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5′-Terminal 7-methylguanosine in eukaryotic mRNA is required for translation

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Unmethylated reovirus and VSV mRNAs are specifically methylated to form 5′-terminal structures of the type, m^7G (5′) ppp (5′) N by protein synthesising extracts prepared from wheat germ and mouse L cells. Reticulocyte mRNA also contains 5′-terminal m^7G. mRNAs having 5′-terminal m^7G stimulate protein synthesis in vitro. *Removal of m^7G by β-elimination abolishes translation of the mRNAs.*

Reovirus and vesicular stomatitis virus (VSV) mRNAs synthesised *in vitro* by their virion-associated RNA polymerase in the presence of S-adenosyl-methionine (SAM) contain the 5′-terminal structures, $m^7G(5')ppp(5')G^m$ (ref. 1) and m^7G (5′) ppp (5′) A^m (ref. 2), respectively, and no other methylated nucleotides. The mRNAs are translated with fidelity in a wheat germ cell-free protein synthesising system[3,4], and their messenger activity is unaffected by addition to the extract of SAM or its analogue, S-adenosyl-homocysteine (SAH)[5]. Viral mRNA made in the absence of SAM also promotes the synthesis of authentic viral polypeptides *in vitro*; this was shown to be due to the ability of the wheat germ extract to methylate the viral mRNAs since translation of unmethylated viral mRNA was enhanced by addition of SAM to the extract but completely blocked by the inhibitor of methylation, SAH[5]. To determine if the methylation of mRNA required for translation is specific, we have analysed reovirus and VSV mRNAs after incubation in cell-free protein synthesising extracts. The viral mRNAs are methylated exclusively at the 5′ termini and the resulting 7-methyl-guanosine (m^7G) is essential for translation. Similarly, rabbit reticulocyte mRNA contains 5′-terminal m^7G and its removal by β-elimination results in the loss of translational activity *in vitro*.

5′-Terminal methylation of mRNA

Reovirus mRNA was synthesised with α-^{32}P-CTP as radioactive precursor in the presence of SAH to minimise methylation during transcription[6]. The 5′ termini of reovirus mRNA synthesised in these conditions are predominantly ppG . . ., but some of the molecules (~ 25%) have blocked, unmethylated 5′-terminal GpppG (our unpublished results). Purified, ^{32}P-labelled mRNA was incubated in a wheat germ cell-free extract with methyl-^{3}H-SAM in conditions of protein synthesis as described previously[3–5]. The mRNA was extracted with phenol, separated from 4S RNA by density gradient centrifugation[5], digested with *Penicillium* nuclease and alkaline phosphatase and analysed by high voltage paper electrophoresis[1]. All the ^{3}H derived from methyl-^{3}H-SAM by methylation of the RNA migrated as a single component in the region characteristic of blocked 5′-terminal structures[1,7] (Fig. 1*a*). No ^{3}H-labelled nucleosides were detected, consistent with the absence of methylated residues within the RNA chains. The enzymatic digestions were complete as shown by the quantitative recovery of the ^{32}P as Pi. The presumptive 5′-terminal material was analysed by DEAE-cellulose column chromatography and had a net negative charge of −2.5 (Fig. 1*b*, inset). The same charge was found for m^7GpppG^m isolated from the 5′ termini of methylated reovirus mRNA. When analysed by paper chromatography, however, the presumptive 5′ termini (peak I) migrated more slowly than marker m^7GpppG^m (Fig. 1*b*). Treatment of the peak I material with nucleotide pyrophosphatase converted all the radioactivity to 7-methylguanylic acid (m^7pG); no 2′-O-methylguanylic acid (pG^m) or other methylated nucleotides were detected (Fig. 1*c*). Alkaline phosphatase treatment after pyrophosphatase digestion converted the radioactivity to m^7G (Fig. 1*d*). The identity of both m^7pG and m^7G was confirmed by paper chromatography as described previously[1]. The results indicate that in the presence of SAM, wheat germ extract methylates the 5′ termini of reovirus mRNA, yielding m^7GpppG. This blocked, methylated structure is similar to the 5′-terminal sequence of reovirus mRNA methylated during transcription[1], but with the notable absence of 2′-O-methylation.

