IN VITRO TRANSLATION OF MENGOVIRUS RNA DEPRIVED OF THE TERMINALLY-LINKED (CAPPING?) PROTEIN

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1. Introduction

The genomic RNA of picornaviruses serves directly as viral messenger RNA (mRNA) and can be efficiently translated 'in vitro' and 'in vivo'. However, there is no 'capping' group in the RNA isolated from purified picornavirions or from the viral polyribosomes ([1–3], unpublished observations), suggesting that host-cell ribosomes can still translate this mRNA in the absence of a 'capping' group. Moreover, the RNA of picornaviruses is far more efficient than host-cell mRNAs. Recent studies have shown that picornavirus RNA outcompetes cellular mRNAs [4–5], possibly because of a higher affinity for the initiation factors [6,7]. Surprisingly enough, the 'cap' analog m^7 GpppGm competitively inhibits the binding of the initiation factor eIF-2 to the 'uncapped' mengovirus RNA, a finding which leads to the proposal [6] of the 'three binding site' model of eIF-2. In the absence of a genuine 'capping' group, the high stability of the complex mengovirus RNA–initiation factor can be explained as a result of a strong interaction with some other structure(s).

Against this background, the reported presence of a protein, covalently-linked to the 5′-end of poliovirus RNA [7,8] seemed to offer a clue: it was tempting to speculate that the terminally-linked protein might functionally substitute for the missing 'capping' group.

The terminally-linked protein seems to 'cap' the 5′-end of the nascent poliovirus RNA chains, still attached to the template in the replicative intermediate [9,10].

It was difficult not to correlate the concomitant absence of a genuine 'capping' group and the presence of a terminally-linked protein at the 5′-end of the viral RNA, suggestive at first glance, of the possible involvement of the protein in a mechanism of translational regulation. Conceivably, the 'cap' protein might react with the initiation factors and secure the predominance of the viral over the cellular mRNAs. Otherwise, per se or by reacting with other viral peptides (primary translation products, for instance), the 'capping' protein might be able to introduce conformational modifications into the viral RNA and by this bias initiation might occur at different sites or at different rates.

However, the 'cap' protein was not found in the viral RNA extracted from viral polysomes [11,12] and it was suggested that the terminally-linked protein might actually interfere with translation: a cellular enzyme, in this case, should remove the 'cap' protein before the viral RNA could enter the initiation complex [12].

Nothing proved, however, that the opposite was not true. No experimental evidence was available to substantiate the notion that the terminally-linked protein actually interfered with the translation of the viral RNA: it was perfectly possible that the protein was even needed at a very early stage of initiation and was subsequently removed from the 5′-end.

Obviously, however, before any hypothesis could be elaborated about the possible involvement of this protein in the processes of assembly, translation or transcription, sound evidence had to be provided that we were facing a general feature, common to the

picornavirus group, and not a characteristic peculiar to poliovirus. Such a situation would be hardly surprising: it is perhaps pertinent to remind here that a long, extra-cistronic (spacer?) poly(C) fragment is present in the genomic RNA of cardioviruses and foot-and-mouth disease virus (FAMD), whereas polio- and enteroviruses lack this tract.

The studies reported in this paper were, therefore, intended to analyse the structure of the 5′-end of the genomic RNA of mengovirus in a search for a similar 'capping' protein and, were this the case, it seemed of obvious interest to investigate whether or not the RNA of a picornavirus deprived of its 'capping' protein could still function as a mRNA.

2. Materials and methods

2.1. *Mengovirus RNA*

A thrice plaque-purified stock of mengovirus was used throughout these studies. ^{32}P-Labelled RNA was extracted from purified mengovirions as in [13]: these buffers and conditions yielded in our hands far more intact, translatable RNA than other procedures tested. All manipulations involving the handling of the viral RNA were done with autoclaved plasticware instead of glassware according to [8].

