EFFECT OF $m^7G^{5'}ppp^{5'}Nm$ ON THE RABBIT GLOBIN SYNTHESIS

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1. Introduction

It has been shown that the 7-methyl guanosine(m^7G) at the 5'-end of mRNAs is required for translation[1–3] and $m^7G^{5'}p$ inhibits the translation of rabbit globin mRNAs in the wheat germ and *Artemia salina* systems [4,5]. The previous work [6] showed that $m^7G^{5'}p$ preferentially inhibited the α-globin synthesis in the rabbit reticulocyte lysate system.

The present work reports the effect of concentrations of $m^7G^{5'}ppp^{5'}Am$, $m^7G^{5'}ppp^{5'}Cm$, $m^7G^{5'}ppp^{5'}Gm$ and $m^7G^{5'}ppp^{5'}Um$ on the α- and β-globin synthesis in the rabbit reticulocyte lysate system. These dinucleotides preferentially inhibited the α-globin synthesis and the inhibition was shown to be at the level of initiation. The data obtained with these nucleotides were very similar to those with $m^7G^{5'}p$ [6], but the 50% inhibition was observed at about 6 times lower concentrations than $m^7G^{5'}p$.

2. Materials and methods

2.1. *Materials*

Rabbit reticulocyte lysate and [^{35}S]Met-$tRNA_f^{Met}$ were the same preparations that were used previously [6]. $m^7G^{5'}ppp^{5'}Am$, $m^7G^{5'}ppp^{5'}Cm$, $m^7G^{5'}ppp^{5'}Gm$, $m^7G^{5'}ppp^{5'}Um$ were purchased from P-L Biochemicals. Concentrations of these dinucleotides were determined by measuring the absorbance at 260 nm. The extinction coefficients of these nucleotides were assumed to be 22.8 for $m^7G^{5'}ppp^{5'}Am$, 16.6 for $m^7G^{5'}ppp^{5'}Cm$, 20.2 for $m^7G^{5'}ppp^{5'}Gm$ and 18.5 mM^{-1} cm^{-1} for $m^7G^{5'}ppp^{5'}Um$ at 260 nm in deionized water. L-[U-^{14}C]Leucine (308 mCi/mmol) and Aquasol-2 were from New England Nuclear.

2.2. *Amino acid incorporation experiments*

Incubation conditions were detailed previously[6]. Aliquots of 20 μl lysate were incubated with 0.125 μCi [^{14}C]leucine at various concentrations of $m^7G^{5'}ppp^{5'}Nm$ in the 50 μl incubation mixture. Product analyses were done for the samples obtained after 30 min incubation at 30°C. The ^{14}C-incorporations into α- and β-globin chains were determined as described[6,7].

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

Experimental conditions were detailed previously [6]. Nascent chains labelled with [^{14}C]leucine in the postribosomal supernatants were released at various concentrations of $m^7G^{5'}ppp^{5'}Nm$ in the incubation mixture for 4 min at 30°C. After incubation, ^{14}C-radioactivities retained on the ribosomal pellets were determined as described[6].

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

Experimental conditions were detailed previously[6]. Aliquots of 40 μl lysate were incubated with various concentrations of $m^7G^{5'}ppp^{5'}Nm$ and with [^{35}S]Met-$tRNA_f^{Met}$ (2.7×10^5 cpm, 1.9×10^6 cpm/A_{260}) in 100 μl incubation mixture for 5 min at 30°C. After incubation, ^{35}S-radioactivities at 40 S and 80 S ribosome regions were analyzed as described [6].

3. Results

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

Rabbit reticulocyte lysate was incubated at various concentrations of $m^7G^{5'}ppp^{5'}Nm$ as described in