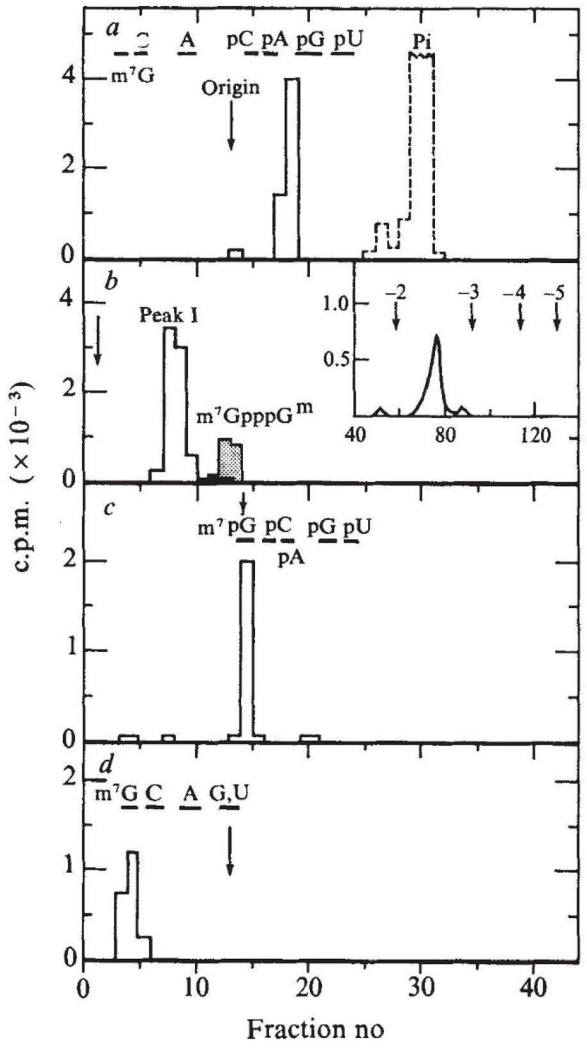

Fig. 1 Methylation of 5′ termini of reovirus mRNA by wheat germ extract. Reovirus mRNA (20 μg) synthesised in the presence of α-^{32}P-CTP and 10 μM SAH was incubated for 20 min in wheat germ cell-free protein synthesising extract containing methyl-^{3}H-SAM (2 μM, 7.3 Ci mmol^{-1})[3,5,6]. ^{32}P-labelled viral mRNA was recovered by phenol extraction and glycerol gradient centrifugation[3]. *a*, After treatment of the RNA with *Penicillium* nuclease followed by alkaline phosphatase, the digest was analysed by high voltage paper electrophoresis (pyridine acetate, *p*H 3.5, 2600 V, 40 min)[1]. *b*, The enzyme-resistant ^{3}H-labelled material in *a* was eluted and analysed by descending paper chromatography in isobutyric acid: 0.5N NH_4OH (10:6 v/v). Inset: an aliquot of peak I material was mixed with 20 A_{260} of a pancreatic RNase digest of yeast tRNA and applied to a 1×20 cm column of DEAE-cellulose equilibrated with 0.05 M Tris buffer, *p*H 8, containing 7 M urea. The column was eluted with a gradient of NaCl (0.0–0.3 M). Samples of 1 ml were counted in methyl-cellosolve and toluene-based scintillant[7]. The arrows represent the positions of the oligonucleotide markers as determined by absorbancy. *c*, Peak I material was digested with nucleotide pyrophosphatase and analysed by paper electrophoresis[1]. *d*, Peak I material was treated with a mixture of nucleotide pyrophosphatase and alkaline phosphatase and analysed by electrophoresis. The papers were dried, cut into 1 cm strips, and counted in toluene-based scintillation fluid[1].

