TRANSLATIONAL DISCRIMINATION OF 'CAPPED' AND 'NON-CAPPED' mRNAS: INHIBITION BY A SERIES OF CHEMICAL ANALOGS OF m^7GpppX

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1. Introduction

During the past year a novel sequence of methylated nucleotides at the 5′ termini of a wide variety of eukaryotic and viral messenger mRNAs has been described [1–9]. Rottman et al. [1] introduced the term 'cap' to describe the structural characteristics of this methylated 5′ terminus. Related or identical 'caps' have been discovered in certain low molecular weight nuclear RNAs [10]. The formation of 'capping' structures in vitro in HeLa nuclear extracts has been reported [11]. Muthukrishnan et al. [12] have demonstrated the requirement of the 5′-terminal 7-methylguanosine for eukaryotic mRNAs translation.

In vitro protein synthesis directed by 'capped' mRNA was inhibited by penicillium nuclease (P1) resistant oligonucleotides, derived from P1 digest of total HeLa cells as well as by G^5ppp^5G [13] isolated from *Artemia salina* (Groner, Y., and Grosfeld, H., unpublished). The inhibitory effect of these compounds is most probably due to their structural similarity to the 5′ termini of mRNAs. As reported here synthetic 'caps' like $m^7GpppGm$ and 'cap' analogs like m^7pG, m^7pGp and m^7GTP were tested and found to be specific inhibitors of initiation on 'capped' mRNA. Translation of non-capped messengers, is resistant to m^7pG, suggesting a mechanism of discrimination between the two classes of mRNA.

Abbreviations: Hb, hemoglobin; EMC, Encephalomyocarditis; SDS, sodium dodecyl sulphate; m^7pG, 7-methylguanosine 5′ monophosphate; m^7pGp, 7-methylguanosine 5′, 2′ (3′) disphosphate; m^7G, 7-methylguanosine; pG, guanosine-5-monophosphate; m^7GTP, 7-methylguanosine 5′ triphosphate; SAH, *S*-adenosylhomocystein.

2. Materials and methods

Preparation of wheat-germ extracts for cell-free translation was as described [14], except that the preincubation step was omitted. Protein synthesis in wheat-germ extract was as previously outlined [14] with the following modifications: Reaction mixture (0.025 ml) contained 0.6 A_{260} wheat-germ S-30 fraction, 2.5 mM MgOAc, 100 mM KOAc, 8 μCi [^{35}S]methionine (250 Ci/mmole) and one of the following messengers: 0.5 μg HbRNA or mengo RNA, 0.4 μg EMC RNA, SV40 mRNA, 0.7 μg SV40 cRNA, 3.5 μg T4 mRNA. Incubation was for 2 h at 25°C. 5 μl aliquots were spotted on Whatman 3 MM filter paper discs and processed for measurement of amino acid incorporation into protein [14]. Products were analyzed by SDS polyacrylamide slab gel electrophoresis [17]. Cell-free extracts from mouse L cells were prepared as previously outlined. [15]. Conditions for protein synthesis in L cell extracts were as described [17] including 1 μg crude initiation factor from rabbit reticulocytes and 8 μCi [^{35}S]methionine per 25 μl reaction. After incubation at 30°C for 1 h, 10 μl aliquots were processed [14]. HbRNA and mengo RNA were prepared as previously described [17]. SV40 mRNA and SV40 cRNA were prepared as reported [18] by E. Gilboa and T4 mRNA by G. Goldberg from this department. EMC RNA was a generous gift from Dr A. Traub, Israel Institute for Biological Research, Ness-Ziona, Israel. pG, m^7pG, m^7G were purchased from Sigma Chemical Co., USA and m^7GTP, $m^7GpppGm$, GpppGm from P. L. Biochemical Inc., USA. m^7pGp was prepared essentially as published [19]. [^{35}S]methionine was obtained from the Radiochemical Center, Amersham, England.

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3. Results

3.1. *Specific inhibition of mRNA translation by 'cap' analogs*

Hemoglobin mRNA and SV40 mRNA, which are known to contain 'capping' structures at their 5′ termini [7,12] are efficiently translated in wheat-germ protein synthesizing systems. When synthetic 'caps' or 'cap' analogs were added to the reaction mixtures, translation of these messengers was strongly impaired (tables 1 and 2).

