DIFFERENTIAL INHIBITION WITH PARTIALLY PURIFIED AND ENDOGENOUS RABBIT RETICULOCYTE GLOBIN mRNA BY 7-METHYLGUANOSINE 5'-MONOPHOSPHATE

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Received August 31,1977

SUMMARY: The effect of 7-methylguanosine 5'-monophosphate ($m^7G^{5'}p$) on translation of partially purified globin mRNA and of polysome-associated endogenous globin mRNA has been studied. Under identical experimental conditions, with 0.4 mM $m^7G^{5'}p$, translation with partially purified globin mRNA is inhibited 50%; translation with endogenous globin mRNA is inhibited 10%. The inhibition of protein synthesis by $m^7G^{5'}p$ occurs at a step before the first peptide bond formation as evidenced by studies with pactamycin; 0.4 mM $m^7G^{5'}p$ inhibited the first dipeptide synthesis 43% when the partially purified globin mRNA was used whereas 15% inhibition was observed with the endogenous mRNA. The inhibition of $m^7G^{5'}p$ appears to be related to the structural integrity of globin mRNA.

Most eucaryotic cellular and viral messenger RNA's are modified at the 5'-termini by a "cap" structure which consists of 7-methylguanosine linked through its 5'-hydroxyl group via a triphosphate to the 5'-hydroxyl group of a ribose methylated nucleotide (1-4). Using a wheat germ translating system, Shatkin and co-workers have demonstrated that the 5'-terminal 7-methylguanosine (m^7G) is important for translation of globin, reovirus and VSV mRNA's (5,6). Furthermore, it was proposed that the 5'-terminal m^7G functions as a recognition signal for the binding of ribosomes to mRNA because efficient binding of mRNA to ribosomes depends on the presence of terminal m^7G at the 5' end (7,8). The importance of 5'-terminal m^7G in translation was further substantiated by showing that the 5'-phosphorylated derivatives of m^7G act as potent inhibitors of "capped" mRNA's (9,10).

However, some animal cells may not rely heavily on a m^7G on the 5' end of mRNA (11). In addition, the 5'-termini of a number of viral mRNA's do not contain the "cap". These mRNA's are translated efficiently in

ISSN 0006-291X

wheat germ extracts. Examples are poliovirus RNA (12,13), picornavirus EMC RNA (14) and satellite tobacco necrosis virus RNA (15). Therefore, in addition to the "cap" structure, other properties of the mRNA must be involved for proper binding of mRNA to ribosomes. These properties may be intrinsically related to the secondary structure of the 5' non-coding region of mRNA or could involve some mRNA associated protein(s) which have a high affinity for ribosomes. Hallerman and Shafritz have found that some protein(s) are tightly bound to globin mRNA (16). A loss of this protein(s) during mRNA purification could conceivably affect the efficiency of translation.

Because the inhibition by $m^7G^{5'}p$ is related to the "cap" present at the 5' terminus of mRNA, studies reported here were done to determine whether the translation of partially purified globin mRNA is more susceptible to inhibition by $m^7G^{5'}p$ than the polysome-associated endogenous globin mRNA. The data clearly show that $m^7G^{5'}p$ exerts a much more pronounced inhibitory effect in the translation of partially purified globin mRNA than it does on the translation of the polysome-associated "native" globin mRNA. The data may provide evidence that the marked difference is attributed to the structural integrity of globin mRNA. Loss of some globin mRNA-associated protein(s) in the purified globin mRNA could explain the increased susceptibility to inhibition by $m^7G^{5'}p$. Apparently, the 5'-terminal m^7G is not the only signal for the binding of ribosomes to mRNA. Our data also show that the action of $m^7G^{5'}p$ definitely precedes the formation of the first peptide bond.

MATERIALS AND METHODS

Chemicals and biological compounds of the highest purity were purchased from Sigma, P. L. Biochemicals, Eastman Kodak and Boehringer-Mannheim. L-[4,5-^{3}H(N)]leucine and L-[^{35}S]methionine were from New England Nuclear. Pactamycin was a generous gift of Dr. S. Pestka, Roche Institute of Molecular Biology. Globin mRNA was purchased from Miles Biochemicals.

