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The mRNA cap-binding protein eIF4E in post-transcriptional gene expression.

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Abstract

Eukaryotic initiation factor 4E (eIF4E) plays central roles in the control of several aspects of mRNA metabolism, and affects developmental processes as well as human diseases. This review explores the relationship between structural, biochemical and biophysical aspects of eIF4E and its function *in vivo*, including both long-established roles in translation and newly emerging ones in nuclear export and mRNA decay pathways.

Introduction

The production of proteins is regulated at many different steps of gene expression in order to control both the amount and the nature of the final product. One of the steps that are subject to tight regulation is the recruitment of the small ribosomal subunit to mRNA in preparation for the initiation of protein synthesis. Decoding of an mRNA during translation proceeds in the 5'→3' direction. The recruitment process therefore needs to facilitate placement of ribosomes at the 5'end of the message. Moreover, the frequency of ribosome binding to the mRNA has to match the amount of the encoded protein required by the cell.

In eukaryotes, two principal pathways are available for attachment of the small ribosomal subunit 5' of the translated region. The first, termed cap-dependent translation initiation, relies on the fact that eukaryotic mRNAs are co-transcriptionally modified by attachment of an inverted, methylated guanine moiety to produce the 5'-terminal cap-structure m^7GpppN (where N is the first transcribed nucleotide[1]). The cap serves as a point of anchorage for a cap-binding protein complex that can mediate recruitment of the small subunit of the ribosome to the extreme 5'end of the mRNA (**Fig. 1**). A second pathway utilizes complex secondary structure elements in the

RNA, so called internal ribosomal entry segments (IRES), to recruit small ribosomal subunits either via direct RNA–ribosome contacts or indirectly via initiation factors that can bind both the IRES and the ribosome. Initiation via this pathway does not rely on the existence of a cap-structure on the mRNA, and is therefore termed cap-independent. The overwhelming majority of eukaryotic transcripts are translated in a cap-dependent manner, and this review will focus only on this mode of translation.

Since the cap-structure is located at the very 5'-end of the transcript, cap-dependent translation initiation recruits the small ribosomal subunit to a point that is separated from the translation start codon by an untranslated region (5'UTR) up to several hundreds of nucleotides long. Following recruitment to the 5'end, the eukaryotic small ribosomal subunit therefore has to locate the start codon by means of a processive 5'→3' scanning process. Once this codon has been reached, the large ribosomal subunit binds to the small ribosomal subunit and protein synthesis can begin (for general reviews on translation initiation see refs. 2–4).

The protein complex that is responsible for recruiting small ribosomal subunits comprises the cap-binding protein eIF4E, the large adaptor protein eIF4G, and the poly(A) binding protein PAB (Pab1 in yeast), which binds to the poly(A) tail present on 3'-ends of most eukaryotic transcripts (**Fig. 1b**). Binding of eIF4E and eIF4G to the cap via the activity of eIF4E is absolutely essential for translation both *in vivo* and *in vitro*. On the other hand, contacts between eIF4G and the poly(A)-bound PAB appear to enhance translation efficiency but are not absolutely required for ribosome recruitment. This stimulatory effect may be linked to circularization of the mRNA that can be mediated by the cap–eIF4F–poly(A) tail bridge. Since any break in the chain cap–eIF4E–eIF4G impairs cap-dependent translation, the assembly of this complex is a potential target for translational control. Evidence for such control has

been found during developmental processes[5] and tumorigenesis[6], and eIF4E has consequently been identified as a potential drug target[7].

Despite the fact that eIF4E was identified as a translation factor more than 25 years ago and considerable effort has been spent on elucidating the function of this key protein, there are still many unanswered questions concerning its biological role. One of the problems is that the regulation of translation is linked to the control of mRNA export and mRNA turnover. Indeed, the involvement of eIF4E in these processes has only recently become evident. It appears that all of the functions of eIF4E are linked to the presence on this factor of binding sites for both the mRNA cap and for a number of proteins, and that the particular function of any individual eIF4E molecule depends on the type of protein ligand it is bound to. In the following, we will therefore review currently available data on the physical basis of eIF4E–mRNA and eIF4E–protein interactions, and then relate these data to biological functions. The influence of phosphorylation on eIF4E function is a controversial issue that has recently been reviewed in some detail[8], and we have accordingly given only minimal consideration to it here.

The interaction of eIF4E with nucleic acids

The structural basis of cap-binding – eIF4E specifically binds the 5'-end cap-structures of mRNAs, which in most eukaryotes are of the types m^7GpppNp, m^7GpppN^{m}p or m^7GpppN^{m}pN^{m}p[1] (where m denotes a methyl-group attached to the respective nucleotide, see **Figure 2a**). A number of three-dimensional structures have been solved for eIF4E–cap analog complexes, from which molecular contacts between eIF4E and mRNA 5' ends were identified[9–14]. The cap-binding site is formed by a cavity in the otherwise roughly spherical body of eIF4E. Two tryptophan

residues are situated near the upper and lower fringes of this cavity. When the cap-analog m^7GTP is bound, the guanosine moiety is stacked between the two tryptophans, and held in place through interactions between the ring systems of the three components (**Fig. 2b**). A strong stabilizing effect is associated with the presence of the methyl group, which introduces a positive charge on this moiety that greatly enhances the stacking interaction[14].

This preferred binding to methylated guanosine is of biological relevance because intracellular levels of GTP are in the millimolar range in logarithmically growing yeast cells[15] and thus three to four orders of magnitude higher than the levels of eIF4E or mRNAs[16]. Efficient binding to unmethylated GTP would thus strongly interfere with mRNA 5'-end binding. The equilibrium affinity for the eIF4E–m^7GTP interaction is almost five orders of magnitude higher than that for the interaction with GTP[14], and thus under equilibrium conditions the majority of eIF4E is bound to cap-structures despite the high GTP concentrations.

