IN VITRO TRANSLATION OF SEMLIKI FOREST VIRUS 42 S RNA

Initiation at two different sites

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1. Introduction

The genome of α-viruses like Semliki Forest virus (SFV) and Sindbis virus consists of a single-stranded RNA molecule of M_r 4–4.5 × 10^6 [1–3]. The genes coding for the non-structural proteins are located near the 5′ (capped) terminus of this 42 S RNA, whereas the information for the structural proteins, one capsid and three envelope proteins, is found near the 3′ terminus [4–6].

Although the 42 S SFV genome contains both the non-structural and structural protein genes, the structural proteins appear to be translated not from 42 S mRNA but from a second viral mRNA of 26 S [4,7,8]. This 26 S RNA is transcribed independently from the 5′ third of the 42 S minus strand in infected cells [9].

The non-structural proteins encoded by the 42 S mRNA, as well as the structural proteins translated from the 26 S RNA, appear to be derived from large precursor molecules by post-translational cleavage [10]. Therefore, like most (if not all) eukaryotic mRNAs the 42 S viral mRNA seems to be monocistronic.

However, studies with cell-free systems to which the 42 S but no 26 S mRNA was added, suggest the existence on 42 S RNA of a second initiation site, which acts as a starting point for the synthesis of structural proteins. In such systems more than one initiating dipeptide [11] and simultaneous production of both structural and non-structural proteins were demonstrated [12–14]. In the latter case, however, the possibility of post-translational cleavage of a large percursor, initiated at the 5′ terminal site was not excluded [12–14].

Here we present further evidence for a two site model for initiation of protein synthesis on 42 S RNA in cell-free systems: we show that when 42 S RNA is added to cell-free fractionated systems derived from rabbit reticulocytes, 'non-structural' as well as structural proteins are produced at a cap-analogue sensitive and a cap-analogue insensitive site, respectively.

It is important to mention the observation that with excess 42 S RNA, initiation of protein synthesis occurs predominantly at the cap-analogue insensitive site, suggesting different affinities of the two sites for one or more of the components of the initiating machinery.

2. Materials and methods

Wild-type SFV was grown on mouse neuroblastoma cells in minimal essential medium supplemented with 3% fetal calf serum. The SFV suspension was concentrated 3 times in an Amicon hollow fiber concentrator, type DC2A. The virus was sedimented in a SW27 rotor for 2 h at 27 000 rev./min, resuspended in BS buffer (0.12 M NaCl, 0.05 M Na_3BO_3, pH 9.0) and further purified on a 20–50% sucrose gradient in the same buffer in a SW 50.1 rotor for 2 h at 50 000 rev./min. The virus-containing gradient fractions were supplemented with lithiumdodecylsulfate and 2-mercaptoethanol to final conc. 0.5% (w/v) and 5% (v/v), respectively. The 42 S RNA was deproteinized

twice with a phenol/chloroform mixture (1:1), precipitated with ethanol and dissolved in H_2O at 1 mg/ml.

Protein synthesis in a fractionated system, derived from reticulocyte lysates was determined as in [15], as modified [16]. A typical 25 μl reaction mixture contained: 20 mM Hepes–KOH (pH 7.6), 1 mM ATP, 0.4 mM GTP, 5 mM creatine phosphate, 1 mM dithiothreitol, 0.05 unit creatine kinase, 2 mM Mg-acetate, 120 mM K-acetate, 100 μM spermine, 0.3 μM [^{35}S]-methionine (600 Ci/mmol), 50 μM each of 19 unlabeled amino acids, 0.1 and 0.3 A_{260} unit of washed 40 S and 60 S ribosomal subunits, respectively, 0.1 A_{260} unit tRNA, 10 μg crude initiation factors, fraction A (see [15]), 12.5 μg fraction B, 7.5 μg fraction C and amounts of SFV RNA as indicated. [^{35}S]-Methionine incorporation was measured after 30 min incubation at 37°C by hot trichloroacetic acid precipitation. Analysis of the polypeptides was performed on 12.5% polyacrylamide gels by the method in [17]. After staining, destaining and drying the gels were subjected to autoradiography [18].

[^{35}S]Methionine and m^{7}GpppGm were obtained from NEN and PL Biochemicals, respectively. Rabbit antiserum was kindly provided by Dr B. A. M. v. d. Zeyst, the SFV structural proteins were immunoprecipitated with this antiserum [19].