2.2. *Pronase treatment of mengovirus RNA*

This was carried out in 85 mM NaCl; 10 mM EDTA; 0.75% SDS; 10 mM Tris·HCl (pH 8.0). Viral RNA and pronase (Calbiochem, Grade B, preincubated at 37°C for 3 h), were adjusted to final conc. 0.2 and 10 μg/ml, respectively. After incubation at 37°C for 40 min, extraction buffer (20 mM Tris·HCl (pH 7.5); 2 mM EDTA; 5 μg/ml polyvinyl sulfate) was added up to final vol. 500 μl and the RNA was immediately extracted 3 times with an equal amount of redistilled phenol as in [13].

2.3. *Conditions for enzymic digestions*

Cleavage of Mengovirus RNA with RNase T_1 was as in [14].

Complete takadiastase/pancreatic RNase A digestion: the RNA was dried under vacuum and redissolved in 10 μl 10 mM Na acetate; 1 mM EDTA (pH 4.5) containing 1 μg/μl phenol-purified, commercial carrier RNA. After heat-denaturation (100°C, 2 min, then quickly chilled in ice), 5 μl of a takadiastase extract mixture (Sanzyme R, Sankyo-Calbiochem) and pancreatic RNase A (20 and 1 μg/μl, respectively) in 1 mM EDTA were added and the sample was incubated at 39°C for 2–3 h.

Complete hydrolysis with nuclease P_1 (*Penicillium*, Calbiochem) was carried out in 25 mM Na acetate (pH 5.0) in the presence of carrier RNA to adjust the ratio of enzyme:substrate to 1:10.

Digestion with bacterial alkaline phosphatase was at 37°C in 10 mM Tris·HCl (pH 8.0) at 1 μg enzyme for 10 μg RNA. To inactivate the phosphatase, the sample was heated at 100°C for 5 min in the presence of EDTA (1 mM) as in [15].

Digestion with snake venom phosphodiesterase (SVP) was carried out in 10 mM Tris·HCl; 10 mM $MgCl_2$ (pH 8.0) at a ratio of 1 μg enzyme for 5 μg phosphatase-treated RNA (37°C, 30 min).

2.4. *Preparation of cell lysates*

Krebs tumour-ascites cells were propagated by intraperitoneal inoculation in female Swiss or Albino mice. Animals were sacrificed by ether anaesthesia, the peritoneal exudate was aspirated and immediately poured onto semi-frozen TK buffer (35 mM Tris·HCl; 140 mM KCl; pH 7.4). After 3 washes with ice-cold TK buffer, cell lysates were prepared as in [16] and stored in small aliquots under liquid air. Their A_{260} usually exceeded 30–40.

2.5. *Cell-free translation of Mengovirus RNA*

In vitro protein synthesis was carried out in final vol. 50 μl containing 35 μl cell lysate and amounts of the reagents to adjust their final concentrations as follows: 20 mM Hepes–KOH (pH 7.4); 100 mM KCl; 3 mM $MgCl_2$; 7 mM mercaptoethanol; 0.5 mM spermidine; 5 mM creatine phosphate; 130 μg/ml creatine phosphokinase; 1 mM ATP; 0.6 mM CTP; 0.25 mM GTP; 60 μM each of the 19 unlabelled amino acids; [^{35}S]methionine (Radiochemical Center, Amersham, 400–500 Ci/mmol) 5–10 μCi/50 μl reaction; unlabelled methionine up to 1.1 μM and 80 μg/ml mengovirus RNA. After 1 h incubation at 32°C, the reaction mixtures were treated 30 min at 37°C with 5 μg RNase A in the presence of 10 mM EDTA. For quantitative analysis, 3 μl samples were spotted on 3 MM paper discs, immersed in ice-cold 10% trichloroacetic acid (TCA) for 20 min, incubated at

90°C in 5% TCA for 25 min, washed thrice with ice-cold 5% TCA, rinsed with alcohol, alcohol–ether, dried and the radioactivity retained on the filters was counted by liquid scintillation. When ^{32}P-labelled mengovirus RNA was used as messenger, the dry filters were first counted as Cerenkov radiation to assess that no traces of residual ^{32}P-labelled RNA remained on the paper discs.