In contrast to GTP and GDP, their methylated counterparts bind to eIF4E with high affinity. m^7GDP is generated during the decapping step in the mRNA degradation pathway (see below). Its interference with the process of translation appears to be prevented in yeast and humans by the existence of a dedicated enzyme pathway that rapidly degrades this compound[17].

In addition to the influence of stacking interactions, mRNA caps are stabilized inside the cap-binding cavity by several van-der-Waals contacts and hydrogen bonds with the three phosphate groups and the ribose of the cap-analog. Comparisons of the free energies of m^7G, m^7GMP, m^7GDP and m^7GTP-binding showed that about half the total binding energy of the m^7GTP–eIF4E-bond derives from van-der-Waals

contacts and hydrogen bonds, with the other half derived from the stacking of the ring systems[14].

Binding of eIF4E to mRNAs with different 5'-end sequences – Since the methylated guanosine is an invariant component present on all capped transcripts, molecular contacts with the cap are identical independent of the mRNA species. In contrast, if contacts with downstream nucleotides exist, the overall affinity of eIF4E for mRNA 5'-ends could be transcript-dependent, and this could theoretically affect the efficiency of translation in a transcript-dependent manner.

NMR-studies and X-ray crystallographic analyses of eIF4E in complex with the larger cap-analog m^7GpppA showed that additional contacts exist between the C-terminus of the protein and the adenine[13,18,19]. Interestingly, Niedzwiecka *et al.* found that the affinity of m^7GpppN cap-analogs differed according to the nature of the second nucleotide[14], following the order $m^7\mathrm{GpppG} > m^7\mathrm{GpppA} > m^7\mathrm{GpppC}$.

In addition, nucleotides downstream of the cap may contribute to eIF4E-binding. Studies with the human protein showed that several of the nucleotides after the cap may contact the body of eIF4E, and that altering the sequence close to the cap produces up to four-fold variations in equilibrium affinity[20,21]. However, NMR experiments using capped trinucleotide RNAs showed no detectable intermolecular contacts beyond the first nucleotide following the cap-structure[19], so that any binding of downstream nucleotides to the body of the protein is likely to be weak. Whether the observed differences in mRNA cap-affinity are sufficiently strong to affect translational efficiency significantly is currently unclear.

Structural differences in cap-bound and apo-eIF4E – A number of observations suggest that small conformational changes in the overall structure of eIF4E are closely linked to cap binding. The loops forming the outer fringes of the cap-binding pocket are relatively flexible portions of eIF4E both according to NMR data[18] and to Molecular Dynamics simulations[13], and a decrease in flexibility is predicted following binding of mRNA ends[13]. Moreover, the secondary structure content of the cap-binding protein appears to be altered in the presence of cap-analogs, since addition of the latter produces subtle changes in CD spectra recorded for eIF4Es from various organisms[22–24]. Consistent with these observations, cap-bound and apo-eIF4E show differing behavior in some biochemical assays[24,25].

These structural differences indicate that some or all of the binding partners of the cap-binding protein may be able to distinguish between its cap-bound and unbound state. Indeed, it has been shown that the association of small translational inhibitors, the 4E-binding proteins (4E-BPs), with human eIF4E is significantly increased for the cap-bound form[25]. 4E-BPs act by competing with eIF4G for binding to eIF4E, thus preventing the formation of translationally active eIF4E–eIF4G–mRNA complexes (**Fig. 1a**). The above observation therefore has important implications for the regulatory properties of the 4E-BPs, because the preferential binding to cap-bound eIF4E increases the proportion of cap-bound versus free 4E-BP–eIF4E complexes.

***Affinity of the cap-interaction* –** A large number of studies using a range of experimental techniques have established that the equilibrium affinity of the eIF4E–mRNA cap interaction is in the range of 0.1–4 μM, depending on the source of the protein, and the pH and salt conditions under which the experiments were

performed[21,26–31]. Kinetically, the interaction is characterized by a very rapid binding-release cycle[14,22]. The rapid association is thought to be the result of strong electrostatic steering of the negatively charged mRNA cap towards the cap-binding site, due to a large dipole moment of eIF4E[32]. The resulting complexes are unstable and decay with a half-live of ~ 0.1 seconds (based on dissociation rates measured for the human protein[31]).

These kinetic characteristics indicate that apo-eIF4E, which is non-functional for the purposes of translation since this protein can recruit ribosomes only in complex with eIF4G, leaves cap-structures again rapidly after binding to them. In contrast, eIF4E in complex with protein ligands has been found to undergo more stable interactions with mRNA 5'-ends using a variety of mechanisms (discussed in more detail below). In consequence, any cellular pool of free eIF4E does not compete efficiently with the translationally active eIF4E–eIF4G complexes for cap binding. Free eIF4E therefore becomes a relevant competitor for the latter only upon binding of a translationally inactive ligand that also increases cap-affinity (e.g. 4E-BP).

Protein-protein interactions of eIF4E

Binding of eIF4G – Two independent studies employing mutants of yeast eIF4E[33] and X-ray crystallography of murine eIF4E[10] identified a coherent epitope formed by residues originating from strand 1, helix 1 and helix 2 (numbering for the human protein[9]) that is required for binding to eIF4G. The binding site thus identified is distal from the cap-binding pocket, and does not contain any known residues involved in cap binding.

