INITIATION OF PROTEIN SYNTHESIS IN NEUROBLASTOMA CELLS INFECTED BY SEMLIKI FOREST VIRUS

A decreased requirement of late viral mRNA for eIF-4B and cap binding protein

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1. Introduction

Semliki Forest Virus causes shut-off of protein synthesis in its mammalian hosts [1,2]. Usually, this shut-off takes place 3–5 h post-infection [2]. In neuroblastoma cells from mouse such an event leads to the exclusive production of late viral (= structural) proteins (e.g., p97, p62, E_1 and capsid protein, see [2–4]). These proteins are synthesized as a large precursor from a sub-genomic template of 26 S [1,5] an mRNA which appears late in infection and is identical to the 3′-terminal part (~1/3rd) of the viral genomic RNA of 42 S [6,7]. This 42 S mRNA serves as a template early in infection for the synthesis of non-structural proteins [2,4]. The genes for these proteins are located at the 5′-terminus of this messenger, whereas the genes for the structural proteins, which are found at the 3′-part of 42 S are silent (see above, [2]). At a late stage of infection, 26 S mRNA is the only messenger recognized by the protein synthesizing machinery, despite of the fact that still considerable amounts of 42 S and host mRNAs are present in the infected cell [1,2,8].

High salt washes of ribosomes (containing the initiation factors of protein synthesis), isolated from neuroblastoma cells at a late stage of infection by SFV, have mostly lost the ability to support the translation in 'in vitro' systems of host- and early viral mRNA, although they are still highly active with viral late- and EMC mRNA [9].

Addition to systems programmed with host- and viral early mRNA, of purified initiation factors eIF-4B (M_r 80 000, [9,10]) and cap binding protein (CBP, M_r 24 000, [9,11]), but none of the others significantly increases the activity of these infection-exposed crude initiation factors.

These findings indicate that the lesion in the protein synthesizing machinery has occurred at the level of initiation factors eIF-4B and CBP, although the precise mechanism by which these factors are incapacitated remains, at present, unclear.

Here we substantiate the ability of purified eIF-4B and CBP to stimulate the activity of infection-exposed crude factors in protein synthesis in an 'in vitro' system programmed with early viral mRNA. We explore why the SFV-induced blockade of eIF-4B and CBP activity results in a preferential recognition of late SFV mRNA by the protein synthesizing machinery in infected cells. We show that optimal translation of 26 S mRNA requires a 2–4-times smaller amount of eIF-4B and CBP when compared to host- and 42 S mRNA. Such a low requirement for eIF-4B and CBP results in a relatively undisturbed translation of viral late mRNA in infected cells in spite of the fact that these factors are gradually inactivated. The reason for the decreased need for eIF-4B and CBP, factors which are thought to be involved in the recognition of the 5′-terminal cap structure of mRNAs [12–14] is not based on a decreased importance of the cap-structure of 26 S mRNA, since chemically decapped 26 S RNA almost completely looses its capacity to serve as a template in pH 5 systems.

2. Materials and methods

2.1. *Materials*

The following components were prepared by the

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methods in [9,10,15–17]: pH 5 enzymes, crude initiation factors from control- and SFV-infected neuroblastoma cells, ribosomal subunits from rat liver, 42 S and 26 S SFV RNS, neuroblastoma poly(A) RNA (host mRNA) and purified initiation factors from rabbit reticulocytes. EMC virus was grown on neuroblastoma cells; purification of the virions was obtained using standard techniques, which have been described for SFV isolation [15]. The EMC RNA was freed from proteins by extraction of the virions with a phenol/chloroform mixture (1:1) in 0.5% lithiumdodecylsulphate, thereafter the RNA was precipitated with ethanol and dissolved in H_2O at 2 mg/ml.

2.2. *Assays*

2.2.1. pH 5 sytem

Protein synthesis was determined in reconstituted pH 5 systems of 10 μl with crude initiation factors of protein synthesis (8 μg fraction A and 12 μg fraction BC, see [9,18]) from SFV-infected or control neuroblastoma cells. The composition of this translation assay system has been described in [9]. Incorporation of [^{35}S]methionine into protein was measured as described after 45 min incubation at 37°C in 5 μl aliquots [9].

2.2.2. Restoring assay

As shown in [9] crude initiation factors of protein synthesis from SFV-infected neuroblastoma cells are unable to support protein synthesis with SFV 42 S mRNA as a template, unless supplemented with eIF-4B and CBP. In this assay we measured the ability of various amounts of eIF-4B and CBP as indicated to stimulate protein synthesis with infection-exposed crude initiation factors and 42 S SFV mRNA as a template. Incorporation of [^{35}S]methionine into protein was measured as in section 2.2.1.

