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INHIBITION OF NONCAPPED mRNA TRANSLATION BY THE CAP ANALOGUE, 7-METHYLGUANOSINE-5'-PHOSPHATE*

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SUMMARY: The cap analogue, 7-methylguanosine-5'-phosphate (pm^7G), inhibits the translation of the noncapped STNV (satellite tobacco necrosis virus) RNA and CPMV (cowpea mosaic virus) RNA in the in vitro wheat germ protein synthesizing system. While the translation of some capped mRNAs is inhibited more strongly by the analogue, other capped mRNAs have a level of sensitivity similar to that of the noncapped RNAs. Evidence is presented demonstrating that the effect of the analogue is exerted at a cap binding site even when it is inhibiting noncapped mRNAs. These results therefore indicate that the cap binding site of the translational system is either part of or is closely linked to another mRNA binding component, this component being specific for a site on the mRNA other than the 5' cap. The observations also suggest caution in the use of pm^7G inhibition to indicate the presence of a 5' cap on a particular mRNA.

Many viral and cellular mRNAs have an $m^7G^{5'}ppp...$ cap at their 5' end, and considerable evidence has been presented indicating that the cap functions to facilitate ribosome attachment to the mRNA (1), and to protect the mRNA from 5' exonucleolytic cleavage (2, 3). One approach supporting the idea of functional significance of the 5' cap was the observation that an analogue, pm^7G, specifically inhibited mRNA translation, particularly in an in vitro protein synthesizing system from wheat germ (4-6). The specificity of inhibition included the demonstration that neither pG nor m^7Gp were inhibitors and that the inhibitory function of the pm^7G was exerted at the mRNA-dependent step in which a 40S·Met-$tRNA_i^{Met}$ initiation complex was converted to an 80S complex. In a limited number of tests, inhibition of translation by pm^7G

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seemed to occur only with capped mRNAs and not with noncapped mRNAs (4, 5). In addition to its practical use in discriminating between capped and uncapped mRNAs, this observation suggested the presence of a specific component in the protein chain initiating system that would be utilized only by a capped mRNA. In pursuing this question further, we have examined in detail the effect of pm^7G on the translation of two noncapped mRNAs. We show here with both of these mRNAs that inhibition can be obtained to an extent even greater than that observed with some capped mRNAs and that neither pG nor m^7Gp are inhibitory. This specificity of inhibition of noncapped mRNA translation indicates a close interaction between the cap and mRNA binding sites of the translation system with both sites perhaps being part of a single component.

MATERIALS AND METHODS

The in vitro translational incubation is that described earlier (6) with the monovalent ion concentration varied as indicated in the legends to the tables. TMV-RNA, globin mRNA, and STNV-RNA were prepared by established procedures (7-9). CPMV-RNA was a kind gift of George Bruening, Department of Biochemistry and Biophysics, University of California at Davis, and AMV-RNA 4 of Lous Van-Vloten Doting, Department of Biochemistry, University of Leiden, Leiden, Netherlands.

RESULTS

The data of Table 1 show the effect of a moderate level of pm^7G (0.5 mM) on the translation of noncapped STNV-RNA and two capped RNAs, globin mRNA and TMV-RNA, under monovalent salt conditions (20 mM KCl, 100 mM K acetate) optimal for all three mRNAs. Two points emerge. While the analogue discriminates between globin mRNA and STNV-RNA (inhibiting globin mRNA translation better than 90%) it is nevertheless clearly able to substantially inhibit translation of the noncapped STNV-RNA. Secondly, when compared with TMV-RNA, the distinction between the capped and uncapped mRNAs is not obvious. Indeed, the inhibition by pm^7G at a subsaturating level of STNV-RNA is considerably greater than that obtained at the higher level of TMV-RNA. Similar, but even more dramatic results are obtained in comparing the inhibition of STNV-RNA translation to that of another capped RNA, RNA 4 of alfalfa mosaic virus (AMV-RNA 4).