Subsequent experiments with larger fragments of eIF4G concluded that contacts between the full-length proteins include additional residues[34]. An NMR study

of a yeast m^7GDP–eIF4E–eIF4G$_{393–490}$ complex showed that the N-terminal tail of eIF4E and the eIF4G-fragment, both of which are unstructured in the apo-proteins, mutually induce extensive folding events that result in a complex, interlocking structure[12] (**Fig. 2c.** The secondary structure elements in the eIF4G$_{393–490}$ fragment shown in blue in this panel are detectable only in the eIF4E-bound form, but not in apo-eIF4G$_{393–490}$[34].) The binding reaction leading to this interlocking structure is thought to occur in two stages, an initial encounter involving the minimal binding site identified earlier, followed by a collapse of disordered parts of eIF4E and eIF4G into the compact, folded structure. Consistent with the extensive binding interface, the resulting complex is stable with a k_D of 2–4 nM and a half-life of five to six minutes ($k_{diss} \sim 2x10^{-3}$ s^{-1})[12,33].

Since the 393–490 fragment of yeast eIF4G1 is unstructured in solution and becomes folded only when in contact with eIF4E, the question arises how the eIF4E binding site is organized in full-length eIF4G. Deletion of N-terminal residues of the cap-binding protein weakens its interaction with eIF4G1$_{393–490}$ *in vitro,* and also reduces the ability of eIF4E to mediate pull-down of full-length eIF4G from cell extracts[12]. It is therefore likely that contacts with the N-terminal tail that are crucial for formation of the interlocking eIF4G$_{393–490}$–eIF4E interface are similarly important for the interaction with full-length eIF4G. If a similar unfolded-to folded transition occurs in the latter as is observed for the fragment *in vitro*, the eIF4E-binding site can be predicted to form an unfolded loop in an otherwise folded protein, since a conserved part of eIF4G adjacent to this site has recently been shown to fold into a HEAT domain[35]. A disordered binding site would be consistent with observations by Berset *et al.* that yeast eIF4G expressed in *E. coli* can be protected from proteolysis by co-expression of eIF4E[36]. However, with only limited structural data available on

the eIF4G N-terminal domain, the exact nature of the eIF4E binding site in the context of the full-length protein remains elusive.

Interactions with other proteins **–** Binding of the 4E-BPs to eIF4E was shown to occur via the region centering around the W73 residue of eIF4E that is also required for eIF4G-binding[10,30]. The interactions of peptides corresponding to minimal eIF4E binding sites on the 4E-BPs mimic the interaction of the corresponding eIF4G peptide closely[10]. 4E-BPs are relatively small proteins (~12 kDa) that show no obvious structure in solution, and undergo a folding transition around the eIF4E binding site upon interaction with this factor. However, parts of the 4E-BPs outside of the binding region remain largely unfolded[37]. Subtle differences in the ways eIF4G and the 4E-BPs contact eIF4E were also observed in two-hybrid studies with mutant cap-binding proteins[30], but it is at present not known whether the different contact surfaces of eIF4E-ligands are the result of differences in the unfolded-to-folded transition.

In addition to the 4E-BPs, various organisms have a number of other 4E-binding partners that compete with the formation of translationally active eIF4E–eIF4G-complexes. These are generally larger proteins than the 4E-BPs, and can assume highly specialized roles during particular developmental stages. Examples for such proteins are Maskin in *Xenopus*[38], and Cup[39] and Bicoid[40] in *Drosophila*. To date, nothing is known about the structural organization of the eIF4E-binding site in these proteins.

In humans, two further proteins have been identified as binding partners of eIF4E that appear to serve as negative regulators of the eIF4E-dependent export of a subset of mRNAs. These are the Promyelocytic Leukemia Protein (PML) and the Proline-Rich Homeodomain protein (PRH)[24,41]. It is known that interactions of these

proteins with eIF4E show the same sensitivity to mutations of the W73 residue on the cap-distal surface of eIF4E as do interactions of eIF4G and the 4E-BPs[24,41], despite the fact that PML and PLH do not contain the conserved minimal eIF4E-binding motif Tyr-X-X-X-X-Leu–ϕ (where ϕ is Leu, Met or Phe)[42] found in the latter proteins. While PRH contains a related motif in which the hydrophobic ϕ is exchanged for a polar Gln[41], the 60-residue region in PML identified as the eIF4E-binding domain does not contain any motif of similar sequence[43]. Thus, all known protein binding partners of eIF4E bind to a common region on the cap-distal side of this protein, although the details of the molecular contacts involved in these interactions may vary considerably.

Ligand-dependent stabilization of the eIF4E–cap interaction

While the interaction of apo-eIF4E with cap-structures is transient, it is significantly stabilized following eIF4G binding[22,27,33,44]. It was originally proposed that this is attributable to the RNA-binding activity of eIF4G[44]. However, an alternative explanation is that eIF4G imposes conformational changes on eIF4E that stabilize cap binding[33]. *In vivo,* a combination of both mechanisms may determine the overall stability of the mRNA 5'-end associated eIF4E–eIF4G–PAB complex.

***Stabilization of cap-binding through conformational changes* –** Slower dissociation of eIF4G-bound than of free eIF4E has been observed from cap-analogs that do not contain additional nucleotides to which eIF4G could bind, and also with fragments of eIF4G that bind to eIF4E but not to RNA[22,27,33]. In both types of experiment, RNA binding activity of eIF4G can be ruled out as the source of stabilization, and allosteric effects on the conformation of eIF4E are the most likely cause of the cap-arrest.

Mapping of chemical shifts in eIF4E following eIF4G binding reveals small changes in the structure or flexibility of key residues within the cap-binding site of eIF4E[12]. While it remains to be formally proven that these changes are causally linked to stabilization of the eIF4E–cap interaction, it was shown that both these changes and the increased binding to capped RNAs depend on the extensive folding events that occur during the eIF4E–eIF4G interaction[12]. Thus, important determinants of the eIF4E–cap interaction are situated outside the consensus binding motifs in both eIF4E and eIF4G, and these determinants are not present in the N-terminally truncated proteins employed in many of the published biophysical studies of human eIF4E.

