**Review Letter**

# FUNCTIONS OF THE 5'-TERMINAL m$^7$G CAP IN EUKARYOTIC mRNA

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## 1. Introduction

All cellular eukaryotic mRNAs studied so far contain at their 5'-terminus a blocked methylated structure often referred to as a 'cap'. Most of the eukaryotic viral mRNAs are similarly modified. The general formula for the capped 5'-terminus of mRNAs may be represented as m$^7$G$^{(5')}$ppp$^{(5')}$X$^{(m)}$pY$^{(m)}$. In this structure the 7-methylguanosine (m$^7$G) and the penultimate nucleoside (X) are joined by their 5'-hydroxyl groups through a triphosphate bridge. Nucleosides X and Y are often also methylated in the 2'-O-position and an $N^6$,2'-O-dimethyladenosine may be found in position X. The 26 S mRNA coded in vivo by Sindbis virus contains, in addition to the usual m$^7$G, also m$_2$$^{2,7}$G or m$_3$$^{2,2,7}$G at its 5'-terminus [2].

This article is concerned with the possible role played by the 5'-terminal cap in eukaryotic mRNA. For more details on the cap structure, its occurrence in different mRNA species, the mechanism of cap synthesis and earlier studies on its function, the reader is referred to the review by Shatkin [3].

Based on extensive in vitro translation studies two main functions have emerged for the 5'-terminal

7-methylguanosine. One is a facilitation of mRNA entry into the initiation complex with eukaryotic ribosome, the second, a protection of mRNA against degradation by cellular nucleases.

## 2. Preparation of mRNA molecules with different 5'-termini

Most of the data on the possible role of methylated nucleosides at the 5'-terminus of mRNAs comes from experiments in which activity of capped methylated mRNAs was compared with the activity of their unmodified or partially modified counterparts. There are several methods for obtaining such mRNA preparations:

(1) The cores of some animal [4–8], insect [9,10] and plant viruses [11] contain all the necessary enzymes for the in vitro synthesis of capped and methylated mRNAs. Viral core-directed mRNA synthesis performed in the presence of the methyl donor, AdoMet, results in the production of mRNA molecules with a m$^7$GpppN$^{(m)}$ terminus. Synthesis without added AdoMet or in the presence of AdoHcy, the inhibitor of methylation, produces mRNA molecules with either ppN or GpppN at their 5'-ends [12–17]. By further adjusting the reaction conditions a selective synthesis of mRNAs bearing either ppN or GpppN ends may be obtained as shown with reovirus cores [18]. The vaccinia core-associated enzymes have also been used for the capping and 2'-O-methylation of RNAs which, as for instance STNV RNA [19], function normally as uncapped molecules bearing (p)ppN at their 5'-ends.

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*Abbreviations:* m$^7$G, 7-methylguanosine; pm$^7$G, 7-methyl-guanosine-5'-monophosphate; N, nucleoside (A,G,U or C); N$^m$, 2'-0-methylnucleoside; AdoMet, *S*-adenosyl-methionine; AdoHcy, *S*-adenosyl-homocysteine; STNV, Satellite Tobacco Necrosis Virus; VSV, Vesicular Stomatitis Virus; TMV, Tobacco Mosaic Virus; CPV, Cytoplasmic Polyhedrosis Virus

m$^7$GpppN$^{(m)}$- and GpppN-terminated mRNA molecules are referred to as capped and blocked mRNAs, respectively. Nomenclature used for the eukaryotic initiation factors is as in [1]

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(2) The treatment of capped mRNA with periodate and aniline leads to the removal by $\beta$-elimination of the blocking $m^7G$ from the mRNA; the resulting RNA has a $pppN^{(m)}$ terminus [4,6,20]. It was pointed out that $\beta$-elimination may lead to some unspecific damage such as degradation or modification of the RNA [20]. In many instances this procedure was, however, applied successfully to functional studies [15,20–26].

(3) The nucleotide pyrophosphatase purified from tobacco cells [27,28] and potato [29] specifically cleaves the pyrophosphate linkages in the 5'-terminal cap of mRNA. Recently a very rapid procedure for the purification of tobacco cell enzyme has been described [30]. Phage T4-induced polynucleotide kinase was also reported to cleave the pyrophosphate bond from a cap structure [31]. However, the utility of this procedure needs to be confirmed [30].