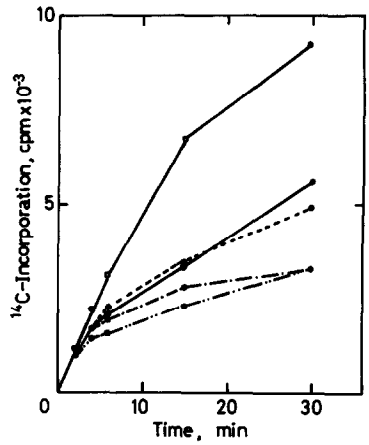

Fig.1. Effect of time on the incorporation of [^{14}C]leucine into TCA-insoluble materials at a given concentration of m^{7}G$^{5'}$ppp$^{5'}$Nm. Experiments were done as described [6]. The total volume of incubation mixture was 50 μl. At a given time, 5 μl incubation mixture were taken to measure the ^{14}C-incorporation into TCA-insoluble materials. (○———○) Without added m^{7}G$^{5'}$ppp$^{5'}$Nm, (●———●) with 230 μM m^{7}G$^{5'}$ppp$^{5'}$Am, (●– · – · –●) with 390 μM m^{7}G$^{5'}$ppp$^{5'}$Cm, (●- - - - - -●) with 250 μM m^{7}G$^{5'}$ppp$^{5'}$Gm, (●– · · –●) 450 μM m^{7}G$^{5'}$ppp$^{5'}$Um.

Materials and methods [6]. Aliquots of 5 μl incubation mixture were taken at a given time and analyzed for [^{14}C]leucine incorporation into hot TCA-insoluble materials as described [7]. Figure 1 shows the effect of time on the [^{14}C]leucine incorporation. During the initial 15 min, the ^{14}C-incorporation increased almost linearly with time without added m^{7}G$^{5'}$ppp$^{5'}$Nm, but not with m^{7}G$^{5'}$ppp$^{5'}$Nm.

Figure 2 shows the effect of concentrations of m^{7}G$^{5'}$ppp$^{5'}$Nm on the ^{14}C-incorporation into α- and β-globin chains. The total ^{14}C-incorporation did not decrease at relatively low concentrations of m^{7}G$^{5'}$ppp$^{5'}$Nm. However, the synthesis of α-chain was perferentially inhibited and the synthesis of β-chain was stimulated. These data are very similar to those with m^{7}G$^{5'}$p. However, the 50% inhibition of α-chain synthesis was observed at 0.22 mM m^{7}G$^{5'}$ppp$^{5'}$Nm and 1.25 mM m^{7}G$^{5'}$p [6]. Interestingly, the base change in the second position of these dinucleotides showed same results in the concentration dependency of the inhibition (fig.2).

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

Ribosomes with labelled nascent chains were released

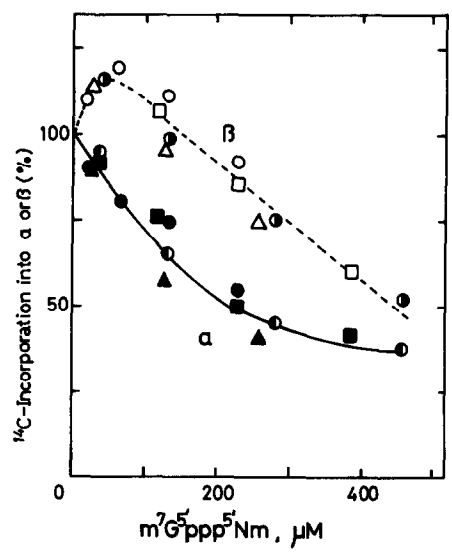

Fig.2. Effect of concentrations of m^{7}G$^{5'}$ppp$^{5'}$Nm on the incorporation of [^{14}C]leucine into α- (●, ■, ▲, ◐) and β-globin chains (○, □, △, ◑). The ^{14}C-incorporation into α- and β-globin chains were expressed as a percentage of the ^{14}C-incorporation into each chain without added m^{7}G$^{5'}$ppp$^{5'}$Nm. Experiments were done as described[6]. (●, ○) With m^{7}G$^{5'}$ppp$^{5'}$Am, (▲, △) with m^{7}G$^{5'}$ppp$^{5'}$Cm, (■, □) with m^{7}G$^{5'}$ppp$^{5'}$Gm, (◐, ◑) with m^{7}G$^{5'}$ppp$^{5'}$Um.