As shown in fig.1 the antiserum is virtually monospecific for the capsid protein (C). The figure demonstrates that only the viral structural proteins, present in SFV-infected BHK-cells, are precipitated by the antiserum (fig.1, lanes 3,4), whereas no precipitate was formed in uninfected cells (fig.1, lanes 1,2).

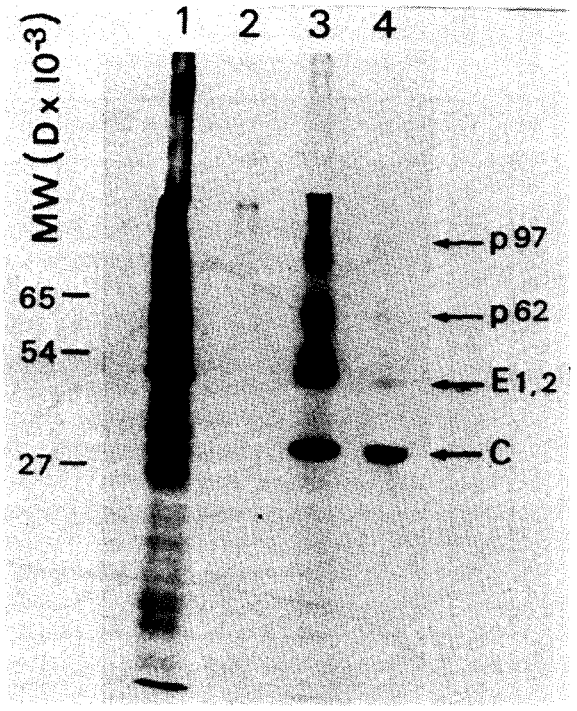

Fig.1. Analysis of proteins labeled in SFV-infected BHK cells. About 10^6 cells were infected with SFV (m.o.i. 50 p.f.u./cell) and labeled at 5–7 h post infection with 20 μCi [^{35}S]methionine (600 Ci/mmol). Cells were lysed in 150 μl Buffer (20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 10 mM NaCl, 0.5% Triton-X100 and 0.5% 1,5-naphtalene disulfonate) and the labeled proteins were subjected to immunoprecipitation and SDS–gel electrophoresis as in section 2. (1) Proteins from mock-infected cells (450 000 cpm); (2) proteins from 1 precipitable with anti-SFV serum (28 000 cpm); (3) proteins from SFV-infected cells (195 000 cpm); (4) proteins from 3 precipitable with anti-SFV serum (71 000 cpm). The gels were dried and subjected to autoradiography for 1 h. The arrows in the figure mark the position of the viral (precursor) proteins (cf [4]).

3. Results and discussion

3.1. *Protein synthesis under direction of 42 S SFV RNA and the effect of cap-analogue*

Addition of 42 S SFV mRNA to a fractionated system for protein synthesis (see section 2) resulted in a 20-fold stimulation of amino acid incorporation over a background obtained in the absence of added SFV RNA. A number of distinct, high molecular weight polypeptides was produced (see fig.2,3). In these systems, protein synthesis under the direction of capped mRNAs, such as 42 S SFV RNA, is inhibited by the addition of cap-analogues like m^{7}GpppGm and m^{7}GpppAm [20–22]. However, little effect of these analogues is observed with non-capped messengers [23].

Therefore it seemed of interest to determine whether the addition of such analogues has any effect on the synthesis of proteins directed by 42 S RNA. Differences in cap-analogue sensitivity might enable us to distinguish between 5′ terminal and internal sites. The results of such an experiment are depicted in fig.2, which shows the effect of m^{7}GpppGm on protein synthesis in the presence of varying amounts