To determine if VSV mRNA is also methylated at the 5′ terminus by wheat germ extract, ^{32}P-labelled viral mRNA was synthesised *in vitro* in the absence of SAM; in these conditions the 5′ termini of the transcription products are GpppA (ref. 8). The RNA was purified and incubated in the wheat germ cell-free system with methyl-^{3}H-SAM. After *Penicillium* nuclease plus alkaline phosphatase digestion of the repurified VSV mRNA, a single ^{3}H-labelled component which migrated toward the anode was obtained by paper electrophoresis (Fig. 2*a*). Paper chromatography of this presumptive 5′-terminal material again revealed that it migrated more slowly than marker m^{7}GpppA^{m}, the 5′-terminal structure of VSV mRNA methylated by the virion-associated enzyme[2] (Fig. 2*b*). Digestion with nucleotide pyrophosphatase and alkaline phosphatase converted all the ^{3}H-labelled material to m^{7}G and, as in reovirus mRNA, no 2′-O-methylated nucleosides were obtained (Fig. 2*c*). The structure of the m^{7}G was confirmed by paper chromatography. Thus, in the presence of SAM, reovirus and VSV mRNAs are methylated exclusively at the 5′ ends by wheat germ extract, yielding the 5′-terminal structures, m^{7}GpppG and m^{7}GpppA, respectively.

The translation of animal virus mRNAs in wheat germ extracts represents a heterologous system, and it was interesting to determine whether cell-free extracts from uninfected animal cells also contain viral mRNA methylating activity. Mouse L cells were used since they replicate reovirus, and cell-free extracts prepared from them have been shown to translate reovirus mRNA[9,10] and VSV mRNA (G. W. B., A. K. Banerjee, and A. J. S., unpublished results). An L cell protein synthesising S10 extract[10] was incubated with ^{32}P-labelled, unmethylated reovirus mRNA and methyl-^{3}H-SAM. The mRNA was methylated as shown by glycerol density gradient centrifugation, and the kinetics of methylation were similar to those observed with the wheat germ extract[5] (data not shown). The *Penicillium* nuclease plus alkaline phosphatase digestion products of the methylated mRNA isolated from the L cell extract were analysed by paper electrophoresis (Fig. 3*a*). The ^{3}H-labelled, presumptive 5′-terminal material migrated between pA and pG,

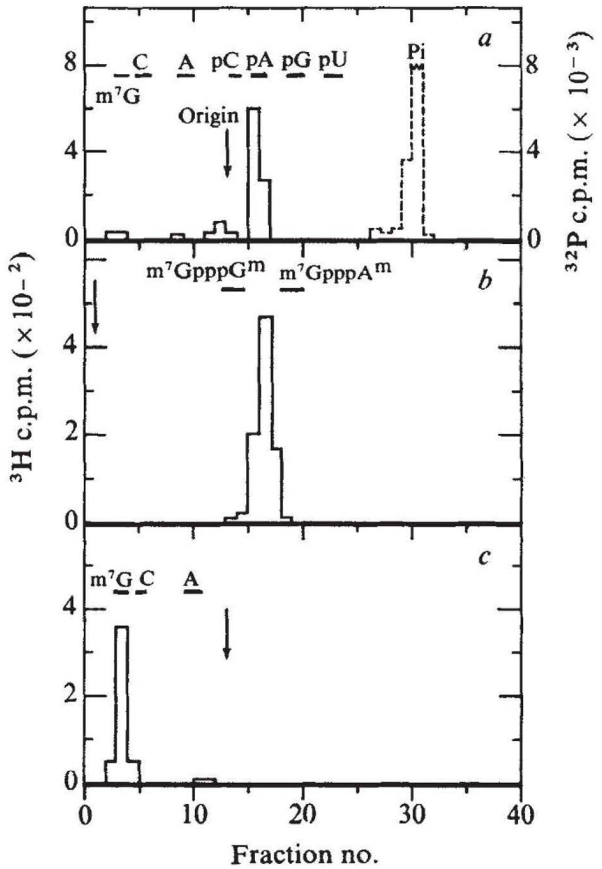

Fig. 2 Methylation of VSV mRNA 5′ termini by wheat germ extract. VSV mRNA (5 μg) synthesised *in vitro*[20] in the presence of α-^{32}P-GTP was incubated with methyl-^{3}H-SAM and analysed as in Fig. 1. *a*, Paper electrophoresis of *Penicillium* nuclease plus phosphatase digest; *b*, paper chromatography of enzyme-resistant, ^{3}H-labelled material from *a*; *c*, paper electrophoresis of nucleotide pyrophosphatase plus phosphatase digest of material in *b*. Marker compounds m^{7}GpppG^{m} and m^{7}GpppA^{m} were purified from *Penicillium* nuclease plus alkaline phosphatase digests of methylated mRNAs of reovirus[1] and cytoplasmic polyhedrosis virus[13], respectively.