(4) Synthetic ribopolymers of variable base composition bearing different 5'-termini may be prepared by priming the polynucleotide phosphorylase-catalyzed reaction with $m^7GpppG^mpC$, GpppGpC or ppGpC [32].

## 3. The role of $m^7G$ in facilitating mRNA binding to ribosomes

The initial observations that a 5'-terminal cap is required for efficient translation of mRNAs come from the comparison of activities of capped and uncapped reovirus, VSV and rabbit globin mRNAs in a wheat germ cell-free protein-synthesizing system [21,33]. Subsequently it was found that the 5'-terminal $m^7G$ is necessary for efficient binding of reovirus mRNAs to the 40 S ribosomal subunits during initiation of protein synthesis [12]. A blocked and methylated 5'-terminus was required for the enhanced activity of reovirus mRNAs. Molecules with GpppN at their 5'-end were translated with low efficiency, as were (p)ppN-terminated mRNAs [12,24,34].

Further studies on the comparison of capped versus uncapped mRNA molecules have demonstrated the importance of the 5'-terminal $m^7G$ for in vitro translation of mRNAs of viruses such as reo [12,21, 24,26,29,34–36], VSV [14,15,20,24], vaccinia

[25,37,38], CPV [28], BMV [22], TMV [28,39] and of cellular messengers like globin mRNA [21,26, 28,29,39], bovine parathyroid hormone mRNA [23], ovalbumin mRNA [40] and *Artemia salina* mRNA [29,41]. Most of these studies were performed in a wheat germ protein-synthesizing system [12,15, 21–25,28,29,33–35,37,39,41]. The other cell-free systems used included the rabbit reticulocyte lysate [15,20,24,25,37–39], *Artemia salina* extract [41], mouse ascites cell extract [14,40] and a fractionated mammalian system of mixed origin [26,36].

An independent approach used to determine the importance of the 5'-terminal cap in translation involves the use of $m^7G$ cap analogs. Compounds such as $pm^7G$, $m^7GDP$, $m^7GpppN^{(m)}$ but not their unmethylated counterparts are specific inhibitors of translation of capped mRNAs [42–47]. The cap analogs act at the level of initiation by inhibiting mRNA binding to ribosomes [45–47]. Their inhibitory effect on the translation or binding to ribosomes of variety of capped mRNAs is documented in wheat germ [15,26,28,35,42–45,47–50], *Artemia salina* [44], L cell [45], HeLa cell [51], mouse ascites cell [40] and rabbit reticulocyte [38,39,46,48,52] extracts as well as in the fractionated mammalian system [26,36].

More recently evidence has been accumulated that, apart from inhibiting the initiation of capped mRNAs, cap analogs may also, although to lesser extent, interfere with translation of uncapped messengers [15,45,53,54]. This can be regarded as an indication that the same cellular component, possibly an initiation factor, involved in interaction with the 5'-terminus of capped mRNAs (see following sections), participates also in translation of uncapped messengers. Cap analogs, when bound to a presumptive $m^7G$-binding site of a factor, might indirectly affect the properties of its other site involved in recognition of an internal sequence in mRNA [53,55].

Much evidence exists that the 5'-terminal $m^7G$ is not absolutely required for mRNA translation but plays rather a facilitating role in mRNA binding to ribosomes [15,20,22,24,26,37,56]. All mRNAs which physiologically function as capped molecules and which have been studied so far may be translated in vitro into the authentic polypeptides also in the decapped or unmethylated form [15,20,22,26,29,35, 37,39]. Furthermore, the RNAs of picornaviruses

[58—62], Feline Leukemia Virus* [63] and plant viruses like STNV [19,65], Cow Pea Mosaic Virus [66] or Tobacco Necrosis Virus [67] are not capped, yet most of them act as efficient templates for protein synthesis [57,68—70]. Moreover, the unmethylated messengers of prokaryotic origin which do not contain caps can be translated in eukaryotic cell-free systems, albeit less efficiently [71—75].