2.2.3. eIF-4B and CBP assay

This assay was used to determine the requirement for eIF-4B and CBP to obtain optimal translation in a pH 5 system [9,18]. The incubation mixture of 10 μl contained: HEPES/KOH, ATP, GTP, creatine phosphate, dithiothreitol, creatine kinase, ribosomes, tRNA and pH 5 enzymes as in [9]. Further additions were: 12 μg fraction BC [18] of non-infected neuroblastoma cells, mRNA as indicated in fig.2 and 1.4 μg high-salt treated eIF-3 (12) and, for CBP assay 2 μg eIF-4B and, for eIF-4B assay 2 μg CBP. Incorporation of [^{35}S]methionine was measured as above.

2.3. *Quantification of the 5′ cap structure on late polysomal 26 S mRNA and viral 42 S mRNA*

2.3.1. Labeling of mRNAs

Mouse neuroblastoma cells were grown and infected with wild-type SFV as in [15]. At 1 h post-infection the inoculum (25 ml) was replaced by 50 ml phosphate-free minimal essential medium, supplemented with 3% fetal calf serum and 1 μg actinomycin D/ml. At 3 h post-infection 10 mCi carrier-free [^{32}P]orthophosphate was added. For the labeling of polysomal mRNA 100 μg cycloheximide/ml was added to the medium at 8 h post-infection. After 15 min the cells were washed twice with phosphate-buffered saline (supplemented with 1 μg cyclohexiymide/ml. Cell lysis and further purification of late polysomal 26 S mRNA has been described in [9].

For the preparation of [^{32}P] viral 42 S mRNA labeling was continued up to 18 h post-infection. 42 S RNA was isolated from the virions as in [15]. 26 S and 42 S mRNA were precipitated twice with ethanol and the total [^{32}P]orthophosphate incorporation was determined by counting the Cerenkov radiation.

2.3.2. Quantification of the 5′ cap-structures of 26 S and 42 S mRNA

The ^{32}P-labeled mRNAs were incubated with RNase T_1 (10 μg), RNase T_2 (3 U) and RNase A (20 μg) in 10 mM Tris–HCl (pH 7.5) and 1 mM EDTA for 2 h at 37°C. The products of digestion were separated on a standard two-dimensional system in [19]. The location of the nucleotide monophosphates and the 'cap' was determined by autoradiography and the absolute amounts of the cap-product were measured by cutting out the spot and counting the Cerenkov radiation.

2.4. *Decapping of mRNAs*

Late polysomal 26 S and viral 42 S mRNA were chemically decapped as in [20]. Under these conditions no internal breaks are introduced in the mRNAs as was determined on agarose gels under denaturating conditions. Of the 5′ terminal cap >80% is removed following section 2.3 (not shown). As control messengers we subjected to the same decapping procedure globin 9 S mRNA and EMC mRNAs which does not contain a 2′,3′-dihydroxyribose at its 5′-end and therefore should not be affected by the procedure.

3. Results and discussion

3.1. *Restoring the activity of infection-exposed initiation factors*

Crude initiation factors of protein synthesis, isolated from Semliki Forest virus (SFV)-infected neuroblastoma cells show a pronounced loss of activity (⩽90%) when tested in pH 5 systems with host- or early SFV mRNA (42 S) as a template (see Introduction in [9]). Protein synthesis with late SFV (26 S)- or EMC mRNA, however proceeds relatively undisturbed in the presence of this fraction [9]. In a preliminary experiment, we showed that purified initiation factors eIF-4B and CBP from rabbit reticulocytes stimulated the translation of 42 S and host-mRNA with infection exposed washes [9]. The dependence of eIF-4B and CBP of the translation of viral early 42 S RNA by infection-exposed crude initiation factors in pH 5 systems (restoring assay) is shown in fig.1. It is clear that eIF-4B alone (fig.1, left and right panel) is able to restore the activity of these washes ⩽70% as compared to control washes. Addition of saturating amounts of CBP restores ~50% of the translational capacity of the infection-exposed washes, in which case addition of eIF-4B results in further stimulation ⩽90%.