Table 1. Effect of pm^7G on the Translation of STNV-RNA, globin mRNA, TMV-RNA, and AMV-RNA 4

mRNA (μg)	^{14}C-leucine incorporated (cpm)		Inhibition
	control	+0.5 mM pm^7G	%
STNV-RNA (1.0)	11,005	5,644	49
STNV-RNA (4.0)	46,577	37,042	20
globin mRNA (2.0)	54,882	1,559	97
globin mRNA (4.0)	77,825	4,547	94
TMV-RNA (1.5)	61,208	6,612	89
TMV-RNA (4.0)	66,150	43,279	34
AMV-RNA 4 (6.0)	130,783	115,923	11

The standard incubation was carried out for 40 min at 25° with 0.32 mM S-adenosylhomocysteine (SAH), 20 mM KCl, and 100 mM K acetate added to all incubations. In the absence of mRNA, 1170 cpm were incorporated.

The data of Table 2 provide an insight into the extent to which STNV-RNA translation can be inhibited by the analogue and the specificity of this inhibition. At a KCl concentration of 100 mM, pm^7G inhibits STNV-RNA translation more than 80 per cent and the inhibition is fully dependent on the structural analogy to the 5' cap. Analogues lacking either the 7-methyl or the 5' phosphate are not inhibitory and the inhibition by pm^7G and $pppm^7C$ is not reversed by excess GMP or GTP.

In studies of competitive translation (to be reported in detail[1]) we have found that STNV-RNA is a "strong" mRNA in that it outcompetes all other mRNAs tested under conditions of *in vitro* competitive translation. It seemed there-

1. Herson, D., Schmidt, A., Seal, S. N., Marcus, A. and Van Vloten-Doting, in preparation.

Table 2. Specificity of pm^7G Inhibition of STNV-RNA Translation

	Additions	^{14}C-leucine incorporated	Inhibition
		cpm	%
1)	STNV-RNA (1.0 µg)	6,887	
	+0.5 mM pm^7G	1,856	73
	+1.0 mM pm^7G	1,126	84
	+0.25 mM $pppm^7G$	1,390	80
	+0.5 mM $pppm^7G$	996	86
	STNV-RNA (3.5 µg)	25,145	
	+0.5 mM pm^7G	10,845	57
	+1.0 mM pm^7G	7,444	70
2)	STNV-RNA (1.0 µg)	5,648	
	+1.0 mM pG	5,459	
	+1.0 mM Gp	6,230	
	+0.5 mM pm^7G+1.0 mM pG	1,625	71
	+0.5 mM pppG	5,952	
	+0.25 mM $pppm^7G$+0.5 mM pppG	1,036	82

The incubation conditions were identical to those of table 1 except that the monovalent salt concentration was 100 mM KCl. In the absence of mRNA, 650 cpm were incorporated. The incubation with 1.0 µg of STNV-RNA of experiment 1 was also carried out in the absence of SAH and resulted in 7060 cpm incorporated.

fore possible that the lack of a more complete inhibition by pm^7G might be due to an unusually strong affinity of STNV-RNA for an mRNA binding site. A "weaker" noncapped mRNA might in turn be inhibited by the cap analogue to a considerably greater extent. CPMV-RNA, a viral RNA that lacks a 5' cap (10) and is translated with a reduced level of efficiency[2], seemed appropriate for

2. Owens, R. A. and Bruening, G., unpublished observation. See also data of table 3.

Table 3. Inhibition of CPMV-RNA Translation by pm^7G

CPMV-RNA	^{14}C-leucine incorporation (cpm)		Inhibition
µg	control	+ 0.5 mM pm^7G	%
1) 20 mM KCl, 100 mM K acetate			
0.5	2,162	841	61
1.0	3,833	1,579	59
2.0	4,795	2,312	52
2) 100 mM KCl			
2.0	1,070	353	67

The incubation conditions were identical to those of table 1 with the monovalent salt concentrations as indicated. In the absence of mRNA, 925 cpm were incorporated in exp. 1 and 424 cpm in exp. 2.