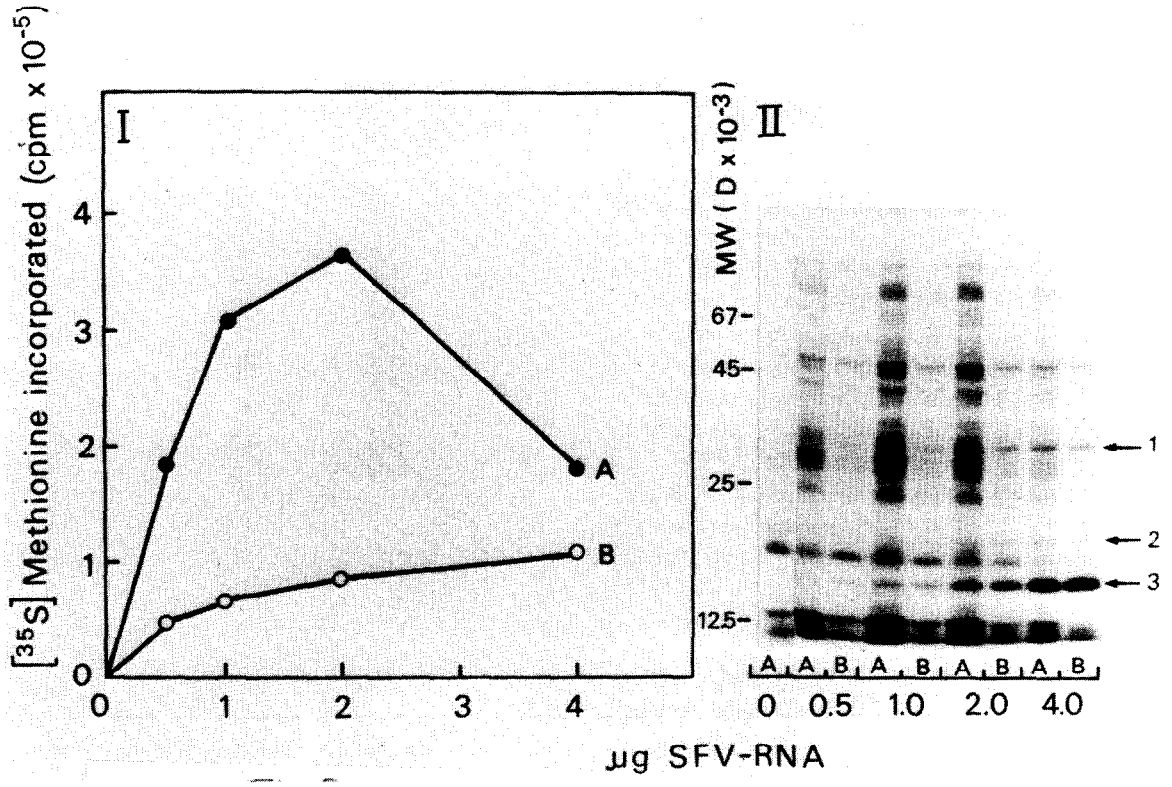

Fig.2. Effect of varying amounts of 42 S SFV RNA on 'in vitro' translation both in the absence and presence of m^{7}GpppGm. (II) Total incorporation (in 25 µl mixtures) of [^{35}S]methionine into proteins in the absence (•-•, A) and presence (○-○, B) of 100 µM m^{7}GpppGm added to cell-free systems as in section 2. (II) Product analysis on a 12.5% SDS gel (autoradiography for 18 h) of expt (I). Lane (A): proteins synthesized in the absence of 100 µM m^7 GpppGm. Lane (B): proteins synthesized in its presence. The arrows (1,2,3) indicate the position to which three cap-analogue insensitive polypeptides migrate (M_r 33 000, 21 000 and 16 000). The faint bands of M_r 50 000 and 18 000 represent proteins synthesized in the system in the absence of added mRNA.

of 42 S mRNA. It is clear that at all RNA concentrations tested, a strong inhibition by m^{7}GpppGm was observed (~85%), particularly at low mRNA concentrations (< 2 µg). The effect with higher amounts of 42 S mRNA, however, was much less severe (~40% inhibition with 4 µg RNA) since high concentrations of mRNA strongly suppress protein synthesis in the absence of cap-analogue, but have little effect in its presence (fig.2, curve A+B). Analysis of the products synthesized under the different conditions is shown in fig.2 (II).

Two interesting observations can be made:

1. The synthesis of the majority of the products is inhibited by m^{7}GpppGm, which is apparent when lanes A and B are compared. However, three polypeptides of M_r 33 000, 21 000 and 16 000 (marked 1,2 and 3, respectively) are produced without a significant effect by m^{7}GpppGm. The sum of amino acid incorporation into these three products essentially accounts for the cap-analogue insensitive protein synthesis as represented by curve B of fig.2 (I).
2. The synthesis of the cap-analogue insensitive polypeptides 1, 2 and 3 is not affected by high levels of SFV mRNA, in contrast to the synthesis of all cap-analogue sensitive products.