The presence of a $m^7G$ at the 5'-terminus of mRNAs increases primarily the rate and frequently also the extent of messenger binding to ribosomes in vitro. The ribosomal binding of capped and uncapped mRNAs of reovirus [24], VSV [15,20,24] and vaccinia virus [25,37] was extensively studied in the wheat germ and rabbit reticulocyte cell-free systems. In all instances the absence of the $m^7G$ cap led to a pronounced decrease of both the rate and the extent of RNA binding. The readdition of $m^7G$, but not of the unmethylated G, to the 5'-terminus of vaccinia virus mRNA subjected previously to $\beta$-elimination resulted in the recovery of the translational activity of this mRNA [25].

Strong support for the facilitating role of $m^7G$ comes from experiments in which the activity of capped and uncapped ribopolymers was compared [24,32,76]. Polymers rich in A and U bind to wheat germ or rabbit reticulocyte ribosomes even in the absence of a modified 5'-terminus. Addition of $m^7GpppG^m$ (but not of GpppG or ppG) to their 5'-end, however increases the extent of polymer binding by ~3-fold; the rate of binding is also markedly enhanced. Certain types of polymers are not able to bind to 80 S ribosomes but interact with the 40 S subunits. This interaction is also strongly promoted by the presence of $m^7GpppG^m$ at the 5'-end [24,32,76].

Further support in favor of a facilitating role of $m^7G$ in mRNA translation is provided by the results of competition experiments. When a mixture of capped and uncapped mRNA molecules is assayed

for the ability to bind to ribosomes, those molecules which bear the $m^7G$ cap at their 5'-end are preferentially bound [24,25]. Such selection by ribosomes of capped over uncapped RNA molecules has been documented for reo [24] and vaccinia [25] virus mRNAs studied in a wheat germ extract and a rabbit reticulocyte lysate, respectively. The preferential translation of capped globin and reoviral mRNAs has also been demonstrated by competition experiments performed with a fractionated mammalian cell-free system [26].

A similar selection of capped over uncapped mRNA molecules most likely occurs also in vivo. In VSV-infected cells all polysome-associated viral mRNA is capped, whereas among cytoplasmic RNAs not bound to ribosomes there is a fraction of viral RNA with uncapped 5'-termini [77]. The translational activity of rabbit globin mRNAs subjected to periodate oxidation or $\beta$-elimination is diminished by ~10-fold in microinjected *Xenopus laevis* oocytes (R. E. Lockard and C. D. Lane, personal communication).

Although the presence of a blocking $m^7G$ seems to facilitate translation of capped mRNAs, addition of 7-methylguanosine to the 5'-end of STNV RNA, an mRNA which normally functions without cap, may not have a similar effect. Smith and Clark [78] have found that capped STNV RNA does not show an enhanced rate or extent of initiation of protein synthesis in vitro as compared to uncapped STNV RNA. However, in the binding of fragmented STNV RNAs to ribosomes, a capped preparation showed better messenger properties than uncapped STNV RNA, indicating that the tertiary structure of STNV RNA may preclude the functioning of the cap in this RNA molecule [57,78].

The apparent importance of the 5'-terminal $m^7G$ for translation of mRNAs is strongly influenced by the conditions of in vitro protein synthesis. Among the factors affecting the requirement for $m^7G$ are: the concentration of mRNA [22,35,39,42] and initiation factors [26,55] and the ionic conditions [37,39,48,54] or temperature [48] used for in vitro translation. Of particular importance is the potassium ion concentration. At low $K^+$ (40—80 mM) little or no dependence on $m^7G$ is found; at higher $K^+$ (~150 mM), which more closely resembles the cellular concentration [79], $m^7G$ at the 5'-terminus stimulates mRNA translation several fold [37,39,48,54].

