EFFECT OF 7-METHYLGUANOSINE-5'-PHOSPHATE ON RABBIT GLOBIN SYNTHESIS

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1. Introduction

It has been shown that the 7-methylguanosine (m^7G) at the 5'-end of mRNAs is required for translation [1–3]. However, vesicular stomatitis virus mRNA lacking 5'-terminal m^7G is translated in the reticulocyte cell free system, but not well in the wheat embryo system [4]. On the other hand, $m^7G^{5'}p$ inhibits the translation of rabbit globin mRNA in the wheat germ and Artemia salina systems [5,6]. Therefore, it was of interest to see whether $m^7G^{5'}p$ inhibits the translation of rabbit globin mRNAs in the rabbit reticulocyte lysate system.

The present work showed that $m^7G^{5'}p$ preferentially inhibited the synthesis of α-globin chain in the rabbit reticulocyte lysate system. The inhibition was shown to be at the level of the initiation of rabbit globin synthesis and a possible mechanism of the inhibition is discussed.

2. Materials and methods

2.1. *Materials*

Rabbit reticulocyte lysate was prepared as described previously [7]. $m^7G^{5'}p$ was purchased from P-L Biochemicals. L-[U^{14}-C]leucine (308 mCi/mmol), L-[^{35}S]methionine (596.87 mCi/mmol) and Aquasol-2 were from New England Nuclear. L-[^{35}S]-Met–$tRNA_f^{Met}$ was prepared from unfractionated rabbit reticulocyte tRNA [8] as described [9]. The L-[^{35}S]Met–$tRNA_f^{Met}$ was determined to be more than 90% pure by the method described [10] and had the specific activity of 3.42×10^6 cpm/A_{260}.

2.2. *Amino acid incorporation experiments*

Each 50 μl incubation mixture contained the following: 20 μl lysate, 30 mM Tris–HCl (pH 7.4), 70 mM KCl, 2.5 mM magnesium acetate, 5 mM creatine phosphate, 2.7 μg creatine kinase, 3 mM mercaptoethanol, 40 μM each of 19 L-amino acids minus leucine, 0.86 mM GTP, 0.36 mM ATP, 0.125 μCi L-[^{14}C]leucine, 30 μM hemin. Product analyses were done for the samples obtained after 30 min incubation at 30°C. The ^{14}C-incorporation into TCA-insoluble materials was determined for 5 μl incubation mixture as described [7]. The rest of the incubation mixture was used to determine the ^{14}C-incorporation into α- and β-globin chains as described previously [7], except that 0.5 ml of each fraction from CM-cellulose column chromatography was counted in 2.5 ml Aquasol-2 in 5 ml scintillation vial.

2.3. *Effect of $m^7G^{5'}p$ on the elongation and/or release of nascent chains*

Aliquots of 0.4 ml lysate were incubated with 2.5 μCi [^{14}C]leucine in 1 ml incubation mixture for 4 min at 30°C as described above. Reactions were stopped by cooling in ice-cold water and by the addition of 2 ml ice-cold buffer A (10 mM Tris–HCl (pH 7.4), 10 mM KCl, 1.5 mM magnesium acetate). Ribosomal pellets of the mixture were obtained by ultracentrifugation through 30% sucrose containing buffer A for 2.5 h at 50 000 rev./min in Hitachi RP65 rotor at 5°C. The pellets with ^{14}C-labelled nascent chains were suspended in 0.4 ml of the post-ribosomal supernatants of the original lysate. Aliquots of 40 μl of the suspension were incubated with various concentrations of $m^7G^{5'}p$ in

100 μl incubation mixture for 4 min at 30°C as described above, except that the mixture contained 40 μM each of 20 L-amino acids. After incubation, ribosomal pellets were obtained as described above and suspended in 0.5 ml distilled water. The suspension was counted in 4 ml Aquasol-2.