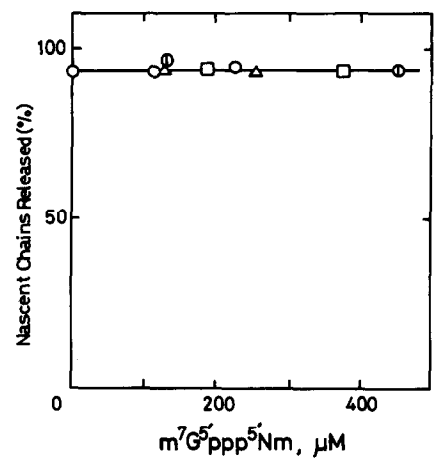

Fig.3. Effect of concentrations of m^{7}G$^{5'}$ppp$^{5'}$Nm on the elongation and/or release of nascent chains. Ribosomes with ^{14}C-labelled nascent chains in the 40 μl postribosomal supernatants were released at various concentrations of m^{7}G$^{5'}$ppp$^{5'}$Nm. The total volume of the incubation mixture was 100 μl. The ribosomes in 40 μl postribosomal supernatants had the radioactivity of 8700 cpm without incubation. Nascent chains were released with m^{7}G$^{5'}$ppp$^{5'}$Am (○), m^{7}G$^{5'}$ppp$^{5'}$Cm (□), m^{7}G$^{5'}$ppp$^{5'}$Gm (△) and m^{7}G$^{5'}$ppp$^{5'}$Um (◇).

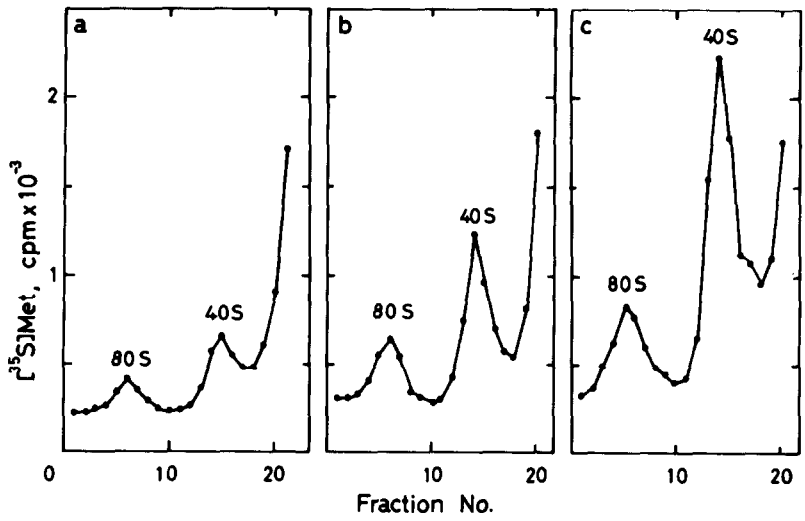

Fig.4. Effect of $m^7G^{5'}ppp^{5'}Gm$ on the initiation complex formation. Aliquots of 40 μl lysate were incubated with 0(a), 26(b) and 260 μM $m^7G^{5'}ppp^{5'}Gm$(c) in 100 μl incubation mixture for 5 min at 30°C. Experimental conditions were detailed previously [6].

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

3.3. *Effect of $m^7G^{5'}ppp^{5'}Nm$ 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 $m^7G^{5'}ppp^{5'}Nm$ with [^{35}S]Met-$tRNA_f^{Met}$ as described in Materials and methods. Figure 4 shows the sedimentation pattern obtained with $m^7G^{5'}ppp^{5'}Gm$. Similar data were obtained with $m^7G^{5'}ppp^{5'}Cm$, $m^7G^{5'}ppp^{5'}Gm$ and $m^7G^{5'}ppp^{5'}Um$. To see the effect of concentrations of these dinucleotides on the formation of the initiation complexes, the ^{35}S-radioactivities at 40 S and 80 S ribosome regions were determined by calculating the total counts in the corresponding peaks (fig.4). As fig.5 shows, the ^{35}S-radioactivities in both 40 S and 80 S ribosome regions increased at relatively low concentrations of $m^7G^{5'}ppp^{5'}Nm$. But the ^{35}S-radioactivities at 80 S region did not increase at relatively high concentrations of $m^7G^{5'}ppp^{5'}Nm$, while those in 40 S region increased. The data in fig.5 were very similar to those with $m^7G^{5'}p$ [6]. As in the case of fig.2, the concentration dependency of the initiation complex formation was almost the same for these dinucleotides.

