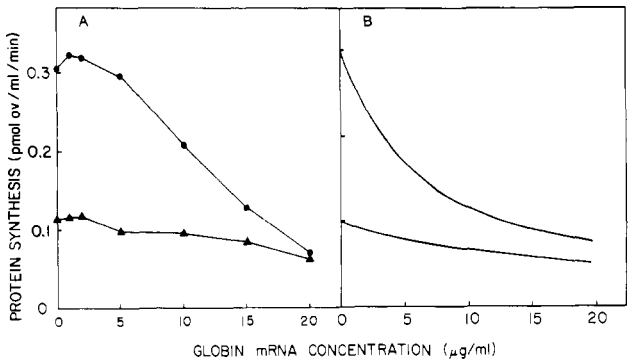

FIGURE 5: Effect of globin mRNA and pppm^{7}G on ovalbumin mRNA translation. (A) Cell-free synthesis data. Ovalbumin was detected by antibody precipitation. One picomole of ovalbumin corresponded to 80 700 cpm. The ovalbumin mRNA concentration was 2.9 μg/mL. (●) No pppm^{7}G added; (▲) 0.2 mM pppm^{7}G added. (B) Data computed by using eq 10. The values of constants were k_1 = 0.44 μM^{-1} min^{-1}, k_e = 26 codons/min, L = 12 codons, N = 385 codons, R_0 = 0.18 μM, K_I^{glo} = 0.025 μM, $I^{pppm^7G}/K_I^{pppm^7G}$ = 2, and m_0 = 0.004 μM. For globin mRNA, 1 μg/mL corresponded to 0.004 μM. (Upper curve) No pppm^{7}G added; (lower curve) plus pppm^{7}G.

effect of globin mRNA on the translation of a second exogenous mRNA, even when the ratio of second mRNA to pppm^{7}G remains constant. To show this, we used ovalbumin mRNA, whose translation product can be measured independently of globin synthesis by immunoprecipitation. As indicated previously (Figure 2), ovalbumin mRNA translation is relatively insensitive to inhibition by pppm^{7}G in the whole lysate system but is sensitive in the depleted lysate system. The results shown in Figure 3B demonstrate that translation became insensitive in the depleted lysate system as the concentration of globin mRNA was increased. With no added globin mRNA, the concentration of pppm^{7}G which produced 20% inhibition was 0.0125 mM, but at 20 μg/mL added globin mRNA it was 0.4 mM. This difference in sensitivity (32-fold) was identical with that observed when comparing whole and depleted lysate (Figure 2). The stimulation of ovalbumin synthesis at low concentrations of pppm^{7}G (Figure 3B) is reproducible but is not understood at the present time. It is similar to the stimulation by cap analogues of STNV (Hickey et al., 1976; Chu et al., 1978) and TVMV (Hellmann and Rhoads, unpublished experiments) RNA, both of which are uncapped.

Inhibition of Ovalbumin Synthesis by Globin mRNA. The finding that globin mRNA affects the inhibition of ovalbumin mRNA translation by pppm^{7}G suggests that globin mRNA, ovalbumin mRNA, and pppm^{7}G all compete reversibly for a common binding site. If so, globin mRNA should also be an inhibitor of ovalbumin mRNA translation. This was shown to be the case in an experiment summarized in Figure 5A (closed circles) where ovalbumin mRNA translation was measured in the depleted lysate restored with different concentrations of globin mRNA. At ~13 μg/mL globin mRNA, translation of ovalbumin mRNA was inhibited 50%. Thus, on a molar basis, globin mRNA was 1000 times as potent as pppm^{7}G in inhibiting ovalbumin mRNA translation.

