Review Letter

INITIATION OF EUKARYOTIC PROTEIN SYNTHESIS

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1. Introduction

The initiation of protein synthesis is defined as the sequence of events, which leads to an 80 S · Met-tRNA · mRNA complex. Several non-ribosomal proteins are required for the formation of this 80 S initiation complex and they are called eIF-1, -2, etc. (eukaryotic initiation factor). They are listed in table 1, with the effect they have on protein synthesis and on partial reactions thereof, as described below.

An initiation factor is defined as a protein which stimulates one or more of these, and only these reactions, and which is released after the completion of an 80 S initiation complex, in contrast with a ribosomal protein, which remains an integral part of the ribosome during all stages of protein synthesis.

Dissociation of 80 S ribosomes is a prerequisite for the initiation of eukaryotic protein synthesis, since the initial binding of Met-tRNA occurs on a 40 S subunit and not on an 80 S ribosome. Spontaneous

Table 1
Biological and physical properties of the eukaryotic initiation factors

	Dissociation of 80 S ribosomes	Met-tRNA binding to 40 S	mRNA binding to 40 S	Joining	Met–puro formation	Affinity for mRNA	Protein synthesis in pH 5 system	$M_r \times 10^{-3}$
eIF-1	–	+	+	+	–	n.d.	+	12
eIF-2	–	+	+	+	+	+	+	122
eIF-3	+	+	+	+	+	+	+	600
eIF-4A	–	–	+	+	–	–	+	50
eIF-4B	–	–	+	+	–	+	+	80
eIF-4C	+	+	+	+	+	+	+	17.5
eIF-4D	–	–	–	–	+	–	–	16
eIF-4E	–	–	+	+	–	+	±	24
eIF-5	–	–	–	+	+	+	+	125–160

Dissociation of 80 S ribosomes was measured by incubating 80 S ribosomes with the protein factors to be studied. The mixture was analyzed on a sucrose gradient; the dissociation level was calculated as in [6]

Met-tRNA and mRNA binding to 40 S subunits was measured by incubating 80 S ribosomes, initiation factors minus eIF-5 to prevent joining to 80 S initiation complexes, with Met-tRNA and mRNA. When mRNA binding was studied ^{125}I-labelled mRNA was added, in assays for Met-tRNA binding [^{3}H]Met-tRNA was included. Analysis was performed on sucrose gradients [6]

The joining reaction: Met-tRNA and mRNA binding to 80 S initiation complexes was studied by adding eIF-5 to assay mixtures for 40 S initiation complex formation as above [6].

Methionyl–puromycin formation was performed as above for 'the joining reaction', except that puromycin was added. After the reaction the amount of methionyl–puromycin formed was determined by extraction with ethylacetate

Affinity for mRNA: See text for details and references

Protein synthesis in a pH 5 system: The incubation mixture contained ribosomes, mRNA, tRNA, elongation and termination factors, synthetases, amino acids and initiation factors. Protein synthesis was measured by estimating the amount of hot trichloroacetic acid-precipitable material [7]

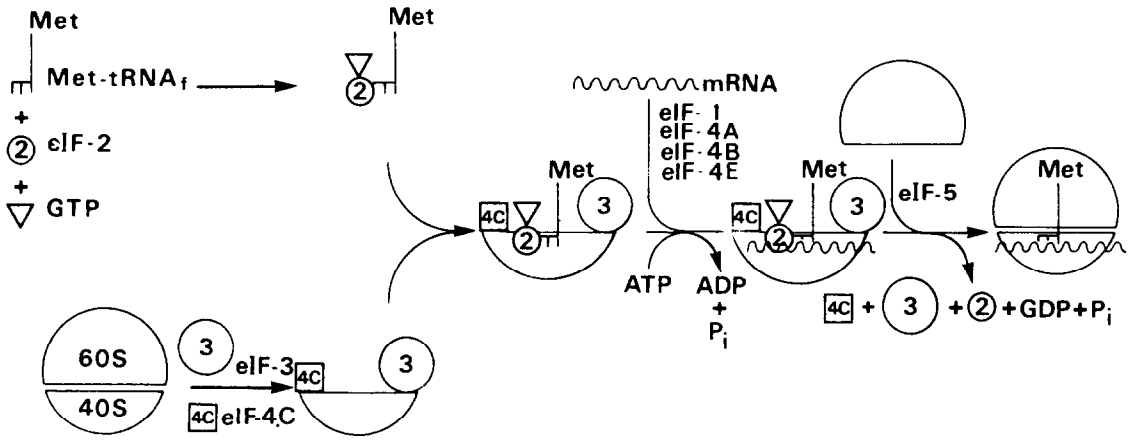

Fig.1. Initiation of protein synthesis. For details see the text. This illustration was kindly provided by Dr J. W. B. Hershey (Davis CA): ②, eIF-2; ③, eIF-3; 4C, eIF-4C; (▽) GTP; (^{Met}) Met-tRNA$_f$.