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* Although the 28 S RNA from the virions of Feline Leukemia Virus (FeLV-Rickard strain) is not capped, the virus-related mRNAs present in infected cells are capped [64]. There are indications that the 28 S RNA does not function as mRNA but corresponds rather to defective RNA molecules and not to the full-length FeLV genome [64]

The relation of $K^+$ concentration to the observed requirement for $m^7G$ partially explains previous reports that translation in a reticulocyte lysate depends much less on $m^7G$ than that in a wheat germ extract [15,20,24]. When translation in these two systems is studied at similar $K^+$ concentrations, the dependence on $m^7G$ becomes comparable [37,39,48]. Likewise, translation of endogenous globin mRNA in a reticulocyte lysate, which had been reported to be relatively resistant to inhibition by $m^7G$ analogs [15,46,52,80], depends on the presence of the $m^7G$ cap as does exogenous globin mRNA, provided the experiments are carried out at comparable $K^+$ concentrations [39,48]. The mechanism of $K^+$-dependent differences in requirement for $m^7G$ is unknown.

Translation in vitro at subsaturating levels of mRNA depends on $m^7G$ more strongly than at higher RNA concentrations [22,35,39,42]. Related to this are the observations that protection of the $m^7G$ cap-containing sequence within a 40 S initiation complex is higher at low mRNA concentrations in a wheat germ extract [81]. On the other hand, the requirement for the $m^7G$ cap decreases when the concentration of reticulocyte initiation factors is raised in a fractionated cell-free system [26,55]. It is conceivable that high mRNA and initiation factor concentrations increase the rate of initiation complex formation to such an extent that a facilitative role of the $m^7G$ cap is no longer strongly apparent.

There are indications that translation of certain capped mRNAs may depend on the presence of $m^7G$ more than that of other messengers. $\beta$-Elimination decreases the ribosome binding activity of reovirus mRNA more than that of VSV mRNA [24]. Similarly, the $m^7G$ cap analogs inhibit translation of rabbit $\alpha$-globin mRNA more strongly than translation of $\beta$-globin messenger [48,52]. The relative importance of $m^7G$ for translation of a given mRNA might depend on how efficiently an mRNA species is able to interact with ribosomes through structural features other than the 5'-terminal cap [20,24,82]. Further studies are required to answer this question.

## 4. $m^7G$ and the ribosomal binding sites in mRNA

Much information about the possible role of the 5'-terminal $m^7G$ in eukaryotic protein synthesis comes from the analysis of mRNA sequences protected by the ribosome during initiation of translation. Particularly extensively studied are the ribosomal binding sites of the 10 different species of reovirus mRNAs [56,81,83–86]; the binding sites of 6 of them have been sequenced [81,84–86]. In the case of all reovirus mRNAs, the $m^7G$ cap constitutes a part of the nucleotide sequence protected within the 40 S initiation complex formed in a wheat germ extract [81,83–85]. The length of the 40 S-protected, nuclease-resistant sequences varies, depending on the mRNA species, from 30 to about 60 nucleotides; all the fragments also contain the AUG codon located 15–33 nucleotides away from 5'-terminal 7-methyl-guanosine [81,83–85].

Fragments protected within the 80 S initiation complexes are substantially shorter, 25–29 nucleotides in length, with the AUG codon positioned centrally, 12–14 nucleotides away from the 5'-end of the fragment. For each individual reovirus mRNA, the fragment protected by the 80 S wheat germ ribosome is the subset of a longer 40 S-protected sequence [81,83–85]. Similar differences in the lengths of 40 S and 80 S-protected fragments were also observed with reticulocyte ribosomes for $\beta$-globin [87] and reovirus mRNAs [86]. In most instances the 80 S-protected fragments do not include the 5'-terminal cap [81,84–86,88]. 80 S ribosomes protect the cap only in those mRNAs in which the distance between the 5'-terminal $m^7G$ and the AUG codon is not longer than ~15 nucleotides, as documented for two of the VSV mRNAs [88] and one of the reovirus mRNAs [85].

The 40 S-protected capped fragments of reovirus mRNAs are able to rebind to wheat germ ribosomes much more efficiently than their uncapped 80 S-protected counterparts [56,83]. Efficient rebinding of the isolated 40 S-protected fragments indicates that they possess all the necessary features required for ribosome attachment. Different subfragments obtained by limited T1 RNase digestion of the 40 S-protected sequences were further analysed for their ability to rebind to ribosomes. Binding of the fragment was substantially reduced by removal of the 5'-terminal region containing the $m^7G$. When the AUG codon was missing the stable complex formation with ribosomes was abolished completely, irrespective of the presence or absence of the $m^7G$ cap [56].