The effect of having both competitive inhibitors, pppm^{7}G and globin mRNA, present simultaneously can also be seen in Figure 5A (closed triangles). This type of plot, in which absolute rates rather than the relative rates of Figures 2 and 3 are used, is the most clear representation of the phenomenon: the apparent potency of one competitive inhibitor (globin mRNA) is diminished if the rate of translation has already been depressed by the other competitive inhibitor (pppm^{7}G). The results shown in Figures 2 and 3B can be explained in

the same terms: the potency of one inhibitor (pppm^{7}G) is diminished if the rate of translation of ovalbumin mRNA has already been depressed by the other inhibitor (globin mRNA).

B. Interpretation of Experimental Data with Kinetic Model

Derivation of Kinetic Model. The effects of pppm^{7}G on translation, such as those shown in Figure 4, are reminiscent of competitive inhibition of enzyme activity. In such a comparison, mRNA would be analogous to substrate, cap analogue to competitive inhibitor, and ribosome to enzyme molecule. However, there are important differences between transformation of a substrate and translation of mRNA. First, the mRNA and the 40S ribosome are present at roughly equal molar concentrations whereas substrate is usually present in vast excess over enzyme. Second, mRNA is not consumed during translation whereas substrate is. Third, multiple ribosomes bind to a single mRNA and are unavailable for reinitiation whereas only one enzyme molecule and one substrate molecule are tied up in the E–S complex. These and other differences make it impossible to treat the foregoing results mathematically by using the classical Michaelis–Menten formulation.

MacDonald et al. (1968) have developed a general kinetic treatment of biopolymerization on nucleic acid templates. Lodish (1974) extended this treatment to formulate a specific kinetic model of globin mRNA translation. This model has led to a simplified interpretation of various experimental phenomena involving preferential translation of one mRNA over another (Lodish, 1976). We have further extended the model to include the situation where the concentration of mRNA becomes high compared with the concentration of 40S ribosomes (see Materials and Methods). This is the case in the whole lysate system where, as we have demonstrated in the foregoing section, the mRNA concentration is at a saturating level. We have also included the effect of inhibitors. Cap analogues are treated as competitive inhibitors of mRNA binding to 40S initiation complexes. This assumption follows from several studies which have indicated that pm^{7}G acts at an early stage in initiation of translation (Both et al., 1975; Hickey et al., 1976; Roman et al., 1976) and that 80S initiation complex formation is inhibited while 40S initiation complex formation is unaffected (Suzuki, 1978).

Assignment of Values to Constants. The rate constant of elongation, k_e, for our particular set of experimental conditions, was calculated from the data presented in Figure 1. The overall rate of globin production was 2.9 pmol/(min·mL) of reaction mixture at 1 μg/mL globin mRNA. By the assumption of 16 pmol of globin mRNA per A_{260} unit of purified mRNA, this rate corresponds to 0.71 mol of globin per mol of globin mRNA per min. If there is an average of 4 ribosomes/polysome (Hunt et al., 1968; L.-Y. Chu and R. E. Rhoads, unpublished experiments), then each ribosome traverses 26 codons/min (see footnote 3).

The number of codons covered by each ribosome, L, is taken as 12, the value used originally by Lodish (1974). There is very little change in the rate calculated with eq 7 using values of L from 8 to 24. The length of the mRNA, N, was assigned a value of 144 codons, the average length of the coding region of α and β rabbit globin mRNAs. Palmiter (1973) has estimated the concentration of polysomal ribosomes in lysate to be 0.44 μM, which gives a final ribosome concentration (R_0) of 0.18 μM since we use 10 μL of lysate per 25 μL of reaction mixture. The rate constant of initiation, k_1, was empirically determined by setting k_e = 26 codons/min, R_0 = 0.18 μM, m_0 = 0.004 μM (1 μg/mL), N = 144 codons, and L = 12 codons and then varying k_1 until Q = 2.84 pmol of globin per mL per min, the observed rate at 1 μg/mL mRNA (Figure 1). In this way, k_1 was found to be 6.6 μM^{-1} min^{-1}.