These results suggest that 42 S SFV RNA contains two initiation sites: a 5′ terminal, cap-analogue sensitive site, which is preferentially recognized at a low mRNA input and an internal, cap-analogue insensitive site, predominantly utilized when excess amounts of mRNA are added to the assay systems. Other exam-

ples of preferential initiation, i.e., one species of mRNA out-competing another mRNA, have been reported [24–26]. The molecular mechanism of such mRNA selection is not fully understood, although a different dependence of mRNAs for limiting amounts of initiation factors such as eIF-4A [24] or eIF-4B [25,26] has been postulated (see also [27]).

3.2. *Immunological characterization of translational products*

In order to determine whether the capsid protein is among the products synthesized under the different conditions, anti-SFV serum (see section 2) was added to the reaction mixtures and the amount of anti-SFV-precipitable products was determined as a function of the 42 S RNA concentration. The results of such an analysis are shown in fig.3 (I) curve A, B. It is clear that at low concentration of 42 S mRNA (< 1 μg) very little antibody-precipitable protein is made (compare curves A and B of fig.3 (I)). However, with large amounts of mRNA a considerable portion (40%) of the synthesized protein was precipitated by the antibody. Gel analysis of the products revealed that proteins 1, 2 and 3 were almost quantitatively precipitated by the antiserum (fig.3 (II) lane A versus B). This result suggests that these three proteins, which were initiated at a cap-analogue insensitive, 'high affinity' site (see fig.2) contain antigenic determinants of the capsid protein. Indeed the molecular weight of product 1 is in good agreement with the molecular