2.6. *Analysis of the in vitro synthesized proteins*

Samples containing the proper amount of radioactivity were first dried under vacuum, then redissolved in 10 μl denaturing buffer (100 mM Na phosphate buffer (pH 6.8); 1% SDS; 1% mercaptoethanol; 10% glycerol and 0.01% bromophenol blue). The samples were heated at 100°C for 3 min, and analyzed by 3–10% polyacrylamide slab-gel electrophoresis (PAGE) as in [13]. Bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome *c* (Schwarz-Mann) were run simultaneously as molecular weight markers. The gels were fixed in 20% sulfosalicylic acid, stained with Coomassie blue, destained, dehydrated with several changes of 55% methanol over 24 h, dried under vacuum and X-ray films were exposed for proper times. The areas of the films corresponding to each lane were excised and scanned with a Joyce-Loeb microdensitometer.

3. Results

3.1. *Search for a protein associated with mengovirus RNA*

^{32}P-Labelled mengovirus RNA was totally hydrolysed by alkali treatment (200 mM KOH; 37°C, 18 h) or digested to completion with a mixture of nucleases (takadiastase/RNase A) and the products were separated by high-voltage electrophoresis on 3 MM paper at pH 3.3, basically as in [9]. Under these conditions, besides the four nucleoside-3′-monophosphates (and a variable proportion of 2′,3′ cyclic monophosphates), a labelled group was always present with a net positive charge (fig.1, lane 1). Its mobility towards the cathodic buffer was 1.1-times that of Cp towards the anode. Treatment of the ^{32}P-labelled mengovirus RNA with pronase eliminated the abnormal spot (fig.1, lane 2), suggesting that the label was indeed associated with a protein. The association of the protein with the viral RNA withstood heat denaturation (100°C, 2 min, in 5 mM EDTA), or boiling in 1% SDS for 3 min (data not shown).