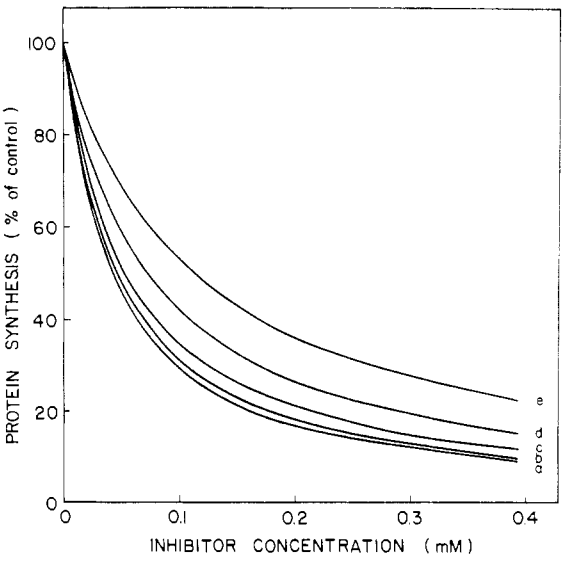

FIGURE 6: Inhibition of protein synthesis by a competitive inhibitor of mRNA binding to ribosomes at different mRNA concentrations as calculated from eq 7. The values used were k_1 = 6.6 μM^{-1} min^{-1}, k_e = 26 codons/min, L = 12 codons, N = 144 codons, R_0 = 0.18 μM, and K_I = 25 μM. The values for m_0 were (curve a) 0.00625, (curve b) 0.0125, (curve c) 0.025, (curve d) 0.05, and (curve e) 0.1 μM.

K_I, the dissociation constant of the R–I complex, was estimated as follows. The values given above for k_1, k_e, N, m_0, R_0, and L were used to compute Q vs. I curves using different values for K_I. The curve which most nearly fits the experimental data, e.g., Figure 2B, corresponded to K_I = 0.025 mM.

Effect of a Competitive Inhibitor on the Computed Rate of Translation as a Function of mRNA Concentration. The expression given in eq 7 was evaluated over a range of inhibitor and mRNA concentrations similar to those used in the experiments described above, and the results are plotted in Figure 6. The rate, expressed as a percentage of that obtained with no inhibitor, fell off with increasing inhibitor concentration at all mRNA concentrations, but the degree of inhibition decreased as the mRNA concentration increased. The theoretical curves are qualitatively similar to the data presented in Figure 3A, indicating that the kinetic model correctly predicts the phenomenon of mRNA concentration-dependent loss of sensitivity to cap analogues.

The theoretical rate of translation, expressed as picomoles of globin per milliliter per minute rather than as the percent of control, is plotted in Figure 7 for a range of mRNA and inhibitor concentrations. With no inhibitor (curve a), the rate of translation approached a maximal value as the mRNA concentration was increased. The concentration of mRNA at which the curve broke and began to plateau, ~20 μg/mL, was similar to that observed in the experimental data (Figures 1 and 4). The effect of adding increasing amounts of inhibitor

[3] This value is somewhat lower than previously published results of 43 codons/min (Lodish & Jacobsen, 1972) and 50 codons/min (Palmiter, 1973). Part of this difference is due to the fact that, since our elongation rate was computed directly from the observed rate of globin synthesis, it includes both the time of elongation and the time of release. The others have subtracted the release time from the transit time, thereby yielding high rates of elongation. Additional factors which could contribute to this being an underestimate of the true rate of elongation are our implicit assumptions that all of the A_{260} in our globin mRNA preparation is active messenger and that the leucine contribution of lysate and [^{3}H]leucine is negligible compared to the 40 μM added leucine.