In addition to the stabilizing effect of eIF4G binding to eIF4E, binding of the poly(A) binding protein PAB to the eIF4E–eIF4G complex further stabilizes the cap-interaction. This effect was shown *in vitro* for the purified proteins from wheat[45,46], humans[47] and yeast[22]. PAB binding to eIF4G is itself enhanced by contacts with poly(A) RNA[48], so that maximal cap-binding activity is likely to depend on full formation of the molecular chain cap–eIF4E–eIF4G–PAB–poly(A). However, weaker stabilizing effects are likely to occur also for subcomplexes in which individual links in this chain are broken.

Allosteric effects caused by other ligands **–** As is the case for eIF4G, reduced dissociation of eIF4E from cap-structures has also been observed in the presence of 4E-BPs[30,31]. In studies of the latter proteins, binding and release kinetics for eIF4E–4E-BP complexes were observed that are equivalent to an apparent half-life for cap-dissociation of ca. 14 minutes ($k_{diss} \sim 8x10^{-4}$, refs. [30,31]).

Interestingly, a modulation of the cap-binding activity of eIF4E has also been demonstrated for PML, binding of which reduces eIF4E's cap-affinity 100-fold[43]. A

somewhat weaker reduction in cap-affinity was likewise found following binding of PRH[41]. This negative effect on cap-binding of eIF4E is likely to be directly linked to the regulatory role of PML and PRH in eIF4E-dependent mRNA export, since mutants of eIF4E with reduced cap-affinity were shown to be less active in this particular type of export than the wild type protein[24].

***Stabilization of cap-binding through mRNA contacts of eIF4G* -** Since the affinity of eIF4G for RNAs is in the nM range[27,36], a direct eIF4G–RNA contact could also make significant contributions to a stabilization of the cap-binding complex on mRNA 5' ends. The overall stability of the cap-binding complex *in vivo* would then be determined by a combination of conformational changes in eIF4E that lead to slower dissociation rates from the cap, and the RNA-binding activity of eIF4G. In yeast eIF4G, it is known that the RNA binding interface is composed of three independent mRNA binding sites[36]. It is currently not clear how this interface is organized in the context of the cap-binding complex, and the relative contributions of allosteric effects and mRNA contacts to the determination of cap-complex stability *in vivo* can therefore not yet be assessed.

A role of eIF4E for limiting translation initiation *in vivo*?

eIF4E is attributed with an often-cited role in limiting the translational activity of cells[49], based on the original observation that it is less abundant than other initiation factors in mammalian cells and reticulocyte lysates[50,51]. The precise relationship between eIF4E levels and translational activity is an important problem, because changes in the abundance or activity of eIF4E occur in many situations, from the adaptation to environmental stresses[52] to developmental decisions[5] and

tumorigenesis[6]. However, although changes in eIF4E abundance or activity appear to correlate with altered translational activity in these situations, it is not always understood whether the two are causally linked.

In theory, biochemical studies on the interactions of eIF4E with its binding partners can provide all the information required to decide whether the availability of this protein limits translation. The questions that need to be answered for this kind of analysis are, what are the relative levels of mRNAs, eIF4E, eIF4G and other eIF4E-binding partners; how often do eIF4E–eIF4G complexes need to form on an mRNA in order for efficient ribosome recruitment to occur, and how well do eIF4E–eIF4G complexes compete with the translationally inactive free eIF4E or with eIF4E–4E-BP complexes for access to mRNA ends.

Relative levels of cap-binding complex components and mRNAs – While it was initially reported that eIF4E occurred at much lower levels than other initiation factors in the reticulocyte system[50], newer results indicate that reticulocyte extracts contain an excess of eIF4E over eIF4G[14,53]. Moreover, in yeast, eIF4E was shown to occur at levels equimolar to ribosomes and most other initiation factors[16,42]. As observed in yeast, eIF4E is more abundant than eIF4G in all developmental stages of *Drosophila* (Rolando Rivera Pomar, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany, pers. comm.).

The relative abundance of 4E-BPs compared to eIF4E and eIF4G has not yet been experimentally determined. However, under active growth conditions the 4E-BPs exist in a hyperphosphorylated state (see e.g. ref. 54) that prevents efficient binding to eIF4E[55]. Under such conditions, the cap-binding protein is therefore likely to exist mainly in complex with eIF4G or in the apo-form. In yeast, reticulocytes and

Drosophila cells, it is thus more likely that the availability of eIF4G (rather than that of eIF4E) limits the frequency with which ribosomes can be recruited to any individual mRNA, or that both factors occur at saturating levels that do not limit translational activity at all.

Under conditions where cells are not actively growing and the requirement for protein synthesis is reduced, the 4E-BPs become dephosphorylated and thus able to bind to eIF4E. The availability of free eIF4E will then be reduced, and this may limit the amount of eIF4E–eIF4G complexes that can be formed. The extent to which this affects translation would depend on the level of 4E-BP dephosphorylation as well as on the efficiency of competition of eIF4E–4E-BP complexes for cap-access with eIF4E–eIF4G complexes.

Frequency of complex formation – While cap-complex formation is known to be essential for cap-dependent translation, there is no clear idea about how often this complex has to be formed for recruitment of a single ribosome to occur. At one extreme would be a situation where the cap-complex is very unstable, and one ribosome-recruitment is on average the result of several cap-binding and -release cycles. Under such conditions, both eIF4E and eIF4G could potentially limit translational activity even if they occurred in excess over mRNAs, since an increase in their concentration could affect the frequency with which cap-binding complexes are formed.