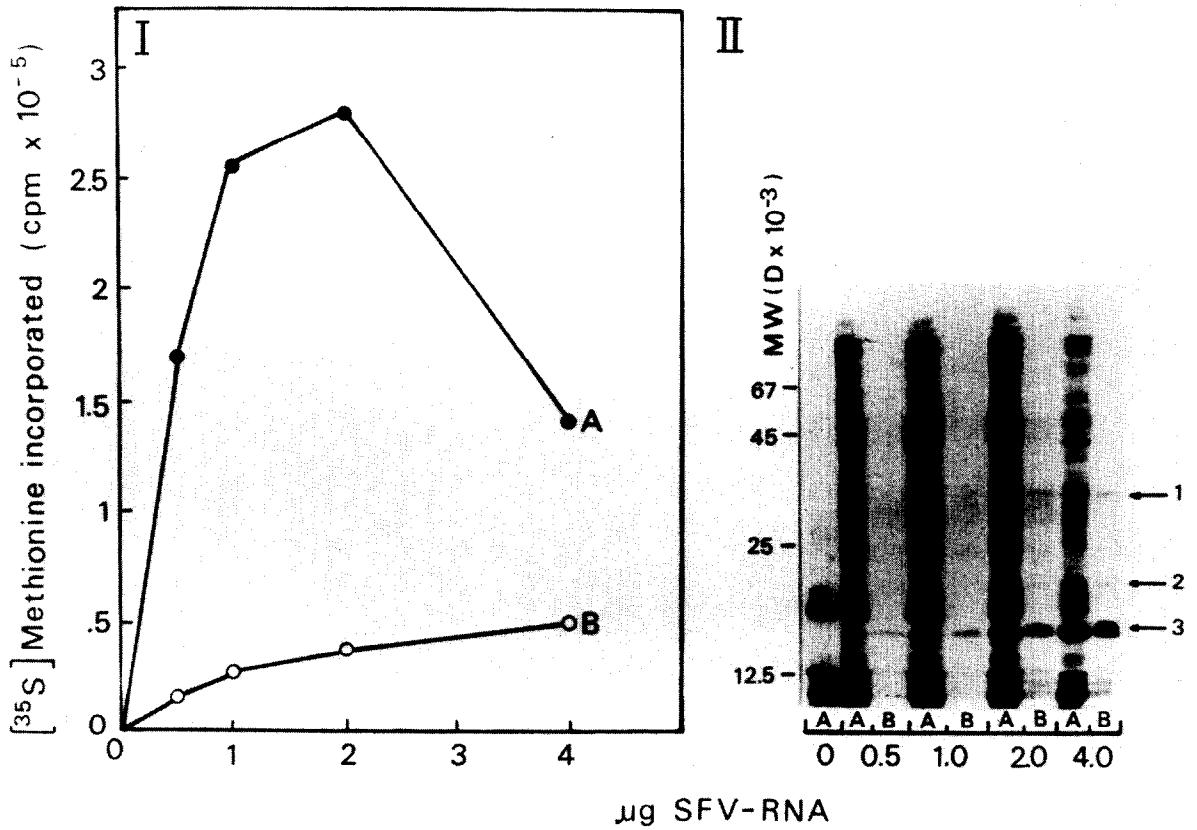

Fig.3. Immunological characterization of translational products. (I, •-•, A) Total incorporation (in 25 μl mixtures) of [^{35}S]methionine into proteins as a function of the concentration of 42 S RNA as in the legend to fig.2 (I). (I, o-o, B) Total amounts of radioactivity incorporated into anti-SFV serum-precipitable material under these conditions. (II) Product analysis on a 12.5% SDS gel (autoradiography for 70 h) of expt (I) (see fig.1,2 and section 2). The arrows indicate the position to which three anti-SFV serum-precipitable polypeptides migrate (M_r 33 000, 21 000 and 16 000).

weight reported for the capsid protein [28,29]. Furthermore, partial proteolytic digestion according to [30] of capsid protein and product 1 by *Staphylococcus aureus* protease (V8) yielded identical fragments, whereas products 2 and 3 appeared to be N-terminal fragments (results not shown). These results indicate that the internal, cap-analogue insensitive site (fig.2) is the starting point for the synthesis of structural proteins, whereas the non-structural proteins are initiated at the 5′ terminal, cap-analogue sensitive site. The extent of the production of structural polypeptides depends on the 42 S RNA concentration used (fig.2,3), a fact which might explain some of the conflicting results reported [12–14, 31–34].

3.3. *Does the internal site originate from RNase action?*

The possibility remains that the expression of the internal site is triggered by RNase action on 42 S SFV RNA, although it seems more likely that such an event would result in spurious initiation at many cryptic sites on the mRNA rather than the selective production of structural proteins [35]. To check this a pulse experiment was carried out in which only those products were labeled which were synthesized during the first 1.5 min incubation, both with low (1 μg) and high (4 μg) amounts of 42 S RNA. The results are shown in fig.4. It is clear that the % of antiserum-precipitable products, labeled during the first 1.5 min, when the mRNA is still virtually intact, is essentially the same as found in the experiment of fig.3, in which the products were labeled for the full 30 min, during which considerable mRNA fragmentation occurs, i.e., ~10% of structural proteins at low versus ~40% of structural proteins at high 42 S RNA concentration. It is also obvious from this figure that