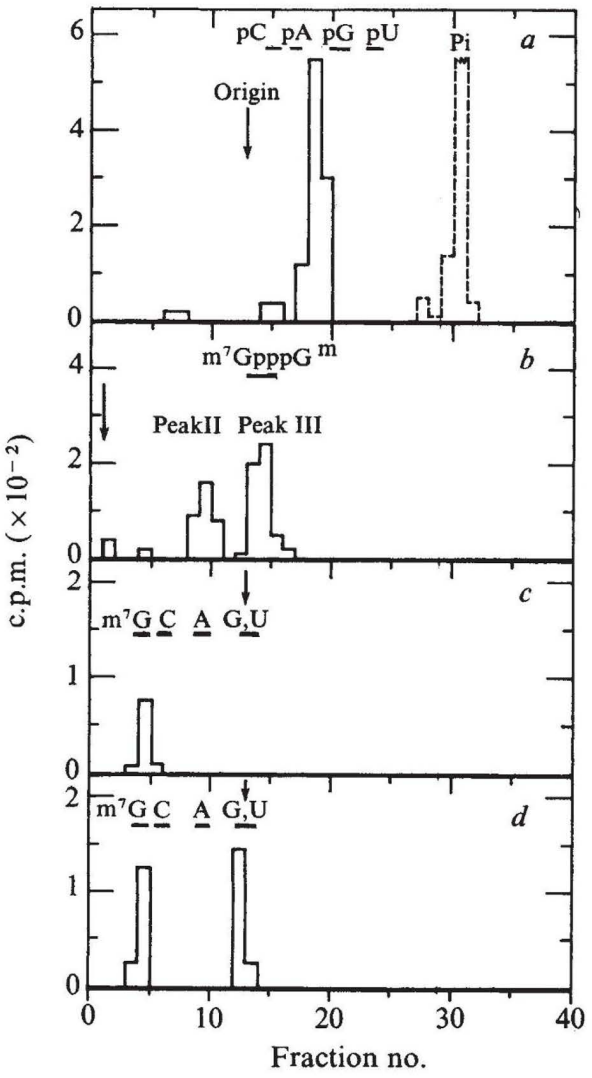

Fig. 3 Methylation of reovirus mRNA 5′ termini by L cell extract. Reovirus mRNA (10 μg) made in the presence of α-^{32}P-CTP and 10 μM SAH[3,6] was incubated under conditions of protein synthesis for 20 min in L cell extract[10] with methyl-^{3}H-SAM (3.4 μM, 7.3 Ci mmol^{-1}). The RNA was isolated and analysed as in Fig. 1. *a*, Paper electrophoresis of *Penicillium* nuclease and alkaline phosphatase digest; *b*, paper chromatography of enzyme-resistant, ^{3}H-labelled material in *a*; *c*, electrophoresis of peak II material after digestion with nucleotide pyrophosphatase and phosphatase; *d*, peak III material analysed as in *c*.

but by paper chromatography two components could be resolved (Fig. 3*b*). The faster component (peak III) migrated with m^{7}GpppG^m and the second component (peak II) migrated more slowly, as observed for m^{7}GpppG (peak I, Fig. 1*b*). Incubation with nucleotide pyrophosphatase plus alkaline phosphatase converted all the radioactivity in peak II to m^{7}G (Fig. 3*c*). By contrast, enzyme treatment of peak III yielded ^{3}H-labelled m^{7}G and an equal amount of radioactivity migrating in the position of G (Fig. 3*d*), which was identified as 2′-O-mG by paper chromatography. Therefore, cell-free extracts of both wheat germ and L cells contain enzyme activities that specifically methylate the N^7 position of the 5′-terminal guanosine. L cell extracts, however, can also methylate the 2′-OH of the 5′ penultimate guanosine in reovirus mRNAs.