The specificity of the inhibitory effect of 'cap' structures was investigated by using a series of structural analogs. Neither pG nor m^7G were inhibitory (table 2). Thus, both the methyl group at the 7th position and the phosphate at the 5′ are essential. On the other hand, addition of a phosphate group at the 3′ (2′) position of m^7pG to give m^7pGp, reduced only slightly the inhibitory effect (table 2); this additional phosphate may reduce the affinity of the compound for the target site (the ribosome or one of the initiation proteins). The inhibition exerted by the non-methylated 'cap' GpppGm (table 1) was abolished in the presence of SAH and was therefore probably due to in vitro methylation of this 'cap' in the wheat-germ system.

The inhibition of 'capped' mRNA translation by 'cap' analogs was observed also in other cell free systems. In L cell extracts, translation of globin and SV40 mRNA was inhibited by $m^7GpppGm$, m^7GTP, m^7pG and m^7pGp although the smaller analogs have slightly less effect than in the wheat-germ system (tables 1 and 3). This may be due to an inactivation of these compounds in L cell extracts by phosphatase or demethylase which removes one of the functional groups.

Table 1
Specific inhibition of mRNA translation by synthetic 'caps'

System and	mRNA		Control	$m^7GpppGm$	GpppGm	m^7GTP
Wheat Germ	Globin	[^{35}S]Meth. cpm incorporated	205 317	13 678	141 317	16 630
		% of control	100	6.6	69	8
L cells	SV40	[^{35}S]Meth. cpm incorporated	86 159	11 647	78 365	13 920
		% of control	100	14	91	16

Translation of globin and SV40 mRNAs in wheat-germ and L cell systems was as described in Materials and methods. The concentration of the synthetic 'caps' added was 5 A_{250} U/ml and of m^7GTP 5 A_{260} U/ml.

Table 2
Specific inhibition of mRNAs translation in wheat germ system by 'cap' analogs

		Control	+ m^7pG	+ m^7pGp	+ pG	+ m^7G
Globin mRNA	[^{35}S]Meth. cpm incorporated	228 000	20 188	55 742	260 935	210 414
	% of control	100	8.7	24	114	92
SV40 mRNA	[^{35}S]Meth. cpm incorporated	55 847	7389	20 288	55 847	44 649
	% of cotnrol	100	13	36	100	81

Translation of globin and SV40 mRNAs in wheat-germ system was as described in Materials and methods. 'Cap' analogs were added at concentration of 0.5 mM.

Table 3
Specific inhibition of mRNA translation in L cells system by 'cap' analogs

		Control	+ m^7pG	+ m^7pGp	+ pG	+ m^7G
Globin mRNA	[^{35}S]Meth. cpm incorporated	82 032	48 458	42 898	84 389	85 381
	% of control	100	59	52	102	104
SV40 mRNA	[^{35}S]Meth. cpm incorporated	96 103	29 236	33 392	88 096	81 457
	% of control	100	30	35	92	85

Translation of globin and SV40 mRNAs in L cell extracts was as described in Materials and methods. 'Cap' analogs were added at concentration of 0.5 mM.

3.2. *m^7pG Discriminates between 'capped' and 'non-capped' messenger RNAs*

As mentioned above, the inhibition of globin and SV40 mRNA translation by 'cap' analogs is related to the structural similarity between these compounds and the 5′ terminus of 'capped' mRNA ($m^7G^{5'}ppp^{5'}N...$). Thus it was interesting to study the effect of these 'cap' analogs on in vitro translation of 'uncapped' messengers.

SV40 cRNA which is transcribed in vitro from SV40 DNA by a purified *E.coli* RNA polymerase contains an unblocked triphosphate 5′ end ($ppp^{5'}N...$). SV40 cRNA is nevertheless active [20] in directing cell-free protein synthesis in wheat-germ extracts, coding for some of the viral polypeptides.

When increasing amounts of m^7pG were added to the wheat-germ system, there was a rapid and complete inhibition in the translation of the SV40 mRNA purified from infected cells. In contrast, translation of in vitro produced SV40 cRNA was insensitive to m^7pG (fig.1). At a concentration of 0.5 mM m^7pG, where inhibition of in vivo SV40 mRNA and globin mRNA (fig.1A) exceeded 90%, protein synthesis