Preparation of rabbit reticulocyte lysate, mRNA dependent lysate, [^{35}S]-met-tRNA$_f^{met}$, and incorporation of tritium-labeled leucine into protein were as described previously (17).

Methionyl-valine was isolated as follows, Protein synthesizing system (300 µl) consisting of 100 µl lysate, 80 µl of master mix (18), 2 µM pactamycin, and [^{35}S]met-tRNA$_f^{met}$ (40 µCi/nmole) was incubated at 24°C for 15 min and diluted with an equal volume of buffer A (100 mM Tris-HCl, pH 7.5, 10 mM KCl, and 1.5 mM $MgCl_2$). The mixture was applied to 5 ml of 1 M sucrose in buffer A and centrifuged at 125,000 x g for 3 hr (19). The ribosome pellet was dissolved in 0.1 ml of 2 M NH_4OH, incubated at 36°C for 30 min and the reaction mixture was applied to a paper chromatogram (Whatman 1 MM). The chromatogram was developed by descending chromatography for 20 hr in n-butanol: acetic acid:water (100:30:35, v/v/v)(20). The chromatogram was cut into 2 cm pieces and counted for radioactivity in triton X-100:toluene (1:2, v/v) scintillation fluid.

RESULTS

Pelham and Jackson have reported an excellent method for converting a rabbit reticulocyte cell-free extract into a mRNA-dependent protein synthesizing system (21). Lysates prepared according to Pelham and Jackson have negligible endogenous amino acid incorporation activity, but show high amino acid incorporation as directed by partially purified globin mRNA. When the effect of $m^7G^{5'}p$ was examined in this mRNA-dependent system, as well as in the whole lysate where protein synthesis is directed by polysome-associated endogenous globin mRNA, a large difference in the degree of inhibition of protein synthesis was observed in the two systems (Figs. 1 and 2). In the whole lysate, there was only a 28% inhibition of protein synthesis at 1.6 mM $m^7G^{5'}p$. This result is in sharp contrast to the mRNA-dependent system where $m^7G^{5'}p$ at 0.4 mM already inhibited protein synthesis 48%; at 1.6 mM, 90% inhibition of protein synthesis was observed.

A possibility exists that with the whole lysate, most of the ^{3}H-leucine incorporation may result from the completion of nascent chains. This would explain the lower susceptibility towards the inhibition of protein synthesis by $m^7G^{5'}p$. An additional experiment was done to clarify this possibility. Pactamycin is a specific inhibitor of protein initiation. At low concentration (10^{-6} — 10^{-7} M), pactamycin