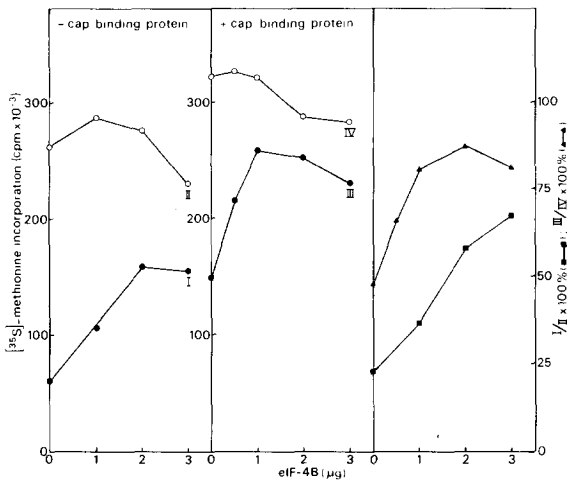

Fig.1. Restoring the activity of infection-exposed initiation factors. Amounts of eIF-4B (as indicated) were added to pH 5 systems (10 μl) with crude initiation factors from control (o—o) and SFV-infected (•—•) neuroblastoma cells and 42 S mRNA (1 μg) as a template, both in the absence (left) and presence (middle) of 2 μg CBP. The relative restoring activity as compared to control incubations (II and IV, = 100%) of eIF-4B (■—■) and eIF-4B plus CBP (▲—▲) is shown on the right. [35S]Methionine incorporation into protein was measured in 5 μl aliquots.

These results are consistent with previous findings and indicate that eIF-4B and CBP cooperate in restoring the activity of SFV-exposed ribosomal washes. It should be pointed out that these factors, although similar in action, are different entities which is demonstrated by the fact that large amounts of CBP do not stimulate in an assay system for eIF-4B (or vice versa; see [9]).

3.2. *Requirement for eIF-4B and CBP of different mRNAs in pH 5 systems*

The observation that the translation of late SFV and EMC mRNA was not affected by the transition from control to viral exposed initiation factors together with the results of fig.1 evoked the important question whether the utilization as a template of these mRNAs is eIF-4B and CPB-independent. Therefore, we set up an experiment in which we compared the requirement for eIF-4B and CBP to achieve optimal translation with host-, EMC-, viral early (42 S)- and late (26 S) mRNA (fig.2). It is striking that optimal protein synthesis with

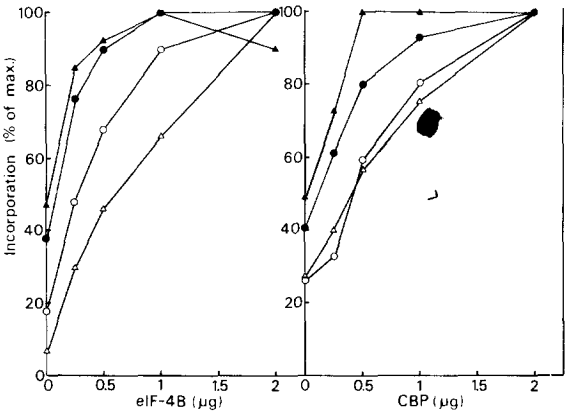

Fig.2. Determination of eIF-4B and CBP requirement to obtain optimal translation of different mRNAs. Increasing amounts of eIF-4B (left) and CBP (right) as indicated in the figure were added to the assay systems for eIF-4B and CBP (see section 2) to determine at which quantity of each factor added, optimal translation of different mRNAs is obtained. The assay systems were programmed with: 0.8 μg EMC-RNA (▲—▲); 1 μg 26 S RNA (•—•); 1 μg 42 S RNA (△—△) or 0.9 μg host mRNA (o—o). Percentages of incorporation relative to the value obtained at maximal translation of each mRNA are shown as a function of initiation factor added. The maximal incorporation of [35S]methionine into protein (in cpm) in the eIF-4B assay are: 86 000; 50 000; 54 500; 41 000 in the case of added 42 S, EMC-, 26 S or host mRNA, respectively. In the CBP assay these values are: 181 000; 52 000; 54 000; 125 000.

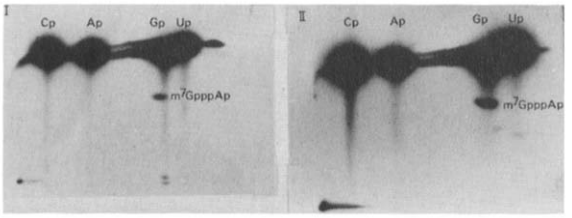

Fig.3. Determination of the molar ratio of the cap structure and 26 S or 42 S mRNA. The molar ratio of cap and mRNA was determined (top) by counting the total radioactivity incorporated in the mRNAs (see section 2) and in the cap containing spots originated from 42 S mRNA (bottom (I)) and 26 S mRNA (bottom (II)). The total digests were separated on a two-dimensional system, [19] and the position of each product was located by autoradiography (see bottom).

Quantification of the cap structure of 42 S and 26 S mRNA.

mRNA	Total ^{32}P inc. (cpm)	Total ^{32}P in 'cap' (cpm)	Ratio (cap/mRNA)
I. 42 S	392 000	125	1.0
II. 26 S	559 200	384	1.0

Table 1

The effect of chemical decapping on the translation of different mRNAs in pH 5 systems

mRNA	Incorporation (cpm X 10^{-3})		
	Non-treated (A)	Treated (B)	B/A X100%
26 S	105	20	19
42 S	172	115	67
Globin 9 S	123	91	74
EMC	67	75	112

EMC-, 26 S, 42 S and globin 9 S mRNA were subjected to a decapping procedure [20] and the effect on the ability to act as a template in pH 5 systems (10 μl) was determined (B). The non-treated mRNAs were translated in control pH 5 systems (A). The systems were programmed with 1 μg globin 9 S or with amounts of the other mRNAs, as indicated in the legend to fig.2. [^{35}S]Methionine incorporation into protein was determined in 5 μl aliquots as in [9].