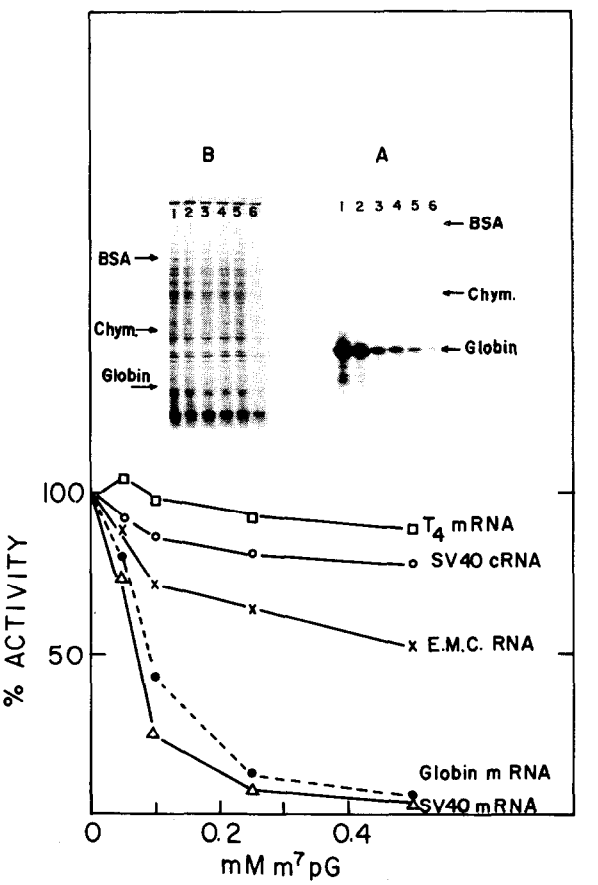

Fig.1. Selective inhibition of mRNAs translation. Protein synthesis in wheat-germ extracts was carried out as described in Materials and methods. Translation in the presence of increasing concentration of m^7pG is expressed as percent of controls minus m^7pG. The control incorporation was 173 820 cpm with globin, 99 728 cpm with SV40 mRNA, 26 220 cpm with EMC RNA, 56 350 cpm with SV40 cRNA and 38 561 cpm with T_4 mRNA. Endogenous incorporation of 12 380 cpm was subtracted. (A) and (B) are autoradiograms of [^{35}S] methionine-labelled polypeptides fractionated on SDS 10–20% polyacrylamide gradient slab gels [17]. (A) Cell-free products directed by globin mRNA in the presence of 0, 0.05, 0.1, 0.25, 0.5, 1.0 mM of m^7pG. (B) Same as in (A) but with EMC RNA. Standards of known molecular weight included bovine serum albumin (68 000), chymotripsinogen (25 700) and globin (15 500).

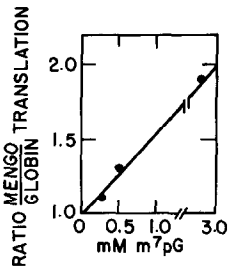

Fig.2. Effect of m^7pG on globin and mengo RNA translation in L cells extract. Hemoglobin mRNA (0.5 μg) and mengo RNA (0.5 μg) were incubated in 0.025 ml reaction mixture as described in Materials and methods. Results are expressed as the ratio of the translation of mengo RNA to globin mRNA against m^7pG concentration. Without m^7pG, mengo RNA and Hb RNA incorporate, 478 800 and 82 800 cpm/10 μl respectively. The endogenous incorporation was subtracted from each point.

Table 4
Selective inhibition of initiation tripeptides synthesis by m^7pG

	[^{35}S]Methionine cpm in tripeptides		
mRNA	Control	+ m^7pG	% of control
Globin	39 400	5626	14
EMC	9092	7310	80

Initiation tripeptides were formed under the usual conditions for protein synthesis as described in Materials and methods except that sparsomycin was added at 200 μM. After incubation, the cell-free system was cooled, extracted with phenol and further processed as described [22]. Paper electrophoresis on Whatman 3 MM was carried out at 3 kV for 3 h in pyridine acetate buffer (pH 3.5). The dried paper was cut into 1-cm strips and radioactivity was determined in scintillation fluid. Marker tripeptides were located by staining with 1% ninhydrin.

directed by SV40 cRNA was reduced merely by 20%. Similarly, the translation of picornavirus EMC RNA, which is 'non-capped' in vivo [21] was less inhibited by m^7pG, as clearly shown by the comparison of product analysis in fig.1B and fig.1A. A prokaryotic mRNA, coliphage T4 mRNA was not inhibited at all (fig.1). The differential effect of m^7pG on 'capped' mRNA translation was observed in mammalian cell-free systems as well. In L cell extracts, translation of globin mRNA was inhibited by m^7pG, whereas 'non-capped' mengo virus RNA translation was even stimulated. As a result, the ratio of mengo virus RNA to globin RNA translation in L cell extracts increased with growing amounts of m^7pG (fig.2).