At the other extreme, cap-complexes would be bound very stably to mRNA ends, sequentially recruiting several ribosomes without dissociating. In such a scenario, and if the 4E-BPs are assumed to be fully phosphorylated, nearly all mRNA ends would be stably bound to eIF4E–eIF4G complexes as soon as the levels of these

complexes match those of mRNAs. If both eIF4E and eIF4G occurred in excess over mRNAs, they would then be present at saturating levels, and the availability of neither protein could limit translational activity.

The biophysical data derived from *in vitro* experiments with recombinant eIF4E, eIF4G and PAB as described above indicate that cap-binding complexes are stable and dissociate relatively slowly from mRNAs. This would indicate that several ribosomes are usually recruited without the cap-binding complex being released and rebound. However, Ray *et al.* showed that, in cap-binding assays employing purified human proteins, the addition of eIF4B (a protein that stimulates the helicase activity of eIF4A[56]) destabilized pre-assembled cap-complexes that also contained eIF4A, and led to an accelerated binding and release cycle[57]. *In vivo*, the action of eIF4B and possibly other trans-acting factors may therefore alter cap-complex stability compared to pure *in vitro* systems.

***Effects of experimentally altered eIF4E levels* -** Given the difficulties in extrapolating kinetic constants estimated *in vitro* in terms of eIF complex stability *in vivo*, the engineering of variations in the abundance of eIF4E within cells is a valuable approach. Results from such experiments indicate that changes in the concentration of eIF4E produce two distinct types of response, relating to bulk translation on the one hand and specific aspects of cellular metabolism on the other.

In yeast, overexpression of eIF4E by up to a factor 100 has only a minor effect on growth rates[58]. Similarly, a 2.5-fold increase of eIF4F levels did not lead to increased general translation rates in feline cardiocytes[59]; while a modest increase in translation rates could be observed in other systems such as *Xenopus* cells[60]. Thus, bulk translation in these systems appears to be quite insensitive to increases in eIF4E

availability. Reduction of the intracellular eIF4E-levels to 30% of wild-type levels is tolerated in yeast without apparent effects on growth rate or amino acid incorporation[16]. Here, translation is affected as soon as eIF4E levels fall close to or below mRNA levels. In other organisms, smaller reductions of eIF4E levels have more dramatic effects, possibly because the natural levels of eIF4E are closer to those of total mRNA than in yeast.

Taken together with the considerations in the previous section, these results suggest that, at least under conditions of high translational activity, eIF4E levels are not limiting general translation.

Interestingly, changes in eIF4E levels that do not or only moderately affect bulk translation can have measurable effects on cellular physiology. Thus, eIF4E overexpression can cause malignant transformation while reduction of eIF4E levels can reverse the transformed phenotype. High eIF4E-levels are observed in an increasing number of cancers, and the level of overexpression often correlates with the severity of the disease[6], although the mechanisms underlying these effects are unknown. In yeast, reductions in eIF4E levels that do not affect methionine incorporation into protein clearly affect cell morphology, ribosome biogenesis, and the cells' ability to progress through the cell cycle[16,61].

eIF4E and transcript-specific regulation of gene expression

One explanation for the striking discrepancy between bulk translation levels and other effects following eIF4E activity changes might be that some growth-critical mRNAs have special requirements with respect to eIF4E function. Specific regulation of this kind has been suggested for CyclinD1[62], ODC[63], FGF-2[64], Pim-1[65] and VPF[66] mRNAs in mammals, as well as for *CLN3* in yeast[61]. Two possible mechanisms have

been put forward in the literature to explain how eIF4E might affect translation of these messages in transcript-specific ways.

Firstly, particular sensitivity to eIF4E-levels might arise from differential affinity of cap-binding complexes for different transcripts. As we described in the beginning of this review, such differences are likely to be only weak, and will not strongly affect mRNA binding if eIF4E and eIF4G are present at saturating levels. As soon as the amount of eIF4E–eIF4G complexes approaches limiting levels, however, lower-affinity mRNA ends are in direct competition for the available cap-binding complex with more tightly bound messages. This could significantly limit cap-complex assembly on less tightly bound mRNAs, while having only a small effect on messages that bind the cap-binding complex with higher affinity. Preferential binding of one RNA species over another at low concentrations of eIF4E and eIF4G has been experimentally shown *in vitro* for different species of Reovirus RNA[67].

Second, mRNAs regarded as sensitive to available eIF4E levels usually feature long and structured 5’ UTRs that interfere with efficient recruitment of the 40S subunits, and it was suggested that this increases the dependency of translation on eIF4A-mediated RNA helicase activity[49]. Since it is one of the roles of the cap-binding complex to recruit eIF4A to mRNA 5’ ends, where this protein is thought to remove secondary structure in preparation of ribosome binding, limitations in cap-complex assembly might also limit 5’-UTR unwinding. At least theoretically, relief from this limitation could affect structured UTRs more than bulk mRNA. In support of this prediction, it was recently reported that the requirement for eIF4A correlates directly with the stability of secondary structures present in mRNA 5’ UTRs[68].

Transcript-specific control of mRNA export – The discussion about transcript-specific regulation via eIF4E is complicated by the fact that nuclear export of some mRNAs is dependent on eIF4E activity, and that there is some overlap between this and translational sensitivity to eIF4E levels (**Fig. 3**). Thus, the human CyclinD1 message and other mRNAs are exported via an eIF4E-dependent mechanism. Although eIF4E-dependent mRNA export is mechanistically still poorly defined, it appears now likely that such a mechanism exists. Such a link might render protein production sensitive to eIF4E levels independently of translational control[41,69].

The two assays usually employed to identify transcript-specific translational control via eIF4E are polysomal gradient analysis and reporter gene measurements, under conditions of normal and increased eIF4E levels. Neither assay can readily distinguish between the utilization of an existing cytoplasmic pool of untranslated or poorly translated mRNAs (which would correspond to direct translational control) and changes in the size of a cytoplasmic pool caused by increased nuclear export. The finding that overexpression in mammalian cells of a W73A mutant of eIF4E, which can not function in translation but is active for CyclinD1 mRNA export, has the same transforming properties as overexpression of the wild-type protein[24], suggests that the oncogenic properties of the cap-binding protein may be coupled to the control of mRNA export.