dissociation of 80 S ribosomes creates a pool of 40 S subunits, which are prevented to associate with 60 S subunits by the binding of eIF-3 and presumably eIF-4C (see table 1 and fig.1). This 40 S · eIF-3 · eIF-4C particle binds the first aminoacyl-tRNA, the initiator tRNA, which requires next to GTP the initiation factor eIF-2.

eIF-1, eIF-4A, eIF-4B and eIF-4E are involved in coupling mRNA to the 40 S · Met-tRNA complex. ATP is hydrolyzed during this reaction. The coupling of the resulting 40 S initiation complex with a 60 S subunit is mediated by eIF-5, leaving an 80 S initiation complex, devoid of all initiation factors (see definition).

Fig.1 shows a schematic representation of the initiation of protein synthesis, together with the role of each initiation factor (see also table 1). Each step will be discussed separately in the forthcoming sections.

2. Dissociation of 80 S ribosomes

As stated above, initiation of protein synthesis can only occur via the dissociation of 80 S ribosomes into 40 S and 60 S subunits [1,2]. The level of dissociation in vitro of 80 S ribosomes is dependent on the salt concentrations [3]: the ionic conditions of the cell favour the association of the ribosomal subunits. Therefore, proteins are required to shift the equilibrium towards dissociation. Two initiation factors, eIF-3 and eIF-4C are able to dissociate 80 S ribosomes [4–6]. The only initiation factor reported to bind to a free 40 S subunit which impairs the dissociation with a 60 S subunit, is eIF-3 (table 1, fig.1) [4,5]. eIF-4C acts probably in the same way, but stable binding to a 40 S subunit needs additional components as will be discussed later [6]. Neither factor binds to 80 S ribosomes.

On the basis of these findings, eIF-3 and eIF-4C are thus called anti-association factors instead of dissociation factors.

The 40 S · eIF-3 · eIF-4C particle provides a target for the binding of other components.

3. eIF-2

eIF-2 has a native M_r = 122 000; it consists of 3 subunits (α,β,γ) with M_r 32 000, 35 000 and 55 000, respectively [9–13]. The molecular mass of the β-subunit has been a point of controversy due to the procedures used.

Barrieux and Rosenfeld have described a procedure to separate the 3 subunits and have tried to ascribe a specific function to each subunit [14,15]. The α-subunit seems to be involved in GTP-binding, the β-subunit in recognizing the Met-tRNA$_f^{Met}$ and mRNA while no function for the γ-subunit could be found. Hershey et al. [16] have tried to substantiate the role of the γ-subunit, knowing that different eIF-2 preparations contain different amounts of this subunit. Although several assays were performed each

eIF-2 preparation was equally active. Even eIF-2 without the γ-subunit was indistinguishable from other preparations.

Recent reports claim [17–19] that eIF-2 only contains two subunits (the α and β). Therefore, the γ-subunit may not be essential for the prominent biological function of eIF-2 (i.e., binding of Met-tRNA) and be only present as a result of the purification procedures used.

4. Ternary complex formation

eIF-2 forms a ternary complex with GTP and $Met\text{-}tRNA_f^{Met}$ [8] (fig.1). No other aminoacyl-tRNA can replace $Met\text{-}tRNA_f^{Met}$ [20]. The ternary complex formation is very rapid and does not show an extensive dependence on $[Mg^{2+}]$ [10,21]. Complex formation can be analyzed on gel-filtration columns [20], as the ternary complex is rather stable (as compared with IF-2 · GTP · fMet-tRNA in *Escherichia coli* [22]). GTP can be replaced by non-hydrolyzable analogues such as GDPCP, but not by other nucleotide tri-phosphates [10]. However, it was suggested that eIF-2 was able to form a binary complex with $Met\text{-}tRNA_f^{Met}$ [20,21]. It remains to be established whether this was due to GTP contamination of the Met-tRNA used or to partly denatured preparations.

It is generally accepted that the components in the ternary complex are present in stoichiometric amounts [10,12,20,21].