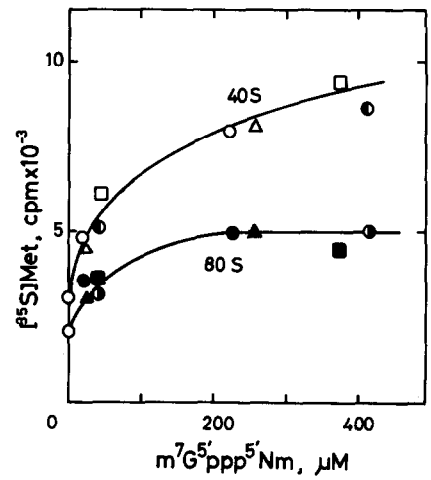

Fig.5. Effect of concentrations of $m^7G^{5'}ppp^{5'}Nm$ on the initiation complex formation. Experiments were done as in fig.4. ^{35}S-Radioactivities in 40 S and 80 S ribosome regions were determined by calculating the total counts in the corresponding peaks. Experiments with $m^7G^{5'}ppp^{5'}Am$(○, ●), $m^7G^{5'}ppp^{5'}Cm$(□, ■), $m^7G^{5'}ppp^{5'}Gm$(△, ▲), $m^7G^{5'}ppp^{5'}Um$(◐, ◑). Radioactivities in 40 S (○, □, △, ◐) and 80 S ribosome regions. (●, ■, ▲, ◑).

4. Discussion

The present work showed that four dinucleotides, $m^7G^{5'}ppp^{5'}Am$, $m^7G^{5'}ppp^{5'}Cm$, $m^7G^{5'}ppp^{5'}Gm$ and $m^7G^{5'}ppp^{5'}Um$ inhibited rabbit globin synthesis in the rabbit reticulocyte lysate system. The inhibition was shown to be at the level of initiation and these dinucleotides preferentially inhibited the synthesis of α-globin chain.

The data obtained with $m^7G^{5'}ppp^{5'}Nm$ (figs 2,3,5) were very similar to those with $m^7G^{5'}p$ [6], therefore the mechanism proposed for the inhibition with $m^7G^{5'}p$ can be applicable for the inhibition with these dinucleotides. In this case, the affinity of $m^7G^{5'}ppp^{5'}Nm$ with 40 S/Met-$tRNA_f^{Met}$ must be greater than that of $m^7G^{5'}p$, since the 50% inhibition was observed with these dinucleotides (fig.2) at about 6 times lower concentrations than $m^7G^{5'}p$ [6].

Filipowicz et al. [5] reported the presence of a protein(s) in *Artemia salina* ribosomal wash that binds with $m^7G^{5'}ppp^{5'}N$. Shafritz et al. [8] reported that $m^7G^{5'}p$ inhibits the interaction of certain mRNAs with purified reticulocyte initiation factor, IF-M_3. The data obtained with $m^7G^{5'}p$ [6] and with $m^7G^{5'}ppp^{5'}Nm$ (the present data) can also be explained by assuming that these nucleotides bind with IF-M_3 and these nucleotides on IF-M_3 can be replaced by β-globin mRNA, but not by α-mRNA.

Several groups [4,5,8–12] reported the inhibition of translation by 'cap' analogs and showed that both the 7-methyl group and 5'-phosphate groups of $m^7G^{5'}ppp^{5'}N$ are essential for the inhibition. In addition to these, the present work suggests that the kind of the second base of the dinucleotide, $m^7G^{5'}ppp^{5'}Nm$, is not essential for the inhibition, since the data obtained with four kinds of dinucleotides were almost the same (fig.2). The data obtained with $m^7G^{5'}p$ and with $m^7G^{5'}ppp^{5'}Nm$ suggest that the different affinity between α- and β-globin mRNAs with 40 S/Met-$tRNA_f^{Met}$, initiation factor(s) or other rate-limiting components of initiation [13–16] must be due to the difference of nucleotide base at an internal position(s) of the mRNAs.

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