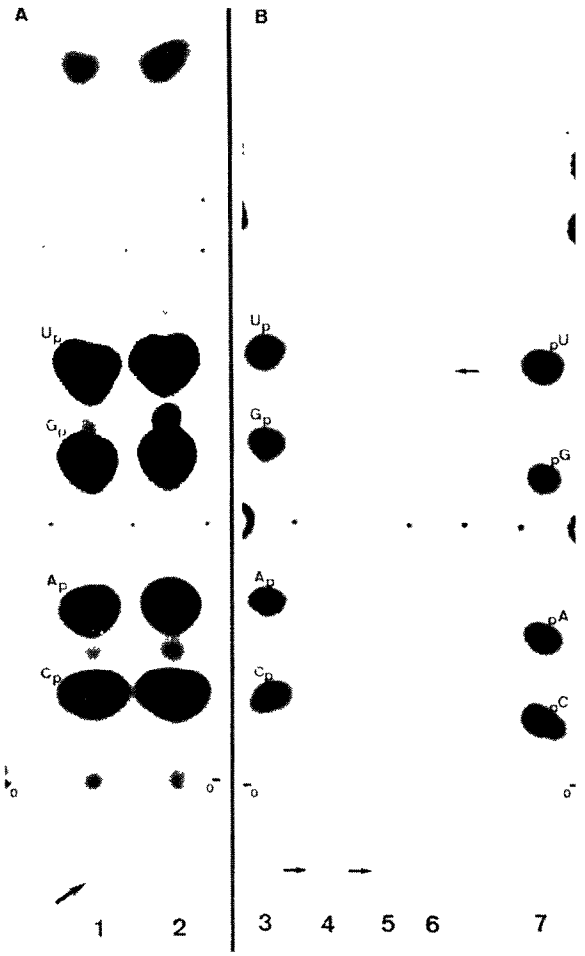

Fig.1A. ^{32}P-Labelled Mengovirus RNA digested with takadiastase/RNase A. Electrophoresis on 3 MM paper at pH 3.3 at 2000 V until the dye (xylene cyanol had moved 10 cm. Lane 1. control; lane 2: pronase-treated mengovirus RNA. (O) indicates the origin. The black arrow points to the 'backwards' moving spot; the white arrow to the orthophosphate. B. The 'backwards'-moving spot in 'A' was eluted and portions were re-run without further treatment (lane 4), after digestion with alkaline phosphatase (lane 5) or alkaline phosphatase and then SVP (lane 6). Lanes 3 and 7 are 3′- and 5′-mononucleotide markers, respectively.

3.2. *Identification of the protein-linked nucleotide*

The protein-associated radioactivity was eluted from the paper with triethyl ammonium carbonate (pH 9.8), dried under vacuum, washed and redissolved in 15 μl of 10 mM Tris·HCl (pH 8.0) containing 25 μg carrier RNA. One third of the sample was kept as untreated control (fig.1, lane 4). The remaining 2/3rds were digested with bacterial alkaline phosphatase (fig.1, lane 5) and SVP after phosphatase treatment (fig.1, lane 6).

After digestion with bacterial alkaline phospatase, 2/3rds of the radioactivity were liberated as orthophosphate and 1/3rd conserved the original mobility towards the cathodic buffer as the untreated sample (fig.1, lanes 4,5).

When the phosphatase-treated sample was further digested with SVP after inactivation of the phosphatase, all the radioactivity was found as orthophosphate and pU (fig.1, lane 6).

Since alkaline phosphatase liberated two phosphate residues, the following possibilities were to be considered:

(i) The protein-associated nucleotide was ppUp, with the protein protecting the phosphate in the 5′-α position;

(ii) Alternatively, the nucleotide could be of the form pUp and the extra phosphate might originate in the protein;

(iii) It was still possible that the 5′- and the 3′-phosphates of pUp could carry a different amount of radioactivity, as a result of an uneven incorporation of the [^{32}P]orthophosphate into the pool of nucleoside-5′-triphosphates, as pointed out [9].

To check whether or not the latter was the case, we determined the specific activity of the 5′-phosphate residues in the 4 constituent nucleotides. We found the same uneven incorporation of the ^{32}P label already reported for the RNA of poliovirus grown in HeLa cells [9]. The amount of ^{32}P in pU was 1.36-times the average incorporation, whereas pG was only 0.64 that value. Since pU was the 5′-mononucleotide with the highest specific activity, the 3′-phosphate residue of a pUp could never have been labelled to a higher extent than the 5′-phosphate.

We were unable to detect traces of the protein-associated radioactivity after digestion with nuclease P_1, a result ruling out the possibility of the protein being the source of the extra phosphate. At first glance, this result would also suggest that the protein-linked nucleotide was pUp. This, however, cannot explain the proportion 2:1 of orthophosphate: protein-pU observed after phosphatase treatment. The most likely possibility is that the association of the protein with the 5′-α-phosphate does not confer absolute protection against the enzyme. But for the time being, other hypotheses cannot be excluded.

Taken together, these results suggest that the protein-associated nucleotide is pUp and, very likely, this represents the 5′-terminus of mengovirus RNA.