Dependence of translation on 5′-m^{7}G

Translation of reovirus and VSV mRNA by wheat germ extracts has been shown to depend on methylation of the mRNA (ref. 5). From the results shown in Figs 1 and 2, it is likely that N^7 methylation of the 5′-terminal guanosine of viral mRNA is sufficient to satisfy the requirement for translation. Since the m^{7}G is in pyrophosphate linkage through its 5′-OH to the adjacent nucleotide[1], it contains free 2′, 3′-hydroxyl groups and can be removed after periodate oxidation by β elimination with aniline[7,11]. The effect of the removal of m^{7}G on reovirus mRNA activity was tested as follows. Reovirus mRNA was synthesised *in vitro* in the presence of SAM. It contained a mixture of molecules with 5′-terminal m^{7}GpppG^m (75%) and ppG (25%) (our unpublished results). After sequential treatment of the mRNA with periodate and aniline[7,11], the extent of removal of the 5′-terminal m^{7}G was 87% as determined with ^{3}H-methyl-labelled mRNA synthesised and treated in parallel. Since the translation of methylated reovirus mRNA is unaffected by SAH[5], the relative messenger activities of β-eliminated and untreated mRNAs were compared in wheat germ extracts containing SAH to prevent methylation of the 5′-terminal guanosine during protein synthesis. As Fig. 4 shows, β-elimination of premethylated mRNA resulted in the loss of about 85% of the mRNA activity compared with removal of most of the 5′-terminal m^{7}G. This loss of activity was not caused by chemical degradation of the RNA since the sedimentation profiles of the β-eliminated and untreated mRNAs in glycerol gradients were identical and included the l, m and s classes of mRNA[3].

Furthermore, the messenger activity of the β-eliminated RNA which consists of a mixture of molecules with 5′ termini of the types: (*a*) pppG^m (87%×75% = 66% of the total), (*b*) m^{7}GpppG^m (9%) and (*c*) ppG (25%), was partially restored when SAM was included in the wheat germ extracts (Fig. 4). The results indicate that chemical treatment did not irreversibly inactivate the mRNA. If, as suggested by these results, m^{7}G is

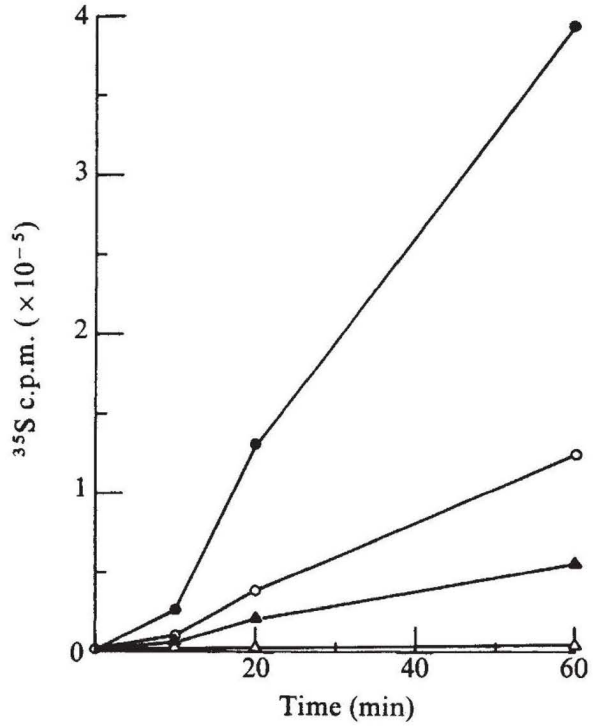

Fig. 4 Effect of removal of 5′-terminal m^{7}G on the ability of reovirus mRNA to stimulate protein synthesis. Methylated mRNA was synthesised *in vitro* with purified reovirus[3]. A sample of this RNA was oxidised with 10 mM potassium periodate, and the 5′-terminal m^{7}G was removed by incubation for 2 h at room temperature with 0.33 M aniline[11,12]. Equal amounts of untreated and treated RNA were used to programme polypeptide synthesis in wheat germ cell-free extracts as described[5]. Incorporation of ^{35}S-methionine into acid-precipitable material was determined. Untreated mRNA in the presence of 160 μM SAH (●); β-eliminated mRNA with 160 μM SAH (▲) or 2 μM SAM (○); and no added mRNA (△).