testing this possibility. At the least, it would provide an indication as to the generality of the inhibition of noncapped RNAs by the analogue. The data of Table 3 show that CPMV-RNA translation is indeed substantially inhibited by pm^7G. The inhibition, however, is at about the same level as that observed for STNV-RNA and does not approach that obtained with globin mRNA. It would seem therefore that the affinity of CPMV-RNA for the mRNA binding site is similar to that of STNV-RNA and that the decreased efficiency of CPMV-RNA translation is due to another rate limiting step in the reaction.

DISCUSSION

The analogue, pm^7G, clearly inhibits the translation of noncapped mRNAs. To exclude possible capping during the *in vitro* translation, SAH (S-adenosyl-homocysteine), a capping inhibitor (11), was included in all incubations (although as reported earlier (6) SAH has no effect on the translation of the noncapped mRNA (see also legend to table 2)). The inhibition by the analogue therefore implies that pm^7G, while utilizing its structural homology to the 5' terminus of the mRNA (table 2), still affects an mRNA binding site in the

in vitro system other than that occupied by the 5' cap of the mRNA. With regard to the mechanism of protein chain initiation, it seems therefore unnecessary to postulate a distinct cap-binding protein. More likely, the translational system contains an mRNA binding factor that has within itself a cap binding site which serves to facilitate attachment of mRNAs having a 5' cap. The major interaction of the factor and the mRNA would, however, involve a site on the mRNA other than the 5' cap.

Reticulocyte factor IF-M3(eIF4B) has been reported to bind both capped histone mRNA and noncapped EMC-RNA with only the binding of the capped mRNA inhibited by pm^7G (12). Granting this factor to be an mRNA binding component, we suggest that the apparent lack of inhibition of the binding of the non-capped mRNA was due to the end point nature of the assay. Since the binding of the mRNA can be expected to be essentially irreversible, substantial inhibition would be seen only in a kinetic assay. A similar explanation would seem relevant to the inability of pm^7G to inhibit STNV-RNA-catalyzed 80S·Met-tRNA complex formation (6). In the latter situation, the high level of mRNA used would also minimize the inhibition (see table 1, line 2). Finally, the report of a protein whose ability to bind the cap structure is completely unaffected by a noncapped mRNA (13), suggests that this protein is indeed a specific cap-binding protein. Should this protein be functional in protein synthesis, our results would suggest that it interacts closely with another mRNA binding component, the latter being specific for a site on the mRNA other than the 5' cap.

While the present communication is the first to consider in detail the significance of the inhibition of noncapped mRNAs by pm^7G, data with EMC-RNA (ref. 5, fig. 1) show such inhibition, although the authors do not comment on it. We have also observed (R. Roman, unpublished results) that TMV-RNA, uncapped by HIO_4 oxidation and β-elimination, is still strongly inhibited by pm^7G (see also ref. 14), indicating that the effect of the analogue on non-capped mRNAs is not specific for particular mRNAs. Recently, Kemper and

Stolansky noted extensive inhibition by pm^7G of the translation of STNV-RNA and uncapped globin mRNA (17). Their interpretation of the effect on uncapped globin mRNA was that it was due to the inhibition of residual capped mRNA. The concept presented here would allow that pm^7G is acting on the uncapped mRNA reaction in such manner as to weaken the attachment of the initiating region of the mRNA.

Notwithstanding the substantial effect of pm^7G on the uncapped mRNAs, we have as yet been unable to inhibit naturally occurring noncapped mRNAs with 0.5 mM pm^7G to a level greater than 75%, and this only under stringent conditions (table 2). An essentially complete inhibition of translation of an mRNA by 0.5 mM pm^7G (e.g. refs. 15 and 16) would therefore constitute reasonable evidence for the presence of a cap structure. Moderate inhibition, however, without additional evidence, should probably not be taken to indicate a capped mRNA.

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