Interestingly, the requirement for eIF4E outside the process of translation provides a potential explanation for the existence of a cooperative cap-binding mechanism. Such a mechanism would enable cells to maintain a pool of free eIF4E that might participate, for example, in nuclear export, yet not compete efficiently with eIF4E–eIF4G complexes for cap-access.

eIF4E and regulation of mRNA turnover

***Pathways of mRNA degradation* -** Removal of the cap-structure from mRNAs is a key step in some pathways of mRNA degradation, namely those that involve 5'→3' exonuclease activities which are blocked by the presence of the cap (reviewed in ref. 70). Dedicated decapping enzymes have been found in a number of organisms, the best characterized of which are the yeast Dcp1 and Dcp2 enzymes. Dcp activity cleaves cap-structures between the second and third phosphate bond, yielding m^7GDP and a 5'-monophosphorylated RNA that is a substrate for 5'→3'exonucleases such as Xrn1. Cleavage of the cap requires access to parts of the cap-structure that are in direct contact with eIF4E, and competition between eIF4E and Dcp activity has been experimentally shown both *in vitro*[71,72] and *in vivo*[72,73]. This suggests that increases in the activity of eIF4E and eIF4G might, at least under certain conditions, lead to mRNA stabilization, whereas the transition from translation to decapping and degradation should coincide with cap-binding complex destabilization.

***Control of mRNA degradation by eIF activity* -** The major pathway of mRNA degradation in yeast requires that decapping be preceded by shortening of the poly(A) tail to approximately 10 nucleotides, which is just below the number of adenine residues to which the poly(A)-binding protein Pab1 optimally binds[48]. A simple, testable model for the control of degradation can accordingly be constructed that uses poly(A) tail length as a master controller for cap-complex stability (**Fig. 4**, left branch). The idea is that a newly exported, polyadenylated mRNA is bound to Pab1, which increases the affinity of the cap-binding complex for this mRNA's cap structure. This leads to efficient recruitment of ribosomes, as well as efficient protection against Dcp activity. During this translation stage, the poly(A) tail is

progressively shortened, until its length drops below the minimum length to which Pab1 can bind. Since Pab1 is then no longer in contact with poly(A), it loses its ability to bind eIF4G and exert a stabilizing effect on the eIF4G–eIF4E–cap interaction. The subsequent dissociation of eIF4E renders the cap accessible to Dcp activity, which may be actively recruited to mRNA 5' ends via interactions of Dcp1 and Dcp2 with other proteins[71].

Although this model is attractive because it can explain many of the features of mRNA turnover observed *in vivo* by a relatively simple mechanism, there are a number of observations suggesting that the connection between poly(A) tail length and mRNA turnover in cells is more complicated. Most importantly, while deletion of the entire *PAB1* gene clearly uncouples deadenylation from decapping[74], both processes operate normally in yeast strains containing mutants of Pab1 that can bind to poly(A) but not to eIF4G[75]. Pab1 may therefore influence decapping via multiple (parallel) routes, only one of which might involve modulation of the access of eIF4E and Dcp to the mRNA cap.

Indirect control of degradation **-** There are several accessory activities required for efficient removal of the cap. Among these are the RNA helicase Dhh1, and the enhancers of decapping Edc1 and Edc2, the Lsm1-7 proteins and the mRNA binding protein Pat1[76–78]. Since, for example, Dhh1 deletion strains show normal deadenylation, but accumulate capped transcripts[76], Dcp-access to the cap is likely to be subject to complex regulation.

The accessory activities named above appear to associate with mRNAs in a large protein complex, and this association appears to occur after deadenylation but prior to decapping[78]. Since mRNAs bound to this complex were shown to be no

longer in contact with eIF4E or eIF4G, it is likely that Lsm1-7 and its associated factors compete with eIF4E for cap-access before the decapping enzymes become active, rather than the Dcps directly competing with eIF4E. Interestingly, one of the proteins implicated in formation of this complex is the RNA helicase Dhh1[76], which may potentially be involved in active dissociation of eIF4E prior to decapping. Thus, a possible sequence of events during the transition from translation to mRNA degradation is that co-translational shortening of the poly(A) tail to around ten nucleotides triggers association of the Lsm1-7 complex with an RNA (**Fig. 4**, right branch). This might then lead to dissociation of the cap-binding complex, allowing the Lsm1-7-bound Dcp proteins access to the cap. It is currently unclear how the exchange of eIF4E–eIF4G for Lsm1-7 is achieved. However, Pat1 activity has been implicated in this process since this is the only Lsm-complex associated protein that has also been detected on eIF4E-bound mRNAs[78].

***Effects of protein localization* -** Attempts to understand the mechanism that converts translationally active mRNPs into substrates for mRNA decay are further complicated by recent observations that translation and degradation may occur in different cytoplasmic compartments. Thus, enzymes involved in mRNA decay are concentrated in cytoplasmic foci (termed P-bodies) in *S. cerevisiae*, and these foci are also enriched for intermediate products of the mRNA degradation pathway[79]. In *S. pombe*, the essential eIF4E1 isoform and the stress-response-linked, translationally inactive isoform eIF4E2 seem to be located in distinct cytoplasmic bodies that are similar in shape to the budding yeast P-bodies[80]. These observations suggest that transitions of mRNAs between different functional states, and the exchange of cap-associated

factors, might involve relocalization within the cell. The role of compartmentalization in the control of posttranscriptional processes clearly requires further investigation.