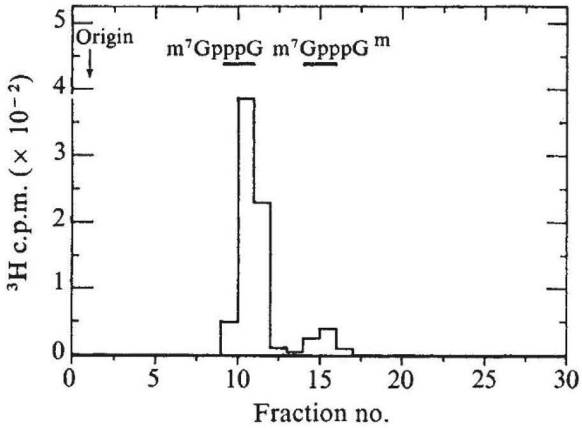

Fig. 5 5′-Terminal structure of β-eliminated reovirus mRNA after methylation by wheat germ extract. β-Eliminated reovirus mRNA was incubated for 20 min in conditions of protein synthesis in wheat germ extract with methyl-³H-SAM (2 μM, 7.3 Ci mmol⁻¹)[5]. The methylated RNA was isolated by phenol extraction and glycerol gradient centrifugation as in Fig. 1. After digestion with *Penicillium* nuclease and alkaline phosphatase, the enzyme-resistant ³H-labelled material was analysed by paper chromatography[1]. The marker compounds were purified by electrophoresis of *Penicillium* nuclease plus phosphatase digests of reovirus mRNA methylated during transcription by virions (m⁷GpppGᵐ) or during protein synthesis by wheat germ extract (m⁷GpppG).

essential for translation of viral mRNA, restoration of the messenger activity of β-eliminated mRNA should be accompanied by the formation of new 5′-terminal m⁷G. To test this possibility, treated mRNA was incubated with the wheat germ extract in the presence of ³H-methyl-SAM in conditions used for protein synthesis. Radioactivity was incorporated into the RNA, and analysis by the procedures described in Fig. 1*a* revealed that all the ³H was present in 5′-terminal structures. To determine which types of 5′ termini are substrates for methylation by the wheat germ extract, the ³H-labelled mRNA was isolated from the extract after protein synthesis, digested with *Penicillium* nuclease and alkaline phosphatase and analysed by paper chromatography (Fig. 5). About 95% of the radioactivity migrated with m⁷GpppG. The 5′-terminal material was further characterised as the monomethyl structure by nucleotide pyrophosphatase and phosphatase digestion followed by electrophoresis as in Fig. 3. The remaining radioactivity (5%) migrated in the position of m⁷GpppGᵐ, indicating that only a small fraction of the predominant type of mRNA molecules, that is those with 5′-terminal pppGᵐ, can be methylated and blocked by the wheat germ extract. The results demonstrate that the recovery of messenger activity of β-eliminated RNA is paralleled by the formation of new 5′ termini containing m⁷GpppG from molecules with 5′-terminal ppG. Since molecules containing 5′-terminal ppG comprise only one-fourth of the total mRNA, this finding is consistent with the incomplete restoration of the activity of β-eliminated mRNA.

5′-m⁷G in reticulocyte mRNA

Globin mRNA, like reovirus RNA[12], is resistant to 5′-terminal labelling by the polynucleotide kinase procedure (R. Williamson, unpublished) suggesting that this cellular mRNA has blocked 5′ ends similar to those found in viral mRNAs. To test for the presence of 5′-terminal m⁷G, rabbit reticulocyte mRNA was oxidised, reduced with ³H-borohydride and analysed as in Fig. 1*a*. In addition to the 3′-terminal adenosine (520 c.p.m.), a peak of radioactivity (350 c.p.m.) was obtained in the position of presumptive 5′ termini (data not shown). Digestion of this 5′-terminal material with nucleotide pyrophosphatase and phosphatase converted all the radioactivity to the trialcohol derivative of m⁷G (Fig. 6*a*). The results indicate that reticulocyte mRNA contains 5′-terminal m⁷G in 5′ pyrophosphate linkage, that is with free 2′, 3′-hydroxyls. 5′-Pyrophosphate structures of this type are sensitive to β-elimination as shown for reovirus RNA[7,12]. If 5′-terminal m⁷G is also required for translation of cellular mRNAs, its removal from reticulocyte mRNA by β-elimination should be accompanied by a loss of polypeptide synthesis in cell-free protein synthesising systems programmed by the treated mRNA. As Fig. 6*b* shows, after β-elimination, reticulocyte mRNA lost 80% of its capacity for stimulating protein synthesis. Treated and untreated mRNA coded almost entirely for a polypeptide which comigrated with globin during sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and its synthesis was reduced by80% following β-elimination of the mRNA.