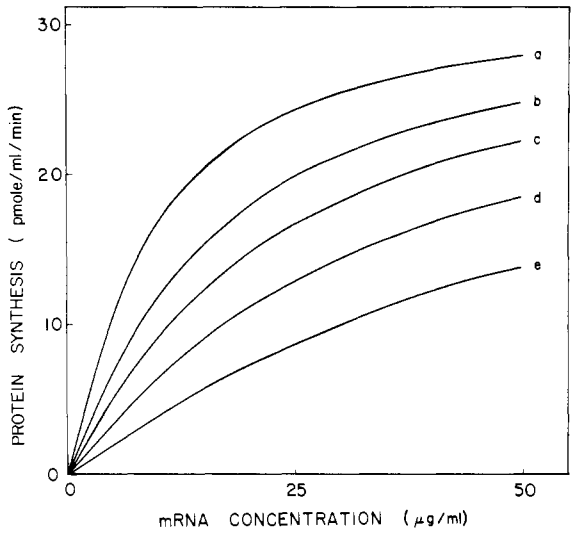

FIGURE 7: mRNA saturation curves at different concentrations of a competitive inhibitor of mRNA binding to ribosomes, as calculated from eq 7. The values of constants used are given in the legend to Figure 6. The concentrations of inhibitor, I, used were (curve a) none, (curve b) 0.025, (curve c) 0.05, (curve d) 0.1, and (curve e) 0.2 mM.

was to shift this curve to the right (curves b–e). Therefore, these curves are qualitatively similar to those presented in Figure 4 and show that the effect of inhibitor is reversed by increasing the concentration of mRNA.

Effect of Globin mRNA on the Inhibition of Ovalbumin mRNA by pppm^{7}G. As was shown in Figure 5A, when two different competitive inhibitors such as globin mRNA and pppm^{7}G are present simultaneously, the effects on ovalbumin mRNA translation are cumulative. This can be described in terms of the kinetic model by reformulating eq 7 as

$$Q_i = k_1 M_0(1 - nL)\left(\frac{R_0 - m_0 nN}{1 + I^i/K_I^i + I^j/K_I^j}\right) \quad (10)$$

where superscript i refers to globin mRNA and superscript j to pppm^{7}G. Ovalbumin mRNA translation is inhibited ~62% by pppm^{7}G at 0.2 mM. This effect can be simulated with the kinetic model by assigning a value of 2 to I^j/K_I^j. Under these conditions, the effect of globin mRNA on ovalbumin mRNA translation is greatly diminished (Figure 5B), in agreement with the experimental data (Figure 5A).

Differences in the Inhibition of Different mRNAs by Cap Analogues. The degree to which globin and ovalbumin mRNAs were inhibited by pppm^{7}G was different, both in the whole lysate and the depleted lysate systems (Figure 2). As noted above, other pairs of messengers behave similarly. Differences of this sort can also be simulated with the kinetic model of translation. One way to do this is to vary the rate constant of initiation, k_1 (Figure 8A). As can be seen, higher values for k_1 resulted in greater resistance to inhibition. A second way is to vary the rate constant of elongation, k_e (Figure 8B). In this case the behavior was just the opposite: higher values for k_e resulted in greater sensitivity to inhibitor. One could formally produce similar effects by varying K_I, but the interpretation of this would be unclear. It is assumed that inhibitor, globin mRNA, and ovalbumin mRNA all compete for a common binding site. Accordingly, the value of K_I for the inhibition of ovalbumin mRNA translation by pppm^{7}G would be the same as the value of K_I for the inhibition of globin mRNA by pppm^{7}G, since only the properties of the inhibitor and its receptor affect K_I. Only the use of a different inhibitor, e.g., pm^{7}G, having a different affinity for the putative cap-binding protein, would lead to a different value for K_I.