In L cell extracts the translational discrimination was seen with the small 'cap' analogs, like m^7pG and m^7GTP, but a complete 'cap' as $m^7GpppGm$ inhibited the translation of both globin and mengo RNA. The molecular basis of the discrimination is currently under investigation.

3.3. *m^7pG Inhibits 'capped' mRNA translation by preventing the formation of the ribosome–mRNA initiation complex*

In order to determine the m^7pG sensitive step in mRNA translation, we studied its effect on the formation of sparsomycin initiation peptides. This antibiotic inhibits polypeptide chain elongation but has little effect on initiation [22]. Translation of globin mRNA or EMC RNA in wheat-germ extracts in the presence of 200 μM sparsomycin resulted in the accumulation of initiation tripeptides. Addition of m^7pG markedly reduced the globin mRNA coded tripeptides, but had little effect when EMC RNA served as messenger (table 4), indicating that m^7pG inhibition occurs at the initial phase of mRNA translation.

Both et al. [23] have recently shown that the binding of reovirus mRNA to 80S ribosomes in wheat-germ extracts requires the 5′ terminal 7-methylguanosine. Based on this information, we tested the effect of m^7pG on the formation of globin mRNA dependent 80S–initiation complex (fig.3). The reaction was carried out in the presence of 200 μM sparsomycin which, as can be seen, prevented the formation of polyribosomes that sedimented ahead of the 80S monomer. Addition of 0.5 mM m^7pG inhibited the formation of the globin mRNA dependent 80S initiation complex by over 70% (fig.3). We concluded from these results that the discriminating effect of m^7pG on 'capped' mRNA translation is expressed at the initiation of protein synthesis, by preventing the assembly of the 80S mRNA initiation complex. A possible effect at the 40S subunit level still remains to be investigated.

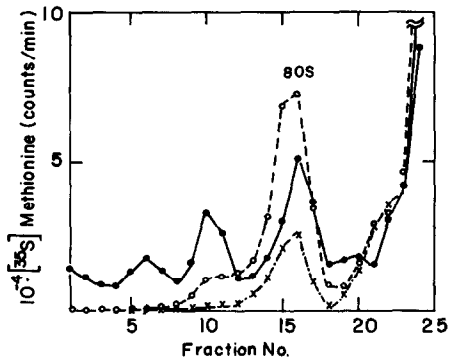

Fig.3. m^7pG Inhibition of 80S–mRNA initiation complex formation. HbmRNA was incubated for 10 min in 0.05 ml of wheat-germ protein synthesizing system. Without sparsomycin (o——o) or with 200 μM sparsomycin. In the absence (o - - - o) and (x - - - x) presence of 0.5 mM m^7pG. After incubation the reaction mixtures were laid over 4.8 ml 10–30% glycerol gradient in 20 mM HEPES pH 7.5, 2.5 mM MgOAc, 100 mM KOAc, and centrifuged in a Spinco Rotor SW 50.1 for 75 min at 42 000 rev/min. Fractions were collected and counted directly by liquid scintillation. Endogenous reactions run in parallel gradients were subtracted.

4. Discussion

Methylated blocked termini ('caps') of the type $m^7G^{5'}ppp^{5'}Nm...$ have been found at the 5′ ends of a large number of viral and cellular mRNAs [1–10]. As we have reported above, synthetic 'caps', as well as 'cap' analogs like m^7pG, m^7pGp and m^7GTP are specific inhibitors of 'capped' mRNA translation. These findings are consistent with previous observations [23] that the 5′ terminal m^7G in reovirus mRNA, is required for the initiation of protein synthesis. The simpler 'cap' analog which still possessed an inhibitory effect was m^7pG, thus the minimal requirements are a methyl group at the 7th position and a phosphate at the 5′. Nevertheless, addition of a phosphate at the 3′ (2′) position of m^7pG did not impair the inhibitory effect.