3.3. *Relationship between the poly(C) tract and the terminally-linked protein*

The genomic RNA of Mengovirus contains a long extra-cistronic poly(C) tract of unknown function. We showed earlier that the poly(C)-containing, T_1-resistant oligonucleotide mapped very close to (if not at) the 5′-end of the viral RNA [14]. It was therefore of interest to check whether or not this T_1-oligonucleotide contained also the protein-linked 5′-end of the molecule. Accordingly, ^{32}P-labelled Mengovirus RNA was digested with RNase T_1 and the products were separated by polyacrylamide gel electrophoresis in the presence of 6 M urea at pH 3.5 as in [14]. Under these conditions the poly(C)-containing T_1-fragment migrated as a well-defined band (fig.2A) in the upper fifth of the gel and sometimes a second band (thought to be a split product of the first one) was visible, moving slightly ahead of the poly(C)-containing oligonucleotide. The two fragments were eluted from the gel, digested to completion with takadiastase/RNase A and the resulting mononucleotides were separated by electrophoresis on 3 MM paper at pH 3.3. As a positive control, full-length, ^{32}P-labelled mengovirus RNA containing in its own poly(C) tract the same amount of radioactivity as was eluted from the gels, was processed and electrophoresed in identical conditions. As expected, digestion of total ^{32}P-labelled mengovirus RNA liberated the protein-associated pUp, clearly visible in fig.2, lane 1. Not even traces of such a positively charged nucleotide originated in the two poly(C)-containing fragments. The obvious conclusion was that the Poly(C) tract does not contain the protein-linked pUp and, therefore, is not terminal.

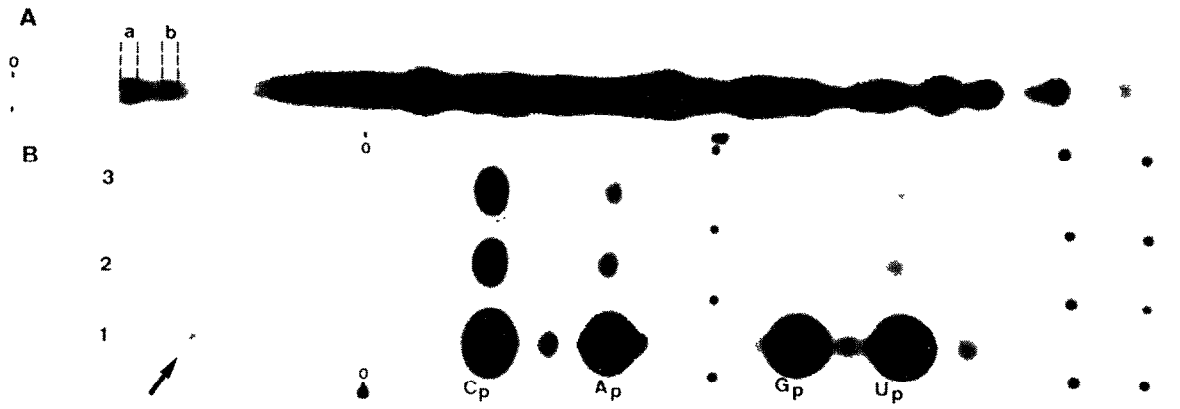

Fig.2A. ^{32}P-Labelled mengovirus RNA (2.5 × 10^6 Cerenkov cpm) was totally cleaved with RNase T_1 and the fragments were separated by PAGE. The poly(C)-containing oligonucleotide (a) and the secondary band (b) were located by autoradiography, and eluted from the gel as in [14]. B. The poly(C)-containing fragments eluted from band a (900 Cerenkov cpm, lane 2) and from band b (720 Cerenkov cpm, lane 3) were digested with takadiastase/RNase A and the products were resolved by paper electrophoresis at pH 3.3 as in fig.1. Lane 1: Total ^{32}P-Labelled mengovirus RNA (9 × 10^4 Cerenkov cpm), totally digested with takadiastase/RNase A and electrophoresed under the same conditions.

3.4. *In vitro translation of mengovirus RNA deprived of the 'capping' protein*

^{32}P-Labelled mengovirus RNA was digested with pronase as in section 2. Under these conditions, removal of the terminally-linked protein was complete. Digestion of the pronase-treated mengovirus RNA with takadiastase/RNase A and separation of the products by electrophoresis on 3 MM paper proved that no traces of the protein were left. The procedure was mild enough to yield intact RNA (identical sedimentation behaviour as the untreated control). Moreover, this treatment did not affect the translation ability of rabbit globin mRNA (data not shown).