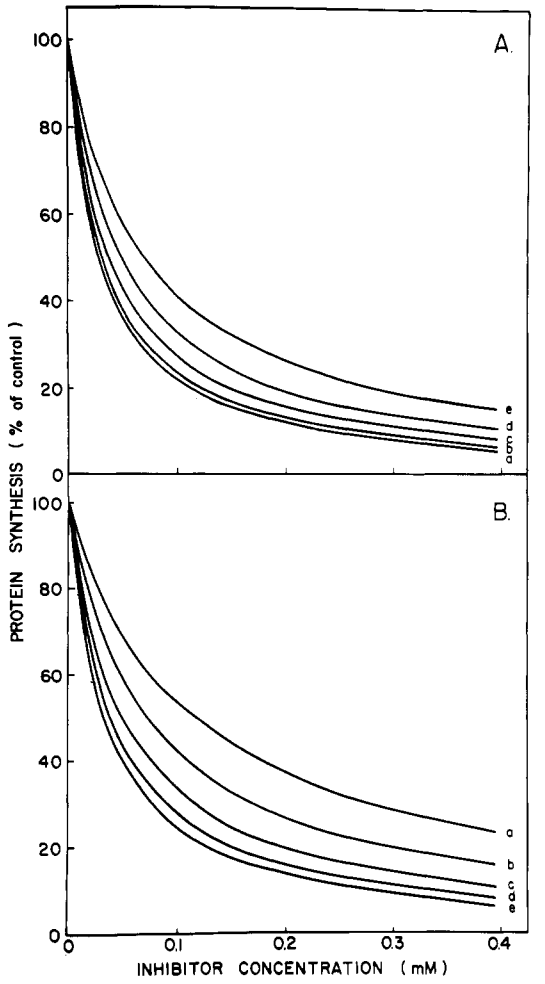

FIGURE 8: Inhibition of protein synthesis by a competitive inhibitor of mRNA binding to ribosomes, as calculated from eq 7. (A) Effect of k_1. The concentration of m_0 used was 0.012 μM. Other values are given in the legend to Figure 7. The values used for k_1 were, in μM^{-1} min^{-1}, (curve a) 1, (curve b) 2, (curve c) 4, (curve d) 8, and (curve e) 16. (B) Effect of k_e. The values used for k_e were, in codons/min, (curve a) 5, (curve b) 10, (curve c) 20, (curve d) 40, and (curve e) 80.

Discussion

In this report we have attempted to explain one of the variables which affects the sensitivity of in vitro translation of mRNA to cap analogues, the difference between whole and nuclease-treated reticulocyte lysate. Our proposal, which is based on both experimental evidence and a kinetic model, is that the total amount of mRNA in a cell-free system strongly affects the sensitivity to inhibition. Wu et al. (1977) and Asselbergs et al. (1978) have put forward a different explanation, that endogenous mRNAs are permanently associated with a cap-binding protein which cannot be displaced with cap analogues while exogenously supplied mRNA are not. Our results indicate, however, that endogenous (Figure 2) and exogenous (Figure 3) mRNAs behave exactly the same in this regard. Therefore, we find it unnecessary to postulate any mechanism for this phenomenon other than competition of mRNA and cap analogue for a common binding site.

Other variables affecting the sensitivity of translation to inhibition by cap analogues include the source of the cell-free extract, the ionic strength, and the type of mRNA. Lodish & Rose (1977) originally presented data showing that the rabbit reticulocyte system was considerably less sensitive to inhibition by cap analogues than the wheat germ system. The

system they used, however, was the whole reticulocyte system which, as we have shown, contains a saturating or supersaturating concentration of endogenous mRNA. Subsequently, we compared wheat germ and *depleted* lysate systems and found that, at the appropriate ionic strengths, the two systems behaved identically with respect to inhibition by pppm^{7}G (Chu & Rhoads, 1978). Most recently, Bergmann & Lodish (1979) compared wheat germ and depleted lysate systems for their ability to distinguish between capped and decapped mRNAs over a range of ionic strengths. The behavior of these two systems was similar but not identical. It is possible that different mRNA concentrations were used in the comparison, and this may have caused some difference. However, it is clear that the results obtained with wheat germ vs. depleted lysate in this study were considerably more alike than the earlier results obtained with wheat germ vs. whole lysate.