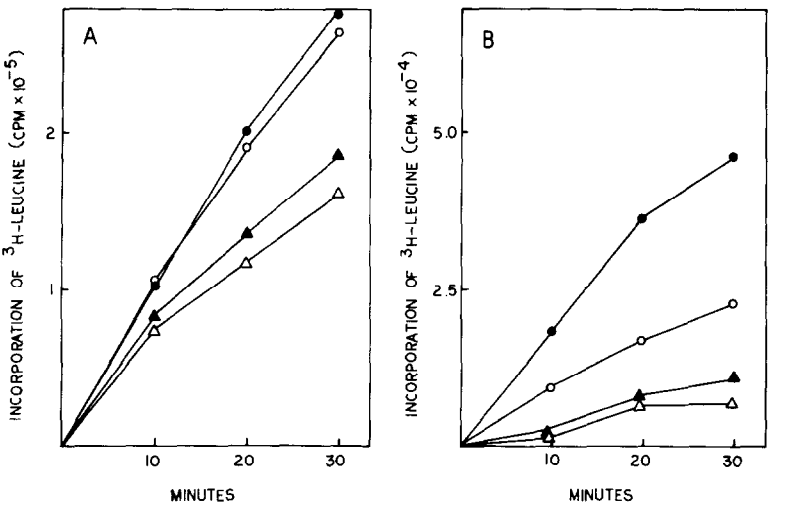


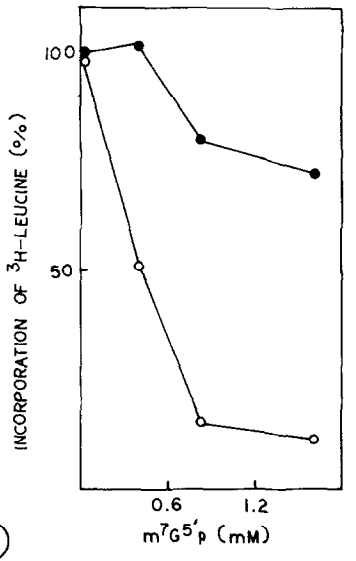
Figure 1. Kinetics of protein synthesis and the effects of $m^7G^{5'}p$. Experiments were done as described previously (17). Temperature of incubation was 25°C. Concentration of hemin was 15 μM. (A) Rabbit reticulocyte lysate; (B) mRNA dependent lysate. The concentration of purified globin mRNA was 0.61 μg/100 μl. ●—●, control; ○—○, 0.4 mM $m^7G^{5'}p$; ▲—▲, 0.8 mM $m^7G^{5'}p$; △—△, 1.6 mM $m^7G^{5'}p$.

causes an accumulation of dipeptides in an active protein synthesizing system (20). Pactamycin (2 μM) when added to the whole lysate completely inhibited the incorporation of ^{3}H-leucine after four minutes (Fig. 3). A similar lag period in the inhibition of protein synthesis was observed with 1.6 mM $m^7G^{5'}p$.

The effect of $m^7G^{5'}p$ on the pactamycin-induced dipeptide accumulation was also examined. Table 1 shows that in the whole lysate, 1.6 mM $m^7G^{5'}p$ gave a 28% inhibition of the first peptide bond formation. However, with the mRNA dependent lysate system, a 78% inhibition of the dipeptide synthesis was observed.

DISCUSSION

The experiments described here show that $m^7G^{5'}p$ is more inhibitory in the translation process with partially purified globin mRNA than with polysome-associated endogenous globin mRNA. $m^7G^{5'}p$ (0.4



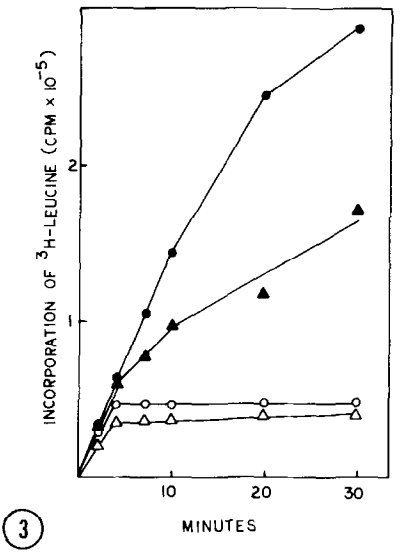


Figure 2. Inhibition of protein synthesis in rabbit reticulocyte lysate by $m^7G5'p$. The experimental results obtained in Fig. 1 after 10 min preincubation have been replotted in this figure.

Figure 3. Effect of pactamycin and $m^7G5'p$ on protein synthesis in reticulocyte lysate. Assays were conducted as described in Fig. 1. ●—●, control; ▲—▲, 1.6 mM $m^7G5'p$; o—o, control + 2 μM pactamycin; △—△, 1.6 mM $m^7G5'p$ + 2 μM pactamycin.

mM) inhibits protein synthesis 50% with partially purified globin mRNA; 1.6 mM $m^7G5'p$ is 90% inhibitory. This is in sharp contrast to the effect of $m^7G5'p$ on the translation of endogenous globin mRNA. In the endogenous globin mRNA system, $m^7G5'p$ (0.4 mM) does not inhibit protein synthesis; 1.6 mM $m^7G5'p$ is only 30% inhibitory. Shafritz et al. also observed that $m^7G5'p$ exerted a greater inhibitory effect on protein synthesis in a reticulocyte messenger-dependent cell free system (22).