Both et al. [23] reported that the 5′ terminal m^7G is required for the binding of the 40S ribosomal subunit to reovirus mRNA. However, neither the short 5′ oligonucleotide m^7GpppG^mpCpUp $(Np)_3Gp$ derived by RNAse T_1 from reovirus mRNA [23] nor the oligomer $m^7GpppGp$ from brome mosaic virus RNA [24] bound to wheat-germ ribosomes, indicating that the 'cap' itself is not recognized by the ribosome. It is possible that the positive charge imposed by the methyl group of the 5′ terminal m^7G is required merely for maintaining a specific conformation at the 5′ end of the mRNA in order to facilitate the binding of the ribosome to the adjacent initiator sequence. Our results would rather suggest a model in which the 'cap' is positively recognized by an initiation protein. 'Cap' analogs would inhibit 'capped' mRNA translation by interacting with the active site of this protein factor and in such a way preventing the binding of mRNA to the ribosome and hence the formation of initiation complex. Studies of this reaction with radioactively labelled 'cap' analogs should help clarify this problem. The inhibition of mRNA translation in wheat-germ and L cell systems by the small 'cap' analogs m^7pG is specific for messages containing blocked methylated 5′ termini, 'uncapped' mRNAs being much more resistant. These mRNAs lacking the 'cap' and in which the 5′ end may be remote from the ribosome binding site, may still be initiated even when the 'cap' binding factor is occupied by the m^7pG inhibitor. It will now be important to determine what role the m^7pG binding factor or ribosome site plays for translational control in eukaryotic cells.

While this work was in progress Hickey, Weber and Baglioni [25] reported similarly that initiation of protein synthesis is specifically inhibited by m^7pG.

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References

[1] Rottman, F., Shatkin, A. J. and Perry, R. P. (1974) Cell 3, 197.
[2] Furuichi, Y. and Muira, K-I. (1975) Nature 253, 374.
[3] Furuichi, Y., Morgan, M., Muthukrishnan, S. and Shatkin, A. J. (1975) Proc. Natl. Acad. Sci. US 72, 362.
[4] Wei-C-M and Moss, B. (1975) Proc. Natl. Acad. Sci. US 72, 318.
[5] Perry, R. P., Kelley, D. E., Friderici, K. and Rottman, F. (1975) Cell 4, 387.

[6] Wei-C-M, Gershowitz, A. and Moss, B. (1975) Cell 4, 379.
[7] Lavi, S. and Shatkin, A. J. (1975) Proc. Natl. Acad. Sci. US 72, 2012.
[8] Furuichi, Y., Morgan, M., Shatkin, A. J., Jelinek, W., Soldittt-Greogieff, M. and Darnell, J. E. (1975) Proc. Natl. Acad. Sci. US 72, 1904.
[9] Adams, J. M. and Cory, S. (1975) Nature 225, 28.
[10] Reddy, R., Ro-Choi, T. S., Henning, D. and Busch, H. (1974) J. Biol. Chem. 249, 6486.
[11] Groner, Y. and Hurwitz, J. (1975) Proc. Acad. Natl. Sci. US 72, 2930.
[12] Muthukrishnan, S., Both, G. W., Furuichi, Y. and Shatkin, A. J. (1975) Nature 255, 33.
[13] Finamore, F. J. and Warner, A. H. (1963) J. Biol. Chem. 238, 344.
[14] Roberts, B. E. and Paterson, B. M. (1973) Proc. Natl. Acad. Sci. US 70, 2330.
[15] Falcoff, E., Falcoff, R., Lebleu, B. and Revel, M. (1973) Journal of Virology 12, 421.
[16] Nudel, U., Lebleu, B. and Revel, M. (1973) Proc. Natl. Acad. Sci. 70, 2139.
[17] Content, J., Lebleu, B., Nudel, U., Zilberstein, A., Berissi, H. and Revel, M. (1975) Eur. J. Biochem. 54, 1.
[18] Gilboa, E., Prives, C. L. and Aviv, H. (1975) Biochemistry 14, 4215.
[19] Yoshikawa, M., Kato, T. and Takenishi, T. (1969) Bull. Chem. Soc. Japan 42, 3505.
[20] Prives, C. L., Aviv, H., Gilboa, E., Winocour, E. and Revel, M. (1975) INSERM 47, 305.
[21] Muthukrishnan, S. and Shatkin, A. J. quoted in: Nuss, P. L., Furuichi, Y., Koch, G. and Shatkin, A. J. (1975) Cell 6, 21.
[22] Smith, A. E. and Wigle, D. T. (1973) Eur. J. Biochem. 35, 566.
[23] Both, G. W., Furuichi, Y., Muthukrishnan, S. and Shatkin, A. J. (1975) Cell 6, 185.
[24] Dasgupta, R., Shih, D. S., Saris, C. and Kaesberg, P. (1975) Nature 256, 624.
[25] Hickey, E. O., Weber, L. A. and Baglioni, C. (1976) Proc. Natl. Acad. Sci. US 73, 19.