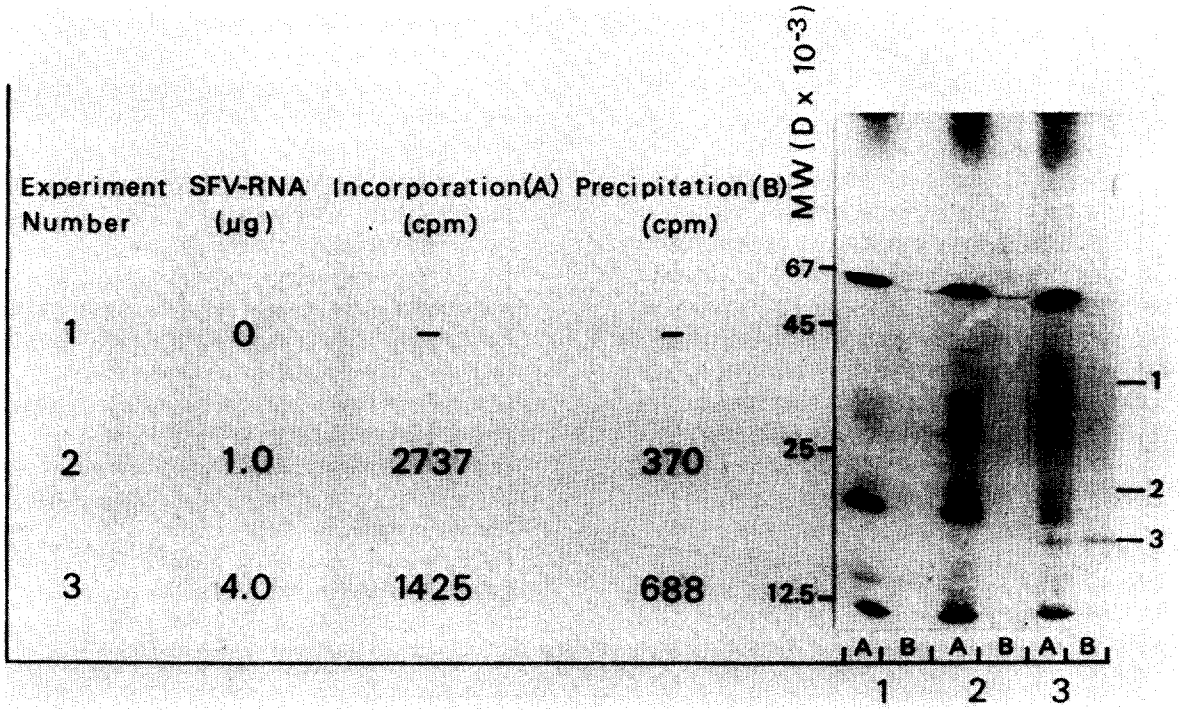

Fig.4. Pulse-chase experiment at low and high 42 S RNA concentration. Proteins were labeled from 0–1.5 min with 50 μCi [^{35}S]-methionine (600 Ci/mmol) in assay conditions for protein synthesis as in section 2, after which the incubation was continued for 5 min with excess non-radioactive methionine (16 mM). Total incorporation of radioactivity into translational products (A) and immunoprecipitable polypeptides (B) was measured as in section 2. Amounts of radioactivity obtained in the absence of mRNA were subtracted. The bands of M_r ~50 000 and ~18 000 are very rapidly labeled as compared with the labeling kinetics of the virus-specific proteins. For that reason the intensities of these bands exceed those of the viral proteins after short incubation times.

product 3, the smalllest N-terminal part of the capsid protein, is the first protein to be labeled. Furthermore, addition of SFV mRNA to a protein synthesizing system derived from wheat-germ [36], in which the mRNA remains undegraded for $\geqslant 15$ min, also results in the synthesis of products 1,2 and 3 in the same relative amounts as observed in the rabbit reticulocyte system (results not shown). These experiments strongly, although not conclusively, argue against the possibility of the second site being potentiated by a random RNase action.

3.4. *Conclusion*

We conclude that 42 S RNA contains two sites for initiation of protein synthesis:

(1) A 5′ terminal cap-analogue sensitive site which acts as a starting point for non-structural proteins
(2) An internal, cap-analogue insensitive site at which the synthesis of the structural proteins (such as the capsid protein) is initiated.

The relative frequency of initiation at either site is determined by the amount of SFV RNA present (see fig.2,3).

It is attractive to make the link to the 'in vivo' situation, where during the early phase of viral infection (with only small amounts of 42 S RNA present) predominantly non-structural proteins are formed. On the other hand, during the later stages of infection the synthesis of these products is gradually suppressed in favour of the structural products [37,38]. This inhibition of non-structural protein synthesis is not exclusively caused by the encapsidation of 42 S mRNA, because the nucleocapsid-negative mutant ts 3 (incapable of encapsidating 42 S RNA) does not synthesize non-structural proteins in the late stage (high 42 S RNA concentration) of infection [39]. The model postulated here would provide an alternative explanation for the occurrence of 26 S RNA late in infection: Preferential initiation at the internal site and subsequent translation of the structural genes would protect the 3′ (26 S) part of the viral messenger against nuclease action, whereas the non-translated 5′ part would be susceptible to degradation.

The precise molecular mechanism of such a preferential initiation remains to be elucidated. Limiting amounts of one or more initiation factors, like eIF-4A, eIF-4B and the cap-binding factor [40] might be one of the principal keys in the regulation. The elegant model of mRNA recognition, proposed [41,42], may be applicable to SFV RNA as far as initiation with all initiation factors is concerned. Alteration or lack of one of the factors might be a clue to open a new regulatory device in order to ensure the synthesis of structural proteins late in infection [43].

The results presented here bear some resemblance to the data obtained with Carnation Mottle virus RNA [44] and with Cowpea Mosaic virus RNA [45] in in vitro translation experiments, whereas in vivo experiments with Kunjin virus also indicated that its viral RNA may exhibit internal initiation sites [46].

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