The comparison of the known 5'-terminal sequences of different mRNAs reveals that the only common features of all these messengers include the presence of a 5'-terminal cap and of an AUG initiation codon positioned 10–69 nucleotides away from the 5'-terminus of the RNA [81,84–86,88–94]. Moreover, it has not yet been established whether an mRNA–rRNA interaction, similar to that involving base pairing between the 3'-end of 16 S rRNA and the ribosomal binding sites of prokaryotic messengers [95,96] occurs also during initiation in eukaryotic systems. Most of the eukaryotic mRNAs appear to contain in the vicinity of the initiator AUG codon sequences that are complementary to the 18 S RNA (discussed in [97]). However, contrary to the situation with bacterial mRNAs, in the case of eukaryotic messengers the distance between the postulated complementary regions and the initiation codon is highly variable, thus weakening arguments for significant mRNA–rRNA interactions in eukaryotic initiation [85,97].

Based on all these data Kozak and Shatkin [56,81,85] suggest that binding of ribosomes to eukaryotic mRNA may not involve the recognition of a specific nucleotide sequence in the mRNA. Instead, they propose that the presence of an AUG codon proximal to the 5'-terminus of the mRNA is of primary importance for the occurrence of an initiation event. Recent experiments involving the use of the antibiotic edeine in a wheat germ extract indicate that the 40 S ribosomal subunits may initially bind to the capped 5'-terminus of mRNA and subsequently move along the mRNA chain until they encounter the first AUG codon [98]. At that point joining of the 60 S subunit would complete the initiation event. Such a mechanism may explain why in numerous eukaryotic messengers with known 5'-terminal sequences protein synthesis starts at the AUG triplet located most closely to the 5'-end [81,84–86,88–94]. Displacement of the 40 S subunit from the 5'-end towards the AUG initiator codon would also explain why in messengers in which the AUG triplet is positioned relatively far from the 5'-terminus the cap is not protected against ribonuclease by the 40 S subunit. No cap protection by the 40 S ribosomal subunit was observed for rabbit β-globin mRNA [86,87] and TMV RNA ([98], W. F. and A. L. Haenni, unpublished results) having

the AUG codons 54 and 69 nucleotides away from the 5'-terminus, respectively [90,93].

The possible role of the terminal $m^7G$ in eukaryotic initiation would be to facilitate initial binding of the 40 S subunit to the 5'-end of mRNA. As recently suggested [56] the $m^7G$ might promote, possibly by interacting with specific protein(s), a loosening of mRNA conformation at its 5'-end. This suggestion is based on the finding that after mild formaldehyde treatment the unmethylated reovirus mRNA becomes able to bind to wheat germ ribosomes and that the uncapped 5'-terminal fragments but not the full-length unmethylated reovirus mRNA, show a low level of binding to ribosomes [56]. Decreased dependence of mRNA translation on the $m^7G$ cap at low $K^+$ [37,39,48] or at high temperatures [48], which conditions possibly also favor a less compact RNA structure, further supports this possibility.

The same AUG codon would, however, be selected by the 40 S subunit irrespective of the presence or absence of the terminal 7-methylguanosine. This was directly determined by fingerprint analysis of the ribosome-protected fragments originating from capped and uncapped reovirus mRNAs [56]; the same sites are protected also by reticulocyte ribosomes in capped and uncapped VSV mRNAs [88]. Hence, the effect of the $m^7G$ cap on mRNA binding would be quantitative rather than qualitative. The possibility of translating uncapped messengers into authentic polypeptide products supports this notion [15,20,22, 26,29,35,37–39]. Interestingly, Rose [88] has noted that in the case of uncapped VSV mRNAs the ribosomes apart from recognizing the proper initiation sites also interact at low efficiency with many non-initiating nucleotide sequences in the mRNA. This result might suggest that the cap at the 5'-terminus helps to restrict the initiation events to the AUG codon located most closely to the 5'-terminus of mRNA. Since it appears that eukaryotic ribosomes are able to initiate translation with Qβ and R17 RNAs [71–74] or bacteriophage f1 mRNA [97a] internally, it would be of interest to know whether the addition of $m^7G$ cap to such RNA molecules affects their translation in vitro in a eukaryotic system.