A unique 5′-terminal structure of the type, m⁷G(5′)ppp(5′)Nᵐ has been found in various viral and cellular mRNAs including those of cytoplasmic polyhedrosis virus[13], reovirus[1,7], vaccinia[14,15], VSV[2], SV40[16], adenovirus (Y.F., A.J.S. and J. E. Darnell, unpublished), mouse L cells (Y.F., A.J. LaFiandra and A.J.S., unpublished results), monkey BSC-1 cells[16] and HeLa cells[17]. A common feature of these mRNAs is the presence of 5′terminal m⁷G. The nature of the adjacent 2′-O-methylated nucleotides apparently varies. In addition, a series of low molecular weight RNAs from Novikoff hepatoma

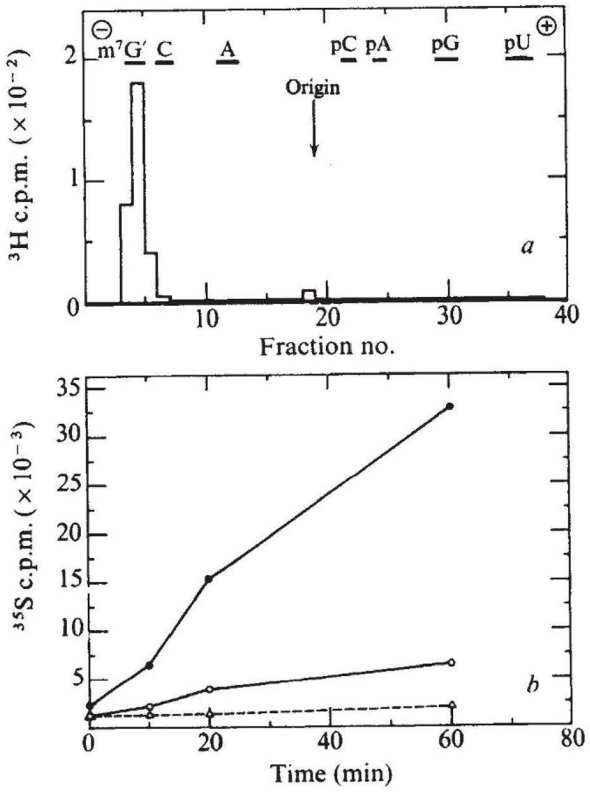

Fig. 6 5′-Terminal m⁷G in reticulocyte mRNA and loss of translational activity after removal of m⁷G by β-elimination. Reticulocyte mRNA was purified from cell lysates by phenol extraction and two rounds of binding to oligo(dT)-cellulose[21]. Aliquots of the eluted mRNA were used for labelling with ³H-borohydride and for translation before and after β-elimination. *a*, mRNA was oxidised with 10 mM potassium periodate, reduced with potassium borohydride-³H (10 mCi, 3.3 Ci mmol⁻¹), and digested with *Penicillium* nuclease and alkaline phosphatase. The presumptive 5′-terminal material was purified by paper electrophoresis, eluted, digested with nucleotide pyrophosphatase and phosphatase and analysed by paper electrophoresis. *b*, Equal amounts of mRNA (0.7 μg per 12.5 μl reaction mixture)[5] were used to stimulate protein synthesis in wheat germ extracts before and after β-elimination as in Fig. 4. Untreated mRNA in the presence of 160 μM SAH (●); β-eliminated mRNA in the presence of 160 μM SAH (○); no added mRNA (△).