2.4. *Effect of $m^7G^{5'}p$ on the initiation complex formation*

Aliquots of 40 μl lysate were incubated with various concentrations of $m^7G^{5'}p$ in 100 μl incubation mixture for 5 min at 30°C. The incubation mixture contained as described above, except that 40 μM each of 19 L-amino acids minus methionine and [^{35}S]Met–$tRNA_f^{Met}$ (4.93 × 10^5 cpm) were used. After incubation, reactions were stopped by cooling in ice-cold water and by the addition of 120 μl ice-cold buffer A. Two hundred μl of the mixture were layered on 5 ml linear sucrose gradient (15–30%) containing buffer A in a centrifuge tube. The tube was centrifuged at 40 000 rev./min in Hitachi RPS65T rotor for 220 min at 5°C. After centrifugation, sucrose gradient was pumped from the bottom through ISCO ultraviolet monitor (model UA4) and 8 drops were directly collected into scintillation vial. To each fraction, 0.5 ml of distilled water and 4 ml Aquasol-2 were added to count the radioactivity.

3. Results

3.1. *Effect of $m^7G^{5'}p$ on [^{14}C]leucine incorporation into α- and β-globin chains*

Rabbit reticulocyte lysate was incubated with $m^7G^{5'}p$ as described in Materials and methods. Aliquots of 5 μl incubation mixture were taken at a given time and analyzed for [^{14}C]leucine incorporation into TCA-insoluble materials. Figure 1 shows the effect of time on the [^{14}C]leucine incorporation. During the initial 10 min, the ^{14}C-incorporation increased linearly with time without added $m^7G^{5'}p$, but not with 1.6 mM $m^7G^{5'}p$.

Figure 2 shows the effect of $m^7G^{5'}p$ on the ^{14}C-incorporation into α- and β-globin chains. The total ^{14}C-incorporation did not decrease with 0.4 mM $m^7G^{5'}p$. However, the synthesis of α-chains was preferentially inhibited and the synthesis of β-chains

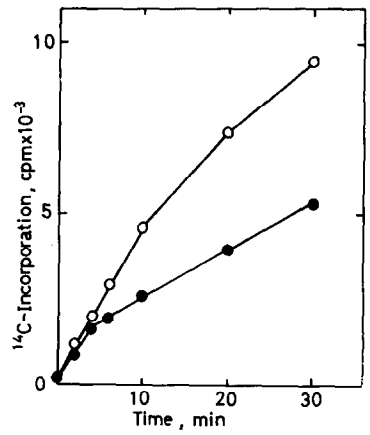


Fig.1. Effect of time on the incorporation of [^{14}C]leucine into TCA-insoluble materials with 1.6 mM $m^7G^{5'}p$. Experiments were done as described in Materials and methods. The total volume of incubation mixture was 50 μl. At a given time, 5 μl incubation mixture were taken to measure the ^{14}C-incorporation into TCA-insoluble materials. (○——○) Without added $m^7G^{5'}p$, (●——●) with 1.6 mM $m^7G^{5'}p$.

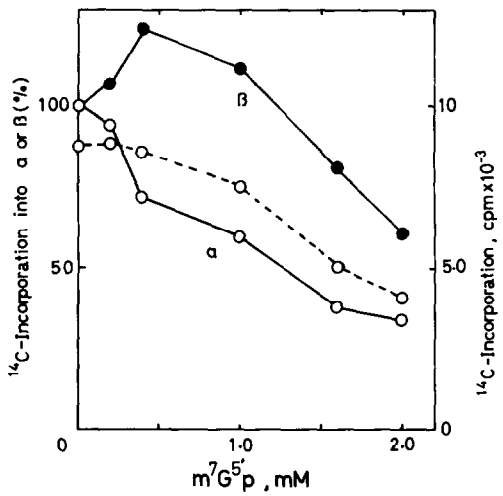


Fig.2. Effect of $m^7G^{5'}p$ on the incorporation of [^{14}C]-leucine into TCA-insoluble materials (○- - -○), α- (○——○) and β-globin chains (●——●). The ^{14}C-incorporation into α- and β-globin chains were expressed as a percentage of the ^{14}C-incorporation into each chain without added $m^7G^{5'}p$. Experiments were done as described in Materials and methods.

was stimulated at relatively low concentrations of $m^7G^{5'}p$.

3.2. *Effect of $m^7G^{5'}p$ on the elongation and/or release of nascent chains*

Ribosomes with labelled nascent chains were released at various concentrations of $m^7G^{5'}p$ as described in Materials and methods. As shown in fig.3, almost 90% of the nascent chains was released with and without added $m^7G^{5'}p$. This means that $m^7G^{5'}p$ does not inhibit the elongation and/or release of nascent chains.