Concerning differences between different mRNAs, we have shown that varying k_1 (Figure 8A) and k_e (Figure 8B) will affect the sensitivity of mRNA translation to pppm^{7}G. Suzuki (1978) has also suggested that varying k_1 will affect the sensitivity of translation to cap analogues. In the case of ovalbumin vs. globin mRNA, a likely explanation for the difference comes from our recent findings that only ~25% of the ovalbumin mRNA molecules are capped (Malek et al., 1979) while 60% of the globin mRNA molecules are capped (Breter et al., 1979). This is presumably due to random scission of mRNA molecules followed by selective purification of 3′-terminal fragments on oligo(dT)–cellulose. Previously, we have shown that translation of ovalbumin mRNA which has been decapped with tobacco acid pyrophosphatase or by β-elimination is considerably less sensitive to inhibition by cap analogues than untreated ovalbumin mRNA (L.-Y. Chu and R. E. Rhoads, unpublished experiments). Since a greater number of the ovalbumin mRNA molecules are uncapped, a relatively larger fraction of the translation is programmed by uncapped mRNA molecules and is therefore insensitive to inhibitors.

The kinetic model of translation presented here extends the models of MacDonald et al. (1968) and Lodish (1974) to include the effect of the free ribosome concentration and the effect of a competitive inhibitor of mRNA binding. The major difference is that the rate of initiation is dependent not only on the availability of free 5′ termini of RNA but also on the availability of free 40S ribosomes. As the mRNA concentration increases, more ribosomes are tied up in polysomes and fewer are available for initiation. Ultimately, this leads to a plateau in the rate of protein synthesis, as seen in Figure 7, which is not obtained with the original equation of Lodish (1974). This modification is necessary to simulate the type of experimental data which we present here, since whole reticulocyte lysate contains a saturating concentration of mRNA. A second modification of the equation is to take into account the effect of a competitive inhibitor of mRNA binding to ribosomes. Other models in which pppm^{7}G was treated as a noncompetitive inhibitor of initiation or as a competitive inhibitor of GTP binding during elongation failed to simulate the experimental data.

An alternate model could be constructed in which the concentration of a free initiation factor becomes depleted at high concentrations of mRNA due to the high concentration of initiation complexes containing this factor, and the scarcity of this factor limits the rate of initiation. This is similar to the approach taken by Suzuki (1978). His model and ours are not mutually exclusive. Rather, they are constructed to explain different phenomena. Whereas ours is intended to describe the effect of mRNA concentration on the sensitivity of translation to cap analogues, measured over a 60-min period, his is intended to describe the effect of Met-tRNA$_f^{Met}$ concentration on the sensitivity of 80S initiation complex formation to cap analogues, measured over a 4-min period. A complete kinetic expression would have to include all the terms used in both of these formulations and many others as well (concentration of GTP, eIF-2, eIF-3, eIF-4B, etc.). Another reason for the difference in kinetic models is that one must be somewhat arbitrary at this time in choosing a pathway for initiation, since not all of the factors and their modes of action have been elucidated. The particular pathway we have used is based on (1) the scheme of initiation proposed by Benne & Hershey (1978) and Benne et al. (1979) in which mRNA binds to a 40S-Met-tRNA$_f^{Met}$–eIF-2–eIF-3 complex and (2) our recent observation that a cap-binding activity copurifies with eIF-3 but is not identical with eIF-3 since it can be dissociated from it at high ionic strength (L.-Y. Chu and R. E. Rhoads, unpublished experiments). Further experimentation will be necessary to decide whether mRNA interacts first with a free cap-binding protein or with a protein which is bound to the 40S initiation complex.