## 5. Proteins binding to the $m^7G$ cap

Several protein initiation factors have been isolated

which are required for binding of natural mRNAs to 40 S initiation complexes [99–101]. It is conceivable that one or more of these proteins may be involved in recognition of the methylated 5'-terminus of the mRNA.

A protein that specifically binds the methylated oligonucleotide $m^7$GpppGpC was identified in a high salt wash of *Artemia salina* ribosomes [44]. As indicated by competition assays, this protein also forms a complex with capped mRNAs. The *Artemia* protein apparently does not correspond to one of the known initiation factors from rabbit reticulocytes since these factors do not form a stable complex with $m^7$GpppGpC [44].

Other studies indicate however that one or more of the known initiation factors may be involved in $m^7$G cap recognition. A preparation of 80–90% pure reticulocyte initiation factor eIF-4B (mol. wt 80 000) binds to capped histone or VSV mRNAs and this binding is specifically inhibited by the $pm^7$G analog [46]. The same factor binds also to uncapped EMC RNA but this interaction is not prevented by $pm^7$G. Hence, it is likely that eIF-4B recognizes the 5'-terminus as well as another sequence in mRNA [46]. eIF-4B is indeed required for translation of both capped and uncapped mRNAs [99,102].

Proteins that are located adjacent to the 5'-terminal $m^7$G within the initiation complex have been identified by chemical crosslinking. In a wheat germ extract three polypeptides of mol. wt 135 000, 93 000 and 26 000 and in a reticulocyte lysate two polypeptides of mol. wt 160 000 and 35 000 crosslink to the periodate oxidized 5'-terminus of reovirus mRNA [103]. Crosslinking of all proteins depends on the formation of an initiation complex; it is inhibited by cap analogs or AMPP(NH)P which decrease the yield of mRNA binding to ribosomes [103]. The polypeptide of mol. wt 160 000 may correspond to the 160 000 mol. wt subunit of the multicomponent initiation factor eIF-3; this factor is necessary for mRNA binding to ribosomes [99–101].

The purified rabbit reticulocyte and mouse ascites initiation factors were also assayed for their ability to interact with the 5'-terminal cap of reovirus mRNA by a chemical crosslinking method. Crosslinking of a 24 000 mol. wt polypeptide, present mainly in eIF-3 and to a lesser extent in eIF-4B preparations, was inhibited by the $m^7$GDP analog, indicating that this

polypeptide binds to the 5'-terminal cap of mRNA [104]. The presence of the 24 000 mol. wt polypeptide in eIF-4B preparations explains perhaps partially the previous findings that eIF-4B binds to the $m^7$G cap in mRNA [46].

In their recent work, Kaempfer et al. [55] present evidence that the initiation factor eIF-2, which forms a ternary complex with initiator Met-tRNA$_f$ and GTP, also binds the mRNA. It is proposed that apart from the site for Met-tRNA$_f$ binding, eIF-2 also has two mRNA binding sites, one of them involved in $m^7$G cap recognition. Attempts to identify specific sites within rabbit globin mRNA based on protection by eIF-2 against ribonuclease digestion were however unsuccessful in that random protection of mRNA sequences was obtained (H. Robertson and T. Hunt, personal communication).

It is difficult at this moment to reconcile these somewhat conflicting reports on the involvement of protein factors in $m^7$G cap recognition. Possibly more than one factor is involved. Moreover, the positioning of the 5'-end of mRNA within the initiation complex may differ at various stages of complex assembly.