Future challenges

Since its identification 25 years ago, eIF4E has become one of the best-characterized translation initiation factors. A major challenge for workers in this area derives from the fact that several intracellular modulators influence the properties of eIF4E, and thus its function(s), *in vivo*. Thus, the presence of competitive binding inhibitors, the various allosteric effects of interacting proteins, as well as the generally overcrowded molecular environment, all contribute to the complex functional behavior of eIF4E in the cell.

Despite this complexity, recently published data on the biophysical characteristics of eIF4E have shed new light on *in vivo* function. Moreover, the broad spectrum of experimental methods described in this review is likely to yield answers to many further important questions. For example, it remains to be determined whether all mRNAs whose translation is apparently eIF4E-sensitive are subject to genuine translational control, as opposed to transcript-specific control at the level of nuclear export or mRNA degradation. Other open questions concern the network of interactions responsible for switching between cap-dependent translation and mRNA decapping and the role of compartmentalization. Detailed quantitative molecular analyses of eIF4E and its interaction partners will undoubtedly continue to provide valuable insight into the mechanisms underlying posttranscriptional control.

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Figure 1. Interactions of eIF4E. (**a**), the organization of the binding sites on eIF4E is shown. A cleft in the otherwise globular protein constitutes the cap-binding site. Distal from this is a binding site for several proteins. Since these proteins connect to the same region on eIF4E, their binding is mutually exclusive. Association of eIF4E with the N-terminal domain of eIF4G leads to translationally active complexes, while association with the 4E-BPs produces translationally inactive complexes. Association with PML or PRH leads to complexes that are less active than free eIF4E for eIF4E-dependent mRNA export (see text for further explanation). Note that further ligands of eIF4E have been described, which appear to compete with eIF4G-binding to this protein in a fashion similar to the 4E-BPs. (**b**), the translationally active eIF4E–eIF4G complex in the context of a translated mRNA. The cap-binding activity of eIF4E tethers several activities that are associated via the C-terminal domain of eIF4G to the mRNA 5'-end: among these are the recruitment to the message of RNA helicase activity in the form of eIF4A or eIF4A–eIF4B complexes, the recruitment of the ribosome itself via contacts with other 40S-associated eIFs, and circularization of the mRNA via contacts with the poly(A) binding protein PAB.

Figure 2. Structural features of the cap-binding complex. (**a**), chemical structure of an mRNA cap (shown for the sequence m7GpppGpGp). Parts of the chemical structure corresponding to the cap-analog m7GDP are colored according to the CPK color scheme in order to aid comparison with panel **b**. (**b**), a detailed view of tryptophan-stacked m7GDP in cap-bound yeast eIF4E, showing the cap-analog in the CPK color scheme and the Trp-residues in yellow. Hydrogen atoms are not shown. (**c**), lateral view of the yeast cap–eIF4E–eIF4G$_{393–490}$ ternary complex. eIF4E is shown in yellow, the eIF4G fragment in blue, and the cap-analog in CPK color scheme. Secondary

structure elements visible in the eIF4G-fragment , as well as the short helix of the eIF4E N-terminal tail visible in the lower right-hand corner of the image, are induced by the interaction of the two proteins since these features cannot be detected in the apo-proteins.

Figure 3. The effect of eIF4E on translation, mRNA degradation and mRNA export is determined by its protein ligands. Stimulatory effects are represented by green arrows, inhibitory effects by red lines. Association of the apo-protein with mRNA caps can stimulate the nuclear export of a subset of mRNAs. This function can be repressed by binding of PML or PRH. eIF4E must bind to eIF4G in order to be translationally active. This association is prevented if eIF4E is bound to a 4E-BP. Based on experiments in yeast, eIF4E–4E-BP complexes could potentially inhibit mRNA degradation since they efficiently compete with decapping. The relationship between eIF4E–eIF4G-cap complex formation and mRNA turnover remains unclear.

Figure 4. Models for the transition from translation to mRNA degradation. Based on the known properties of the cap-binding complex and of mRNA degradation intermediates, two pathways of deadenylation-dependent mRNA degradation can be proposed. Both start with a capped, adenylated mRNA that is bound to the cap-binding complex and efficiently translated. Following co-translational poly(A) shortening, the contact between the poly(A) tail and PAB is lost. Whether loss of the poly(A) tail leads to the immediate dissociation of PAB from the cap-binding complex as depicted, or whether PAB can remain bound to eIF4G in this state, is not yet fully understood. In one model (left branch), the loss of contact with the poly(A)

tail reduces the affinity of the cap-binding complex for the mRNA 5'-end sufficiently to lead to accelerated dissociation from the cap-structure, thus making the latter accessible to the action of the decapping enzymes DCP1 and DCP2. In an indirect model (right branch), the dissociation of PAB from the mRNA 3'-end allows the formation of an intermediate complex comprising the decapping enzymes Dcp1 and Dcp2, and several additional activities like the enhancers of decapping Edc1 and 2, the RNA helicase Dhh1 and the LSm1-7 protein complex (these proteins are here collectively denoted as "Decapping factors"). This intermediate complex induces dissociation of the cap-binding complex, while at the same time stimulating the decapping activity of the Dcps. Upon removal of the cap-structure, the RNA is rapidly degraded by 5'-3' exonucleases. A second pathway of mRNA degradation that proceeds in 3'→5' direction independent of decapping[81] is not considered here.