When the RNA extracted from purified mengovirions was used to programme the 'in vitro' protein synthesis of a lysate of Krebs cells, incorporation of [^{35}S] methionine into the hot-acid precipitable fraction increased 8–15-times over the background of endogenous activity. In a freshly prepared lysate of Krebs cells, mengovirus RNA deprived of the 'capping' protein was as efficient a messenger as the untreated control. After 1 h incubation there was no significant difference in the amount of [^{35}S]methionine incorporated by the lysates programmed by mengovirus RNA with or lacking the 'capping' protein (table 1).

The kinetics of incorporation during the first 30 min was also the same, suggesting that the lysate was able to initiate protein synthesis in both RNAs with comparable efficiency. Apparently, the pronase-treated RNA had no advantage over the control.

3.5. *Analysis of the in vitro products*

While the presence or absence of the 'capping' protein seemed not to affect quantitatively the translation ability of mengovirus RNA, nothing proved

Table 1
Messenger RNA activity of mengovirus RNA deprived of its 'capping' protein

Messenger RNA added	[^{35}S]Methionine incorporated (cpm) after incubation at 32°C for	
	20 min	60 min
None	2277	3780
	2392	3784
Mengo RNA with	8715	29 616
'cap' protein	9009	32 154
Mengo RNA without	7330	27 554
'cap' protein	7678	32 061

Cell free protein synthesis was carried out as described in the text. After incubation for 20 and 60 min at 32°C, 2 μl samples (in duplicate) were spotted on 3 MM paper discs and processed as described

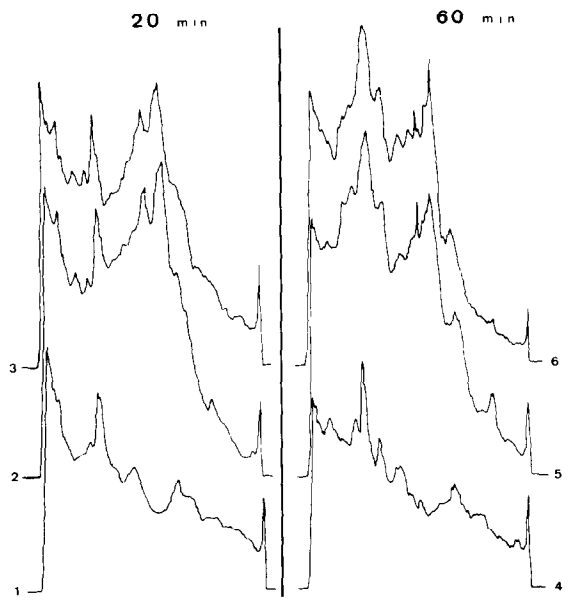

Fig.3. PAGE of the [^{35}S]methionine-labelled peptides synthesized 'in vitro' by lysates programmed by Mengovirus RNA with (traces 2,5) or without (tracings 3 and 6) the terminally-linked protein. The residual activity of the lysate is shown in the lower tracings (1,4). Migration is from left to right.

that the proteins synthesized in both cases were the same. Accordingly, we analyzed the [^{35}S]methionine-labelled proteins synthesized 'in vitro' by the cell lysates programmed by mengovirus RNA with or without its 'capping' protein. The 'early' and 'late' products (20 and 60 min incubation, respectively) were denatured under reducing conditions and the peptides were separated by PAGE.

Mengovirus RNA deprived of its 'capping' protein directed the synthesis of the same set of [^{35}S]-methionine-labelled peptides as did the untreated control (fig.3). No difference could be observed in the electrophoretic mobility of the 'early' products, suggesting that the initiation of translation was not modified, but obviously more work is needed to substantiate this assumption.