5. Binding of Met-$tRNA_f$ to the 40 S subunit

The ternary complex binds to the 40 S subunit [10,21,23,24], facilitated by the presence of eIF-3 and eIF-4C on the 40 S subunit (table 1, fig.1). eIF-4C stabilizes this binding further and its presence results in a 1.5–2-fold stimulation of Met-$tRNA_f$ binding to the 40 S subunit [6,23–27].

When using 80 S ribosomes as the only source of 40 S subunits eIF-1 stimulates the Met-tRNA binding 2-fold [28], while eIF-4A and eIF-4B do not have any effect. eIF-2 and eIF-3 are stoichiometrically present on the 40 S · Met-tRNA · GTP complex [4,5,10,24,76]. Stoichiometric binding of eIF-4C to this complex could only be measured in the presence of mRNA and eIF-1 [6].

Whether the Met-tRNA binding to the 40 S subunit precedes or follows the mRNA binding is no longer questioned [29]. A simple indication is that Met-tRNA binds to 40 S subunits without mRNA, while mRNA cannot be bound without Met-tRNA. However, in prokaryotes the exact order remains to be established [30].

The role of initiation factors in the binding of mRNA is much more obscure. Attention will be paid to the structure of mRNA before a more detailed description of the mRNA binding is presented.

6. Properties of eukaryotic mRNAs

Eukaryotic mRNAs have several structural peculiarities which are absent in prokaryotic messengers. The 3′-end generally contains large stretches of poly(A) from 50–200 nucleotides [31–33]. The function of this poly(A)-tail is not entirely clear, although it is believed that mRNAs containing poly(A) are more stable than mRNAs lacking poly(A). Especially when injected into *Xenopus laevis* oocytes, the difference in stability is noticeable, since $poly(A)^+$-mRNA is still translated after considerable time (56 h), whereas $poly(A)^-$-mRNA is not [34,35].

Specific proteins may bind to the poly(A) segment and protect it from nuclease digestion [36–38].

The 5′-ends of most eukaryotic mRNAs have a so-called 'cap'-structure: $^7mG(5')ppp(5')X(m)pY\ldots(3')$, (X and Y denote nucleotides) [39]. The cap-structure protects the mRNA at its terminus against attack by phosphatases and exonucleases and promotes mRNA binding during initiation of translation ([39,40], review [41]). As binding of the mRNA to the 40 S subunit occurs at the 5′-end of the mRNA (see below) an involvement of the 'cap'-structure in initiation was expected [42].

Eukaryotic viral mRNAs without the 'cap'-structure sometimes carry a protein at the 5′-end, covalently linked to the terminal nucleotide [43]. This protein, called VPg, probably fulfils a role during RNA replication and not, unlike the 'cap'-structure, during protein synthesis [44].

The number of nucleotides between the 5′-end and the starting codon AUG (leader sequence) varies from a few to several hundreds [45–47]. Attempts have been made to determine whether the leader-sequence is able to basepair with the 18 S rRNA of the 40 S subunit to coordinate mRNA binding, as is described for the 16 S rRNA of the 30 S subunit of *E. coli*

[48,49] but up till now common sequences have not been demonstrated in the leader region of different messengers [47].

For ovalbumin mRNA it was postulated that loops are formed in the leader sequence in such a way, that base-pairing can occur between the looped mRNA and 18 S rRNA [50]. Because of these hairpin loops, a comparison of the nucleotide sequences of different mRNAs might not reveal common sequences, which are complementary to sequences in 18 S RNA.

Eukaryotic mRNAs appear to be monocistronic [47,51,52]: protein synthesis is initiated exclusively at the 5′-proximal AUG initiation codon, although some reports indicate that initiation at an internal AUG may occur [53–56].

7. Initiation factor binding to mRNA

While the effect of initiation factors on the Met-tRNA binding to the 40 S subunit is relatively well understood, the binding of mRNA is much more obscure (table 1).

The affinity of eukaryotic initiation factors for mRNA was investigated; eIF-2, eIF-3, eIF-4B, eIF-4C and eIF-5 [15,19,57–61] were shown to bind to mRNA. However, the physiological relevance of this binding remains obscure, since these initiation factors also bind to other RNAs like 18 S rRNA [57,62]. Moreover, the observed interaction was too weak to survive sucrose-gradient analysis and binding of initiation factor to RNAs had to be measured by retention of radiolabelled RNA on Millipore-filters (see also [62]).