cell nuclei have been found to contain 5′-terminal $m_3^{2,2,7}G(5')pp(5')N^mN^mN$, but their biological function has not been established[18]. Our results suggest that in addition to a possible role in mRNA processing[19], the 5′-terminal m^7G in mRNA is essential for translation. Unmethylated VSV and reovirus mRNAs, which are inactive in a wheat germ protein synthesising system in the presence of SAH, become active when methylated[5]. Methylation occurs specifically in the 5′-terminal guanosine at the N^7 position. Removal of the m^7G by β-elimination of methylated mRNA results in a concomitant loss in the ability to stimulate protein synthesis. The presence of a 5′-terminal 2′-O-methylated nucleotide is not required for mRNA functions since the β-eliminated mRNA which contains 5′-terminal $pppG^m$ is inactive and molecules containing m^7GpppG or m^7GpppA are active in protein synthesis. Reovirus mRNA synthesised in the presence of SAH contains 25% of the molecules with 5′-terminal GpppG (our unpublished results), and VSV mRNA made in the absence of SAM contains 5′-terminal GpppA (ref. 8). In each case, the mRNAs with blocked but unmethylated 5′ ends are inactive and become functional only after conversion to m^7GpppN. Reticulocyte RNA contains 5′-terminal m^7G and its removal by β-elimination results in the loss of its ability to stimulate polypeptide synthesis *in vitro*. It will be interesting to determine if 5′-terminal m^7G is a structural feature of all eukaryotic mRNAs that is required for a specific step in protein synthesis that is, ribosome binding.

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Novel initiation of RNA synthesis *in vitro* by vesicular stomatitis virus

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The mRNA synthesised in vitro *by the virion-associated RNA polymerase of vesicular stomatitis virus contains the novel 5′-terminal structure G(5′)ppp(5′)Ap....*

ALL messenger RNAs isolated from prokaryotes and from bacteriophages seem to be initiated by triphosphates at their 5′-termini[1–4]. In contrast, the 5′-terminal structures of natural eukaryotic mRNAs are believed to be different since they are resistant to phosphorylation by polynucleotide kinase after prior treatment with alkaline phosphatase[5]. The mRNAs synthesised *in vitro* by the RNA polymerases associated with the viral cores of several animal viruses including vaccinia virus[6], reovirus[7], vesicular stomatitis virus (VSV)[8] and silkworm cytoplasmic polyhedrosis virus (SCPV)[9], however, have either di- or triphosphates at their 5′-termini. Each of these viruses has been shown to possess an RNA methylase activity which transfers methyl groups from S-adenosyl-methionine (SAM) to the 5′-termini of mRNAs during their synthesis *in vitro*[10–13]. Furthermore, these methylated mRNAs all contain at their 5′-termini a blocked structure consisting of 7-methylguanosine linked by a pyrophosphate bridge to a 2′-O-methylated purine, having the general form $m^7G(5')ppp(5')Pup^m$... (refs 14–16). In the case of VSV the 5′-terminal structure is $m^7G(5')ppp(5')Ap^m$...[17].

During investigation of the mechanism of formation of this unusual structure, we discovered that in certain conditions, the virion-associated RNA polymerase of VSV synthesised RNA *in vitro* which terminated with a new type of structure, which was blocked but not methylated. This type of structure may be an intermediate in the formation of the 5′-termini of eukaryotic mRNAs.

Blocked 5′-terminal phosphates

The virion-associated RNA polymerase of VSV can be activated by non-ionic detergents to synthesise RNA products *in vitro* which are complementary to the genome RNA[18], contain poly(A) sequences at their 3′-termini[19] and are biologically active as messenger RNAs in a cell-free protein synthesising system[20]. Using Triton-disrupted VSV, we observed previously[12] that β,γ-^{32}P GTP was incorporated into the 5′-termini of the product RNA, but not β,γ-^{32}P ATP. This was unexpected since Roy and Bishop[8], using core particles derived from VSV by a polyethylene glycol-dextran phase separation procedure, found that most of the product RNA molecules contained 5′–pppA. VSV mRNA was synthesised *in vitro* using β,γ-^{32}P GTP as the labelled substrate, purified, and the 12-18S mRNA[21] was recovered as described in the legend for Fig. 1. After digestion with RNAase T_2 (which degrades RNA to 3′-nucleotides), the products were analysed by DEAE-cellulose chromatography as shown in Fig. 1. The major peak of radioactivity eluted at the position of tetranucleotides (−5 charge) and a minor peak (10–15%) at a slightly higher salt concentration. The presumptive 5′-terminal oligonucleotide contained in the major peak migrated to a position between the two mononucleotide markers, pG and pU, when analysed by paper electrophoresis (Fig. 2*a*). When digested with nuclease P_1 from *Penicillium citrinum*[22] (which degrades RNA to 5′-nucleotides) and alkaline phosphatase, all the ^{32}P-radioactivity was retained in a product