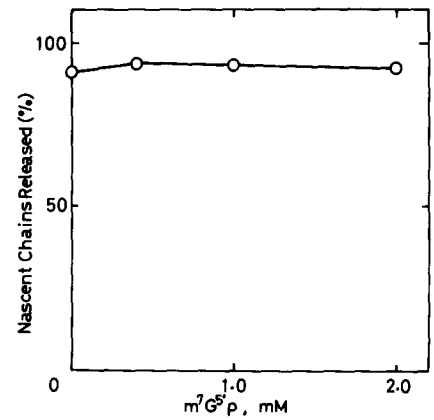


Fig.3. Effect of $m^7G^{5'}p$ on the elongation and/or release of nascent chains. Ribosomes with ^{14}C-labelled nascent chains were released at various concentrations of $m^7G^{5'}p$ as described in Materials and methods. The ribosomes in 40 μl postribosomal supernatants had the radioactivity of 13 230 cpm without incubation. (o——o) Nascent chains released (%).

3.3. *Effect of $m^7G^{5'}p$ on the formation of both a 40 S/Met–$tRNA_f^{Met}$ complex and an 80 S/Met–$tRNA_f^{Met}$ complex*

Aliquots of 40 μl lysate were incubated with various concentrations of $m^7G^{5'}p$ and with [^{35}S]-Met–$tRNA_f^{Met}$ as described in Materials and methods. As figure 4 shows, the ^{35}S-radioactivities in both 40 S and 80 S ribosome regions increased with 0.4 mM $m^7G^{5'}p$. But the ^{35}S-radioactivities at 80 S ribosome region did not increase by the further addition of $m^7G^{5'}p$, while those in 40 S ribosome region increased.

4. Discussion

The present work showed that $m^7G^{5'}p$ inhibited the rabbit globin synthesis in rabbit reticulocyte

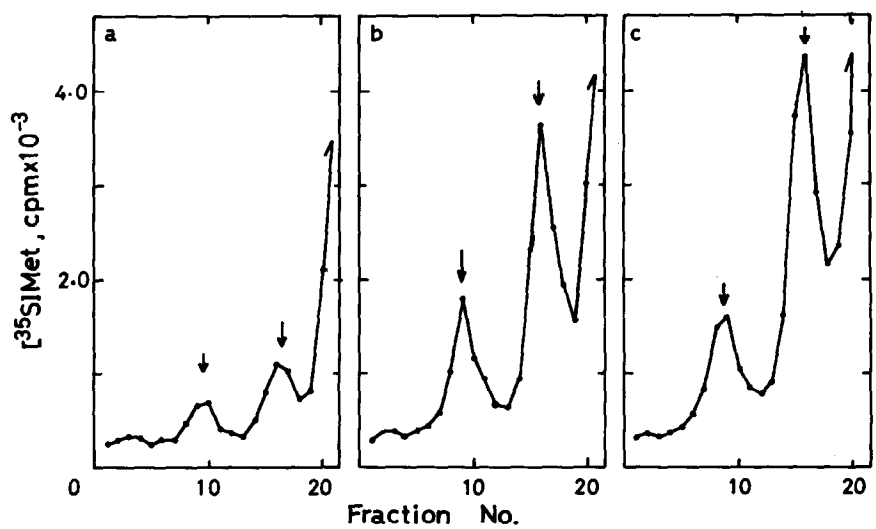
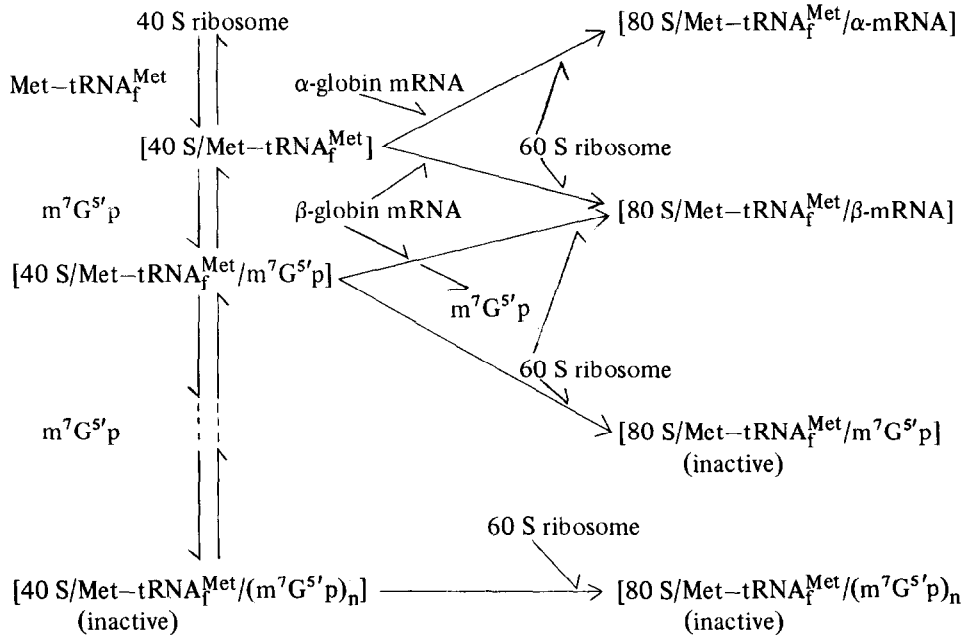