Cap-analogues such as ^{7}mGDP or ^{7}mGMP are thought to inhibit protein synthesis by competing with the cap-structure of the mRNA for initiation factors. Therefore, ^{7}mGMP and ^{7}mGDP were used to show specific binding of initiation factors to the cap-structure of mRNAs. Shafritz et al. [63] showed that eIF-2, eIF-4B and eIF-5 bound to mRNA, while only the binding of eIF-4B was sensitive to the presence of ^{7}mGMP. It was concluded that eIF-4B was a cap-binding protein. However, Sonenberg and Shatkin [62] indicated that inhibition by cap-analogues, as measured on Millipore filters, is not a stringent test for putative cap-specific binding between proteins and mRNA as even the binding of *E. coli* RNA polymerase to 18 S RNA is blocked by ^{7}mGMP [62].

Therefore, Sonenberg et al. developed another method to detect interactions between proteins and the cap-structure of mRNAs [61,64]. By a cross-linking method they discovered that eIF-4B and subunits of eIF-2 and eIF-3 bound to the 5′-region of mRNAs, whereas other initiation factors did not.

The binding of eIF-2 was not cap-specific as cross-linking between eIF-2 and the cap-structure was still possible in the presence of ^{7}mGDP. They concluded, as others did based on similar data [15,19,60], that eIF-2 binds to an internal site rather than to the cap.

The binding of eIF-3 and eIF-4B to mRNA is more complex. eIF-4B and several subunits of eIF-3 can be cross-linked to the cap-structure [61]. However, only the binding of one polypeptide (with M_r 24 000) was sensitive to the addition of ^{7}mGDP. Since the molecular mass did not correspond to the one of eIF-4B or of a subunit of eIF-3 (see table 1) it was concluded to be a contaminating protein of eIF-3 and eIF-4B preparations. The reason that it was not detected before must be that the amount present in the eIF-3 and eIF-4B preparations is low. After its discovery in the eIF-3 and eIF-4B preparations the 24 000 M_r protein was isolated and purified exploiting the fact that it binds to ^{7}mGDP [65–67].

So, of the initiation factors involved in mRNA binding (eIF-1, eIF-4A, eIF-4B and the 24 000 M_r protein) only eIF-4B binds to mRNA, whereas the 24 000 M_r protein, which should be called eIF-4E [68], binds specifically to the cap-structure. Furthermore, two other factors, eIF-2 and eIF-3 show affinity for mRNA, although they are not directly implicated in mRNA binding to the 40 S subunit.

No binding of eIF-4A to mRNA could be demonstrated which is puzzling since eIF-4A is absolutely necessary for mRNA binding (see table 1) [23,25,69,70].

8. Binding of mRNA to the 40 S subunit

Since 18 S rRNA has no complementary sequences with mRNA (see above also) another explanation must exist for the recognition between mRNA and the 40 S subunit. Kozak showed that under certain circumstances (addition of inhibitors of protein synthesis, or lowering the $[Mg^{2+}]$) more than one 40 S subunit is bound to a messenger, suggesting that these circumstances prevented the 40 S subunit from recognizing the start codon [71–73].

A model has been proposed to account for some

of the peculiarities of mRNA binding to 40 S subunits as discussed in section 6. The model states [47] that a 40 S ribosomal subunit binds at the 5′-end of an mRNA, the binding being facilitated by the cap-structure, and subsequently advances until it encounters the first AUG triplet. At this point the 40 S subunit halts and joining with the 60 S subunit occurs. This is consistent with the assumption that eukaryotic mRNAs are monocistronic and with the fact that circular mRNAs without free 5′-ends cannot be translated in a wheat-germ cell free system for protein synthesis [71]. Furthermore, whereas *E. coli* ribosomes recognized the linear and circular form of the leader-region of tobacco mosaic virus RNA, wheat-germ ribosomes only bound to the linear form [74].

The effect of the initiation factors in this process has not been studied. ATP could be involved in the movement of the 40 S subunit along the mRNA and it is tempting to speculate that eIF-4A and eIF-4B are participants in this process, as these factors might be involved in ATP hydrolysis [75,76] (see below also).

The mechanism of action of eIF-1, eIF-4A, eIF-4B and eIF-4E during the binding of mRNA to the 40 S subunit is only partly understood (see table 1 and fig.1). mRNA binding to the 40 S subunit is decreased to 20–50% by omission of eIF-1, eIF-4A or eIF-4B. It has been suggested that eIF-1 induces the repositioning of the Met-tRNA, facilitating the codon–anticodon interaction, which results in a stable binding of mRNA. Thus, the primary effect of eIF-1 appears to be on Met-tRNA binding [28].