Curves computed with the kinetic model are only in qualitative agreement with the experimental data. They fail to agree quantitatively in two ways. First, experimental Q vs. I curves fall more steeply at low inhibitor concentrations than theoretical curves (cf. Figures 3A and 6). Stated another way, experimental curves show more residual translation at high inhibitor concentrations than would be predicted from the initial fall in activity. This could be due to translation which is not dependent on caps, either due to the presence of uncapped mRNA molecules in our preparations (Malek et al., 1979; Breter et al., 1979) or due to the recognition of internal signals on capped mRNAs by the initiation machinery. A second way that theoretical and experimental data disagree is in Q vs. m curves (cf. Figures 4 and 7). With experimental data, the rate of translation saturates more sharply with mRNA than the theoretical. This may be due to the fact that we have used a mass balance equation only for 40S ribosomes (eq 3). It would probably be more correct to assume that at high concentrations not only ribosomes but also Met-tRNA$_f^{Met}$, eIF-2, eIF-3, etc. become depleted and begin to limit the rate of initiation. Taking these factors into consideration would lead to an expression with a higher order dependence on mRNA concentration, from which one would compute a sharper saturation curve.

Acknowledgments

We are indebted to Drs. Mary Barkley, George Schwert, and Robert Jacob for helpful comments during the preparation of this manuscript and to Ginger Hodges and Donna Wilkie for expert secretarial assistance.

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Magnetic Osmometry: Association of Two Peptic Fragments from Bovine Serum Albumin at Micromolar Concentrations†

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ABSTRACT: The magnetic osmometer was employed to study at micromolar concentrations the known association of the two nearly equal sized fragments of bovine serum albumin (BSA) formed by peptic cleavage between amino acid residues 306 and 307. An association constant of $\sim 1.8\ \mu M^{-1}$ was determined at pH 8.6 (0.1 M KCl, 20 °C); similar association constants were obtained by equilibrium sedimentation and from the catalytic decomposition of a Meisenheimer complex (MC) by mixtures of the fragments where the substrate, MC, served as a reporter group (intact BSA exhibits a similar activity). A volume change for the association (~−80 mL/mol) was determined by density experiments, suggesting that nonpolar rather than ionic interactions predominate in the formation of the association complex. This was supported by titration experiments which showed that the net charge on the parent BSA was conserved in the isolated peptic fragments and in the association complex. Intrinsic viscosity values, however, indicated that the complex had a substantially larger hydrodynamic volume than the parent BSA (viz., 5.6 vs. 4.2 mL/g); the C-terminal peptic fragment from BSA exhibited a similarly large value, whereas that for the N-terminal fragment was more BSA-like. Dissociation of the complex was observed in high centrifugal fields and also by osmotic pressure and electrophoresis when the fragments had been exposed to decane; the N-terminal peptic fragment when so exposed exhibited a changed behavior in electrophoretic and centrifugal fields and it was catalytically incompetent, whereas these tests with the other fragment and with BSA, after similar exposure, revealed little or no change. These results and those reported by others illustrate that this BSA system provides a useful model for comparing struction–function relationships in the quasi-independent domains of two compositionally alike macromolecular entities (BSA and the complex).

The osmotic pressure is a direct measure of the change in chemical potential when adding macromolecules to a system. The osmotic method, however, has not been generally useful at macromolecule concentrations of much less than 10^{-4} M, thus excluding many of the association reactions of interest to protein chemists. The magnetic osmometer introduced recently by Beams et al. (1973) can be used in the micromolar range of concentrations with useful precision. A modified version of this osmometer was employed here to elucidate the putative association of the two nearly equal sized fragments of bovine serum albumin (BSA)[1] as obtained by limited pepsin

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‡ Predoctoral Fellow under Training Grant 5-T32-GM-07294 from the National Institutes of Health.

[1] Abbreviations used: BSA, bovine serum albumin; MC, 1,1-dihydro-2,4,6-trinitrocyclohexadienate (a Meisenheimer complex); BTP, 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane (also called Bistris-propane).

0006-2960/80/0419-0191$01.00/0