Fig.4. Effect of $m^7G^{5'}p$ on the initiation complex formation. Aliquots of 40 μl lysate were incubated with 0(a), 0.4(b) and 2.0 mM $m^7G^{5'}p$(c) in 100 μl incubation mixture for 5 min at 30°C. Experimental details are described in Materials and methods. In each figure, left and right arrows indicate the sedimentation positions of 80 S and 40 S ribosomes, respectively.

lysate system. Other workers reported an inhibition of rabbit globin synthesis with $m^7G^{5'}p$ in wheat germ and Artemia salina systems [5,6]. However, the concentration of $m^7G^{5'}p$ for 50% inhibition was high in the present work. An inhibition of the transition of a 40 S/Met-$tRNA_f^{Met}$ complex to an 80 S/Met-$tRNA_f^{Met}$ complex with $m^7G^{5'}p$ was reported by using wheat germ ribosomes and TMV or AMV RNA [11]. But, the stimulation of the formation of these complexes was observed in the present work (fig.4). These conflicting data might be due to the differences among these ribosomes in their affinities with $m^7G^{5'}p$ and/or mRNAs.

The present work also showed that $m^7G^{5'}p$ preferentially inhibited the α-globin synthesis, but stimulated the β-globin synthesis at its low concentrations (fig.2). The inhibition was shown to be at the level of initiation (fig.3). But the stimulation of the formation of both a 40 S/Met-$tRNA_f^{Met}$ complex was observed with added $m^7G^{5'}p$ (fig.4). Several explanations may be possible for the contradiction among these data. However, we propose the following model by taking into account the previous works on the initiation of globin synthesis [12,13].

In this model, the steps leading to an 80 S initiation complex were described, since $m^7G^{5'}p$ inhibited at the level of initiation (fig.3). It is assumed that a 40 S/Met–$tRNA_f^{Met}$/$m^7G^{5'}p$ complex has lower reactivities than a 40 S/Met–$tRNA_f^{Met}$ complex, and that steps to form an 80 S/Met–$tRNA_f^{Met}$/$(m^7G^{5'}p)_n$ complexes are relatively slow. At relatively low concentrations of $m^7G^{5'}p$, the concentration of a 40 S/Met–$tRNA_f^{Met}$ complex decreases, but that of a 40 S/Met–$tRNA_f^{Met}$/$m^7G^{5'}p$ complex increase to form 80 S complexes, so that ^{35}S-radioactivities increase in both 40 S and 80 S ribosome regions (fig.4). This would result in the increase of the β-chain synthesis, but in the decrease of the α-chain synthesis (fig.2). At relatively high concentrations of $m^7G^{5'}p$, concentrations of both a 40 S/Met–$tRNA_f^{Met}$complex and a 40 S/Met–$tRNA_f^{Met}$/$m^7G^{5'}p$ complex decrease to form 40 S and 80 S inactive complexes, so that ^{35}S-radioactivities increase in 40 S ribosome region, but remain almost constant in 80 S ribosome region (fig.4). This would result in the decrease of both α- and β-chain syntheses (fig.2). It is apparent, however, that further experiments are required to ascertain the present model.



The $m^7G^{5'}p$ and/or compounds, such as $m^7G^{5'}pppA^m$ and $m^7G^{5'}pppG^m$ might be useful in the treatment of β-thalassemia, since the α-chain synthesis was preferentially inhibited, while the β-chain synthesis was stimulated with $m^7G^{5'}p$.

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