Furthermore, the presence of mRNA and eIF-1 causes (possibly via the stabilized Met-tRNA binding) the stable association of eIF-4C to the 40 S initiation complex [6].

The role of eIF-1 in the mRNA-binding process as suggested by Kozak [47] may be to stop the migration of the 40 S subunit along the mRNA by co-ordinating the interaction between Met-tRNA and the initiation codon [28].

So far, it has not been shown that eIF-4E has a stimulatory effect on mRNA binding with purified initiation factors. The reason for this may (obviously) be explained by the fact that eIF-4B and eIF-3 (or other preparations) still contain saturating amounts of eIF-4E.

The function of eIF-2 and eIF-3 in the initiation process in relation to mRNA binding is still controversial. Whether their presence is needed only for Met-tRNA binding and, strictly, not for mRNA binding has not been established. As optimal Met-tRNA binding is only possible in the presence of eIF-2 and eIF-3, the suggestion of Kaempfer et al. [60] that eIF-2 also helps in binding needs further experimentation. The fact that both factors have affinity for mRNA (see above) suggests that they play a role in mRNA binding [5].

The process that regulates the selection of specific mRNAs will be discussed later.

After completing the formation of a 40 S · Met-$tRNA_f$ · mRNA complex, this particle has been shown to contain equimolar amounts of eIF-2, eIF-3, eIF-4C, Met-tRNA, mRNA and GTP [4,6,10,24,25,77]. The reason that no other initiation factors were found may be due to failure of the methods used to detect the interaction between these factors and the initiation complex [25]. Of course, the possibility remains that initiation factors do not bind to the 40 S initiation complex (eIF-4A) or are already released before the assembly of the 40 S initiation complex is completed [7].

9. Coupling of a 40 S initiation complex with a 60 S subunit

The joining of a 40 S initiation complex with 60 S subunit (see fig.1) is catalyzed by eIF-5 [9,23,26,78,79]. This transition is accompanied by the release of the initiation factors eIF-2, eIF-3 and eIF-4C [4–6,10,24,25,77] and hydrolysis of the bound GTP [80]. Because no stable interaction of eIF-1, eIF-4A, eIF-4B, eIF-4D and eIF-5 to the initiation complexes could be demonstrated [25], it must be assumed that the 80 S initiation complex is devoid of initiation factors.

Peterson et al. [78] suggested that the action of eIF-5 is to remove eIF-2 and other initiation factors (although their presence was not demonstrated) from the 40 S initiation complex, a process which requires GTP hydrolysis. The resulting 40 S · Met-tRNA complex combines with 60 S subunits.

Which of the two pathways is physiologically relevant remains to be elucidated.

The 80 S initiation complex formed is the end product of the initiation process.

10. Energy used during initiation

The binding of Met-tRNA to the 40 S initiation complex is accompanied by the binding of GTP (via

the ternary complex). The binding of mRNA requires ATP [23,25,81].

If GTP is replaced by its non-hydrolyzable analogue GDPCP, Met-tRNA and mRNA can still be bound to the 40 S complex, but the transition to the 80 S initiation complex is prevented [24,25]. Merrick has shown [80] that the assembly of an 80 S initiation complex requires the hydrolysis of one molecule of GTP.

The Met-$tRNA_f$ binding to the 40 S subunit is unaffected when ATP is replaced by ADPCP or even completely omitted, whereas the mRNA binding decreases to 20–40% [23–25]. The same decrease of mRNA binding occurs when 80 S initiation complex formation is studied, suggesting that mRNA binding only occurs in the presence of ATP.

Recently, Kozak has found that the scanning of the 40 S subunit along the mRNA [47] is dependent on ATP [82,83]. In the absence of ATP, 40 S subunits are bound at the 5′-terminal nucleotide; upon addition of ATP the 40 S subunit migrates towards the initiation codon where coupling with the 60 S subunit can take place. It is likely that 40 S migration along the mRNA requires the hydrolysis of more than one ATP molecule [47], but the ATP requirement of the mRNA binding has not yet been quantified.

11. eIF-4D

The role of eIF-4D is obscure [26,84,85]. eIF-4D stimulates the formation of the dipeptide-analogue methionyl–puromycin and the poly(U)-dependent polyphenylalanine synthesis [25,84,85]. It slightly lowers the $[Mg^{2+}]$ for optimal protein synthesis [9], but does not affect the level of translation [9,25]. No involvement of eIF-4D in protein synthesis with natural messengers could be demonstrated.