## 6. The structural requirement for $m^7$G cap recognition

Studies with the different cap analogs have helped to gain some insight in the structural requirements for cap recognition during the assembly of the initiation complex. The minimal requirement for guanosine analogs to act as inhibitors of capped mRNA translation is the presence of the 7-methyl and 5'-phosphate groups; 7-methylguanosine, guanosine-5'-phosphate or 7-methylguanosine-2',3'-phosphate do not inhibit translation [42,45,47]. 7-Methylguanosine-5'-diphosphate is more inhibitory than the monophosphate counterpart, while $m^7$GTP, $m^7$GpppN or $m^7$GpppN$^m$ show identical inhibitory activity to $m^7$GDP [43,44]. Nuclear magnetic resonance analysis indicates that 7-methylated 5'-phosphorylated guanosine may assume a defined rigid conformation because of the electrostatic interaction between the positively-charged imidazole moiety and the negatively-charged phosphate group. This conformation is more strongly favored in $m^7$GDP than in $pm^7$G, further phosphate addition being without effect [43,105].

7-Ethyl- and 7-benzyl-GDP are as active in inhibi-

tion of reovirus mRNA binding to ribosomes as $m^7GDP$. Hence, it is not the nature of the substituent but rather a positive charge resulting from 7-alkylation which is required for inhibitory activity of the analog [106]. The data on the activity of other $m^7GDP$-related compounds also speak in favor of the importance of a positive charge on the imidazole ring and its involvement in interaction with the phosphate group [106]. The cap structure within the mRNA may have a conformation similar to that of the $m^7G$ cap analogs. By replacing AdoMet by its analog, $S$-adenosylethionine, Furuichi and Shatkin [107] have synthesized in vitro reovirus mRNAs bearing 7-ethylguanosine at the 5'-termini. The ethylated mRNAs bind to ribosomes to the same extent as methylated mRNAs and are translated in vitro into authentic reovirus polypeptides [107].

The 2-amino group of $m^7GDP$ is also important for inhibitory activity, since 7-methylinosine phosphates are less inhibitory than $m^7G$ analogs [43,106] and 7-methylxanthosine-5'-diphosphate is inactive [106]. Moreover, since 1,7-dimethyl-GDP and 6-Cl-$m^7GDP$ are also inactive as inhibitors, it is conceivable that during assembly of the initiation complex the N-1 and $N^2$ atoms of $m^7G$ are used for hydrogen bond formation with an as yet unidentified cellular component [106].

## 7. $m^7G$ and mRNA stability

The cap structures at the 5'-end of mRNA have an important stabilizing effect on mRNA injected into *Xenopus laevis* oocytes or added to wheat germ or L cell protein synthesizing extracts [34]. In all these three systems reovirus mRNAs containing $m^7GpppG^m$ or GpppG at their 5'-termini are degraded more slowly than molecules bearing $pppG^m$, pppG or ppG ends. Degradation kinetics of molecules containing $m^7GpppG^m$ or GpppG termini are identical indicating that blocking of the 5'-end with guanosine, without methylation, is sufficient for protection of the RNA against nucleases. Since the same GpppG-terminated molecules are not active as messengers in vitro it seems that protection of the RNA is not a consequence of its participation in protein synthesis, but rather an intrinsic property of RNAs with blocked 5'-termini [34]. Similar preferential degradation of

uncapped RNA molecules by a wheat germ extract was also established for CPV mRNA [28].

The unblocked mRNAs are most likely degraded in a 5'→3' direction exonucleolytically since the primary products of RNA hydrolysis appear to be 5'-mononucleotides, and since almost no degradation products of intermediate size (poly- or oligonucleotides) are found [28,34]. Contrary to the three systems mentioned above the rabbit reticulocyte lysate does not contain the nuclease(s) involved in the preferential degradation of unblocked reovirus mRNAs [34].

The presence of a terminal 7-methylguanosine is most likely not the only factor determining mRNA stability. Viral RNAs which do not contain the cap are nevertheless active messengers both in vitro and in vivo. Similarly, the enzymatically-decapped TMV RNA, but not decapped rabbit globin mRNA, seems to be a relatively stable RNA molecule, as inferred from its messenger activity in a wheat germ extract [39]. As already suggested [34] some of the uncapped RNA molecules may be protected against exonuclease degradation by interaction with proteins or by a specific conformation at their 5'-termini.

## 8. Other possible functions of the modified 5'-end of mRNA

Facilitation of mRNA binding to ribosomes and protection of RNA against nucleases seem to be relatively well established functions of the 7-methylguanosine that blocks the 5'-terminus of eukaryotic mRNAs. It is not excluded however that the 5'-terminal cap also plays some role during transcription or in mRNA processing and transport from the nucleus to the cytoplasm [108].