4. Discussion

A group of methylated nucleotides 'caps' the 5'-end of almost all mRNAs active in eukaryotes [17]. The genomic RNA of picornaviruses constitutes an important exception, as it serves as viral messenger, although lacking the 'capping' group. This seems not to impair its translation, which is even more efficient than that of the 'capped' host-cell mRNAs. Conceivably, other structures might substitute for the missing 'capping' group at the 5'-end of the RNA molecule. The possible involvement of this area of the genome in the process of initiation of protein synthesis further increases the interest in a detailed knowledge of its structure.

As already reported for two other members of the picornavirus group, namely poliovirus [8,9] and FAMD virus [18], a protein was found covalently-linked to the genomic RNA of mengovirus, of the cardiovirus subgroup. After complete alkali hydrolysis or total digestion of ^{32}P-labelled mengovirus RNA with takadiastase/RNase A, high-voltage paper electrophoresis at pH 3.3 separated the 4 constituent 3'-monophosphate nucleosides and a labelled compound with a 'backwards' mobility.

Oligonucleotides with a net positive charge at this pH are well known, the best example being the CCA_{OH} terminal of tRNAs [15]. The positively-charged compound of mengovirus RNA, however, disappeared upon pronase treatment, an indication that a protein was indeed responsible for its abnormal position in the electrophoretogramme. Attempts to modify the mobility of the abnormal spot by boiling the ^{32}P-labelled mengovirus RNA in 5 mM EDTA or in the presence of 1% SDS were unsuccessful. We had to conclude that (as already proved for poliovirus) the pronase-sensitive material was linked to the genomic RNA of mengovirus by a true chemical bond. Available evidence seems to point to a tyrosine residue being involved in a link of the form protein – tyr–O–pU [19]. Any model, however, must take into account two properties of the bond: its sensitivity to nuclease P_1 and the partial protection conferred to the phosphate in the 5'-α-position.

The function(s) of the terminally-linked protein are not known. Its location, however, at the place of the missing 'capping' group pointed at its possible involvement in the process of translation. Since the protein is absent in the mRNA isolated from viral polysomes [11,12], it has been postulated that the host-cell must remove the 'capping' protein before

translation initiates. Thus the 'capping' protein would introduce a sort of negative control over translation. However, the absence of the 'capping' protein in the polysomal mRNA per se did not prove that removal of the 'cap' occurred prior (rather than after) initiation took place.

Two kinds of experiments could be designed to clarify this issue: In the first type one could protect the linkage between the viral RNA and its associated protein. Since the chemical modifications needed to 'fix' the protein are likely to introduce breaks in the RNA moiety, the meaning of a negative result would be difficult to evaluate.

In our view, the opposite approach looked far more promising. Accordingly, we tried to determine whether or not mengovirus RNA deprived of its terminally-linked protein would still keep its ability to direct the 'in vitro' protein synthesis in a cell-free system.

As reported here, a freshly prepared lysate of Krebs cells was able to translate mengovirus RNA lacking its 'capping' protein as efficiently as the untreated control. Since in our 'in vitro' system the viral mRNA was present in sub-saturating amounts, this result cannot be ascribed to traces of protein-linked RNA that escaped the pronase–phenol treatment.

The rate of incorporation of [^{35}S]methionine during the first 30 min was the same, suggesting that initiation was not affected. The analysis by PAGE of the 'in vitro' products further supported the idea that the lysate of Krebs cells translated the added mengovirus RNA, with or lacking the 'capping' protein, in the same form.

We had to conclude that if the absence of the 'capping' protein constituted a defficiency, the fresh lysate of Krebs cells was able to make up for it.

Obviously, from the opposite standpoint it can be said that the lysate was able to cleave-off the 'capping' protein of the untreated RNA so efficiently as to abolish any possible difference in the translation ability of the two messengers. While this view is not in contrast with the experimental evidence presented here, we would like to emphasize on the first aspect: the 'capping' protein was not needed to initiate translation.

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