Benne and Hershey [25] showed that effects of eIF-4D are also measurable after the completion of an 80 S initiation complex. In this respect it should be questioned whether eIF-4D is an initiation factor or rather a protein which is in some way involved in the formation of methionyl–puromycin (see also [7] and [9])

12. Hemin-regulated inhibition of protein synthesis

When reticulocyte lysates are incubated in the absence of hemin, the rate of protein synthesis drops to 10% ([86,87], reviews [88,89]). The arrest in protein synthesis is due to a block in initiation [90]. The effects of hemin-deficiency are caused by the formation of an inhibitor, called HRI (hemin-regulated inhibitor) [91,92]. HRI is capable of phosphorylating the α-subunit of eIF-2 [93–97]. Since this discovery attempts were made to show that phosphorylated eIF-2 was inactive in initiation of protein synthesis, but no concluding evidence was obtained [24,98]. Phosphorylation of eIF-2 seemed to have neither an effect on its activity in model assay systems [24,98,99], nor on its capacity to recycle [100]. It was concluded that the phosphorylation of eIF-2 is not the sole cause of cessation of protein synthesis.

However, recently we have found that eIF-2 occurs as a complex with a protein, designated anti-HRI [99]. This complex is 5–6-fold more efficient during methionyl–puromycin formation than eIF-2 alone, resulting in the recycling of eIF-2. Phosphorylation of eIF-2 by HRI prevents its association with anti-HRI, leading to a stoichiometric use of the factor in initiation complex formation. Furthermore, a strong inhibitory effect of HRI has been demonstrated in this assay system, employing eIF-2 · anti-HRI complex (H. Goumans, personal communication).

13. Regulation of mRNA binding

The regulation of mRNA selection by the translational machinery in the cell remains an intriguing problem. Some reports in the literature indicate a role for initiation factors of protein synthesis in this process.

Kabat and Chappell described [101] that a discriminating initiation factor exists in their IF-M3 and IF-M4 preparation (containing eIF-3, eIF-4A and eIF-4B), which binds more strongly to β-globin mRNA than to α-globin mRNA and that addition of this preparation resulted in stimulation of the synthesis of α-globin and not of β-globin.

Golini et al. showed that EMC-RNA (a viral RNA) outcompetes host cellular mRNAs and that this could be overcome by addition of excess eIF-4B [102].

Heywood and coworkers have indicated that an initiation factor of 15–18 S from embryonic chick muscle contains components which cause a specific stimulation of the translation of myosin mRNA in a muscle cell-free system [103,104]. This activity was later ascribed to an mRNP protein [105].

Whether these three examples can be explained by the fact that eIF-4E is present in eIF-3 and eIF-4 preparations remains to be elucidated.

Most animal viruses are able to induce the protein synthesizing machinery to translate the viral mRNA and to shut-off the host mRNAs. The level of protein synthesis is decreased [106,107], with some exceptions as in the case of SV40 [108] and reovirus [109]. Much work has been done to elucidate the mechanism of host shut-off, but so far an explanation of this phenomenon is lacking. Host mRNAs are not degraded or inactive in cell-free systems after viral infection [110]. HeLa cell extracts prepared from polio-virus infected cells only translate polio mRNA (which is uncapped [39]) [65,66]. Trachsel et al. reported that the translation of capped mRNAs was restored upon addition of eIF-4E, while the synthesis of polio-proteins was not affected [65]. Thus, it appears that polio-virus infection results in the inhibition of cellular protein synthesis by inactivation of some crucial property of the eIF-4E. However, Van Steeg et al. showed that the known initiation factors, including eIF-4E, have not become inactivated upon infection of neuroblastoma cells with SFV [111]. If the protein synthesizing machinery is unchanged, the possibility remains that upon infection an inhibitor of translation is formed [111] which may be lost after purification of initiation factors [111–113].

Whatever the case, a viral mRNA must have a different affinity towards the protein synthesizing machinery since at some stage discrimination of viral and host mRNA occurs. This indicates that some features in the structure of viral mRNAs exist which differ from those of host mRNAs (see section 6). This may be related to the differential effect of eIF-4E on capped and uncapped mRNAs.

As long as the molecular events occurring during and after the action of each initiation factor in mRNA binding are not fully understood, the study of the shut-off of host protein synthesis will be handicapped.

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