Molecules of heterogenous nuclear RNA (HnRNA) also contain capped methylated 5'-termini and considerable evidence exists that, in many instances, caps present in HnRNA are conserved in the corresponding cytoplasmic mRNA molecules [109,110], most likely as a consequence of the splicing events. The transfer of 5'-terminal precursor segments to the mature mRNA molecules may be considered as a further indication of the importance of the cap for mRNA translation or stability.

Furuichi [111] has noted that the in vitro tran-

scription of CPV depends on the presence of AdoMet. The requirement for AdoMet is however not due to its activity as a methyl donor since AdoHcy, an inhibitor of methylation, also stimulates synthesis of CPV mRNA. More likely AdoMet or related compounds act as allosteric effectors influencing the RNA polymerase and/or the capping enzymes [17]. Some early events in cap formation are nevertheless required for CPV mRNA synthesis. This is supported by experiments in which it was shown that CPV RNA polymerase cannot initiate the transcription process when ATP, incorporated normally as the 5′-terminal nucleotide, is replaced by its $\beta,\gamma$-imido analog, AMPP(NH)P [17]. Under physiological conditions, the first step during CPV mRNA cap formation most likely involves the removal of the $\gamma$-phosphate from the 5′-terminal ATP, catalysed by a virion-associated nucleotide phosphohydrolase. The $\beta,\gamma$-imido analog of ATP cannot be hydrolysed by the enzyme. Surprisingly, its presence prevents not only cap formation but inhibits also the whole transcription process [17].

A relation between methylation at the 5′-end and the polyadenylation at the 3′-end of mRNA was observed during in vitro transcription of VSV virions [112]. The VSV mRNAs synthesized in the presence of AdoHcy in place of AdoMet contain abnormally large poly(A) segments, up to 2000 nucleotides in length. It is not known however whether the synthesis of long poly(A) tails is a genuine consequence of an inhibition of mRNA methylation or rather a direct result of the presence of AdoHcy [112].

Unfertilized oocytes or dormant embryos of a variety of organisms contain latent maternal mRNAs; during the early stages of development the stored mRNAs become active templates for protein synthesis. The methylation of pre-existing mRNAs could serve as a simple mechanism for 'turning on' protein synthesis. Indeed, it has been shown that mRNAs isolated from the unfertilized oocytes of the insect, tobacco hornworm, contain at their 5′-end the blocked, but unmethylated structure GpppN, suggesting that translational inactivity of mRNA prior to fertilization may be related to the absence of $N^7$-methylation [113]. However, this does not seem to be a general phenomenon since direct or indirect evidence exists that 'dormant' mRNAs isolated from sea urchin and *Xenopus laevis* oocytes as well as from

dry cysts of *Artemia salina* do contain the m⁷G caps at their 5′-termini [41,114,115].

While much data has accumulated on possible functions of 5′-terminal 7-methylguanosine little is known about the role of the ribose methylations present in the second and third nucleotides from the 5′-end of many mRNAs. Moreover no function has as yet been ascribed to the methylated nucleosides, mainly 6-methyladenosine, occurring internally in many mRNA species (reviewed in [3,110]). Studies of Muthukrishnan et al. [24,25,76] indicate that 2′-O-methylation of the penultimate nucleoside may have a positive influence on mRNA binding to ribosomes. Most of the evidence in favor of this possibility comes from experiments with synthetic polynucleotides bearing $m^7GpppG^m$ or $m^7GpppG$ termini [24,76]. This stimulatory effect of 2′-O-methylation on polymer binding to ribosomes is particularly evident when ribopolymers with weak affinities for ribosomes are employed in binding experiments [24,76]. This may explain why only little or no preference for 2′-O-methylated mRNAs is observed when capped vaccinia virus mRNAs (which are rather strong ribosomal 'binders') bearing the $m^7GpppN$ or $m^7GpppN^m$ termini are compared for their ability to bind to wheat germ or rabbit reticulocyte ribosomes [25]. Further studies are required in order to elucidate a function of the penultimate and internal methylations in eukaryotic mRNAs.

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