Several possibilities can be offered to explain these observations. The possibility that a component required for the binding of mRNA to a ribosome (either a protein factor or a low molecular weight compound) is lost during the preparation of the mRNA-dependent translating system seems unlikely because the addition of purified mRNA to the whole lysate

TABLE 1

Effect of $m^7G^{5'}p$ on the accumulation of pactamycin-induced methionyl-valine[a]

System	$m^7G^{5'}p$ mM	Total radioactivity per strip cpm	[^{35}S]Met-val cpm	Met-val %	Control %
mRNA-dependent lysate[b]	0.0	8053	1080	13.4	100
	0.4	7250	557	7.7	57.5
	0.8	5500	276	5.0	37.3
	1.6	6001	181	3.0	22.4
whole lysate	0.0	7096	1622	22.9	100
	0.4	6542	1278	19.5	85.2
	0.8	4712	735	15.6	71.0
	1.6	4156	685	16.5	72.1

[a]Incubation conditions and analysis by paper chromatography were as described in Materials and Methods.

[b]Prepared as described by Pelham and Jackson (21).

results in an intermediary degree of inhibition when $m^7G^{5'}p$ is added (data not shown). This suggests that the translation of purified globin mRNA in the whole lysate is also more susceptible to $m^7G^{5'}p$. Another consideration is that the commercial mRNA used in our studies is preferentially enriched with α-mRNA. Suzuki showed that α-globin synthesis is more susceptible to inhibition by $m^7G^{5'}p$ than is β-globin synthesis. At 0.4 mM and 0.8 mM $m^7G^{5'}p$, the inhibitions reported were 28% and 37%, respectively (23). In our experiments, the inhibitions reported at 0.4 mM and 0.8 mM $m^7G^{5'}p$ are 50% and 90%, respectively, with the partially purified mRNA. Therefore, even if our source of mRNA contained only α-globin mRNA, the 90%

inhibition of protein synthesis at 0.8 mM $m^7G^{5'}p$ is 2.4 times greater than that reported by Suzuki. A third possibility to be considered is that some protein(s) tightly bound to globin mRNA is (are) removed during the purification of globin mRNA; therefore the binding of purified globin mRNA to ribosomes becomes absolutely dependent on the 5'-terminal m^7G. The importance of mRNA-associated protein in protein synthesis has been extensively documented (24-27). The transfer of met-$tRNA_f^{met}$ from a 40S ribosomal subunit to an initiation complex containing the 80S ribosome by IF-II with natural mRNA's as templates require the mRNA-associated proteins for proper orientation of the mRNA (28). Lebleu et al. have reported that one of the mRNA-associated proteins is necessary for binding rabbit globin mRNA to 40S ribosomal subunit obtained from rabbit reticulocytes (29). Ilan and Ilan demonstrated that at least one protein from messenger ribonucleoprotein is necessary for translation of natural mRNA's in an insect cell-free system derived from Tenebrico (30). Liautard showed that proteins of the polysomal messenger ribonucleoprotein are responsible for association with the 40S ribosomal subunit in HeLa cells (31). The suggestion has been made that these mRNA-associated proteins play an integral role in the initiation of eucaryotic protein synthesis and may regulate the translational efficiency of the mRNA's (28). Therefore, the difference in inhibition by $m^7G^{5'}p$ between the purified globin mRNA and the endogenous globin mRNA tends to support the idea of a globin mRNA-protein complex as an additional property in establishing the importance of the 5'-terminus of mRNA as related to efficient translation. The pactamycin data further establish that the action of $m^7G^{5'}p$ precedes the formation of the first peptide bond.

ACKNOWLEDGEMENTS: This research was supported by a research grant from the U.S. Public Health Service (NIH AI 12066). Inquiries regarding this work should be addressed to Dr. Suhadolnik.

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