SFV early and host mRNA requires 2–4-times the amount of eIF-4B than translation of SFV-late and EMC mRNA (see fig.2, left).

A similar observation was obtained in the determination of the CBP requirement for optimal translation with the different mRNAs (fig.2, right). In this assay smaller amounts of CBP are needed with late SFV and EMC mRNA than with 42 S early- and host mRNA. As a control experiment we determined the requirement in protein synthesis for other initiation factors such as eIF-2 and eIF-3. We observed an equal need for these factors with different mRNAs (not shown).

It is clear, therefore, that the translation of viral late mRNA requires a considerably smaller amount of eIF-4B and CBP than that of host and viral early mRNA. This observation provides an explanation for the fact that infection-exposed washes in which CBP and eIF-4B are partially inactivated are still fully capable of supporting translation of late SFV mRNA.

3.3. *26 S polysomal mRNA is fully capped*

CBP and eIF-4B are initiation factors which are thought to be involved in the recognition of the 5′-terminal cap-structure present on most eukaryotic mRNAs [12–14]. Although EMC RNA is uncapped and carries covalently linked to its 5′-end a protein which is not involved in protein synthesis [21], this mRNA also needs a small amount of eIF-4B and CBP for optimal translation (fig.2, [13]).

26 S late mRNA is almost equally dependent on eIF-4B and CBP for its translation as EMC-RNA, suggesting that the 26 S mRNA is uncapped also. Nevertheless, cellular 26 S RNA is capped, but a precise quantification is lacking [22]. In view of these observations it seemed of interest to determine to what extent actively translated 26 S mRNA, present in polysomes late in infection, is capped. Therefore, we purified both 26 S and 42 S mRNA (uniformly labeled with [^{32}P]orthophosphate) from SFV-infected neuroblastoma cells and SFV virions, respectively (section 2). RNase digestion of the messengers and 2-dimensional separation of the originated nucleotides revealed after autoradiography 5 major spots (fig.3). Four of these spots comigrate with the four 2′(3′)-nucleotide monophosphates. The material in the remaining spot was eluted and counted, thereafter it was digested by alkaline-phosphatase, resulting in the production of P_i and a product comigrating with the optical marker m^7GpppA_{OH} (not shown). These results stongly indicate that the material from this last spot originates from the 5′-terminal cap structure of both 26 S and 42 S mRNA. In order to calculate the molar ratio of cap structure and 26 S or 42 S mRNA, we counted the total radioactivity present in the starting material and in the 'cap' containing spot. The results are summarized in fig.3. We find that both polysomal 26 S and viral 42 S mRNA are fully capped (see section 3.4).

3.4. *Translation of 26 S mRNA strictly depends on the presence of the 5'-terminal cap structure*

Since 26 S RNA is capped (fig.3), but displays a low need for cap recognizing factors (fig.2) the question arises whether its cap-structure is important for its recognition by the translational machinery. In order to provide an answer we have compared in pH 5 systems the activity of chemically decapped 26 S mRNA to that of untreated 26 S mRNA. The results are given in table 1: it is obvious that decapping of 26 S RNA significantly reduces its ability to serve as a messenger in pH 5 systems. The activity of the EMC mRNA is not affected by the decapping procedure, indicating that no degradation of mRNA occurs. The decapping of other capped mRNAs such as 9 S globin- and 42 S early SFV mRNA also resulted in an (albeit less pronounced) decrease of messenger activity in the pH 5 systems.

This result indicates, that protein synthesis with 26 S mRNA strongly depends on the presence of an intact 5'-terminal cap structure.

The reason why 26 S RNA requires small amounts of eIF-4B and CBP remains at present obscure. It is possible that this messenger has some extraordinary structural features which make it an mRNA easily recognizable by the translational machinery even with low amounts of eIF-4B and CBP (see fig.2).

The relatively high dependence on the cap structure for efficient translation of 26 S RNA (table 1), as compared to other capped mRNAs, indicates that these extraordinary structural features are present in the 'cap'. Indeed, polymethylated cap structures on 26 S RNA of the closely related Sindbis virus have been described [23].

Further characterization of the cap structure of 26 S RNA of SFV and unraveling of the action of eIF-4B and CBP, might be a clue to a better understanding at the molecular level of the processes occurring in neuroblastoma cells after infection with SFV.

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