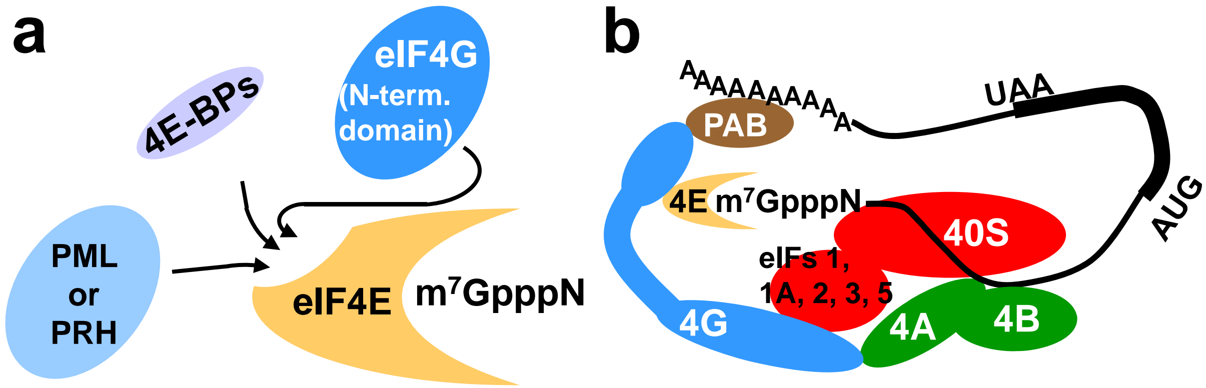
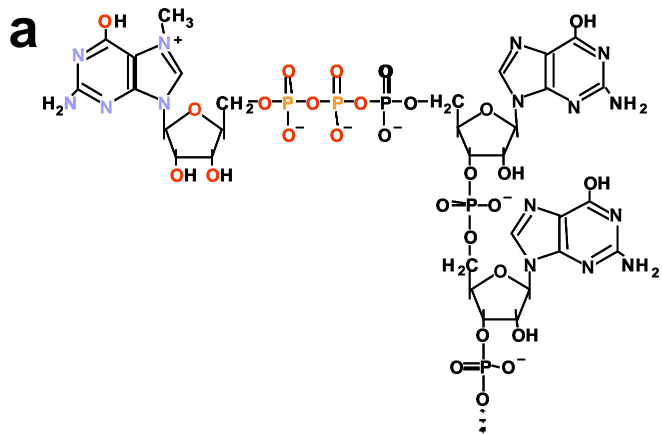
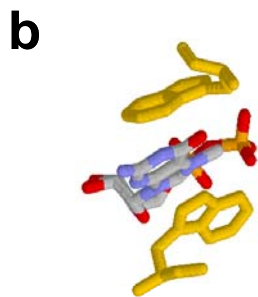


Figure 1





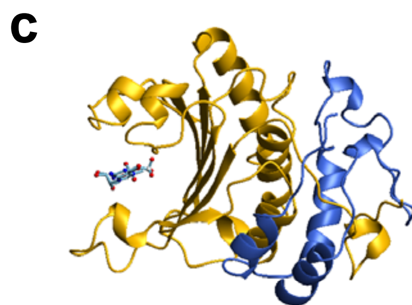


Figure 2

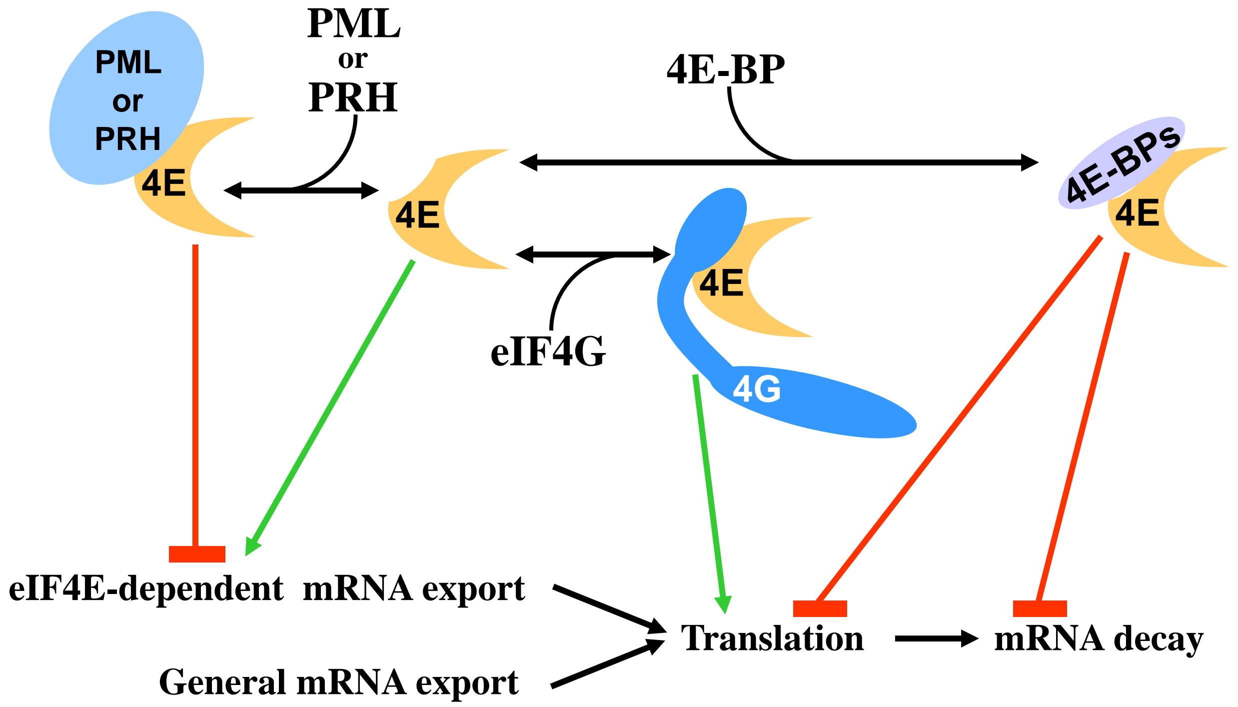


Figure 3

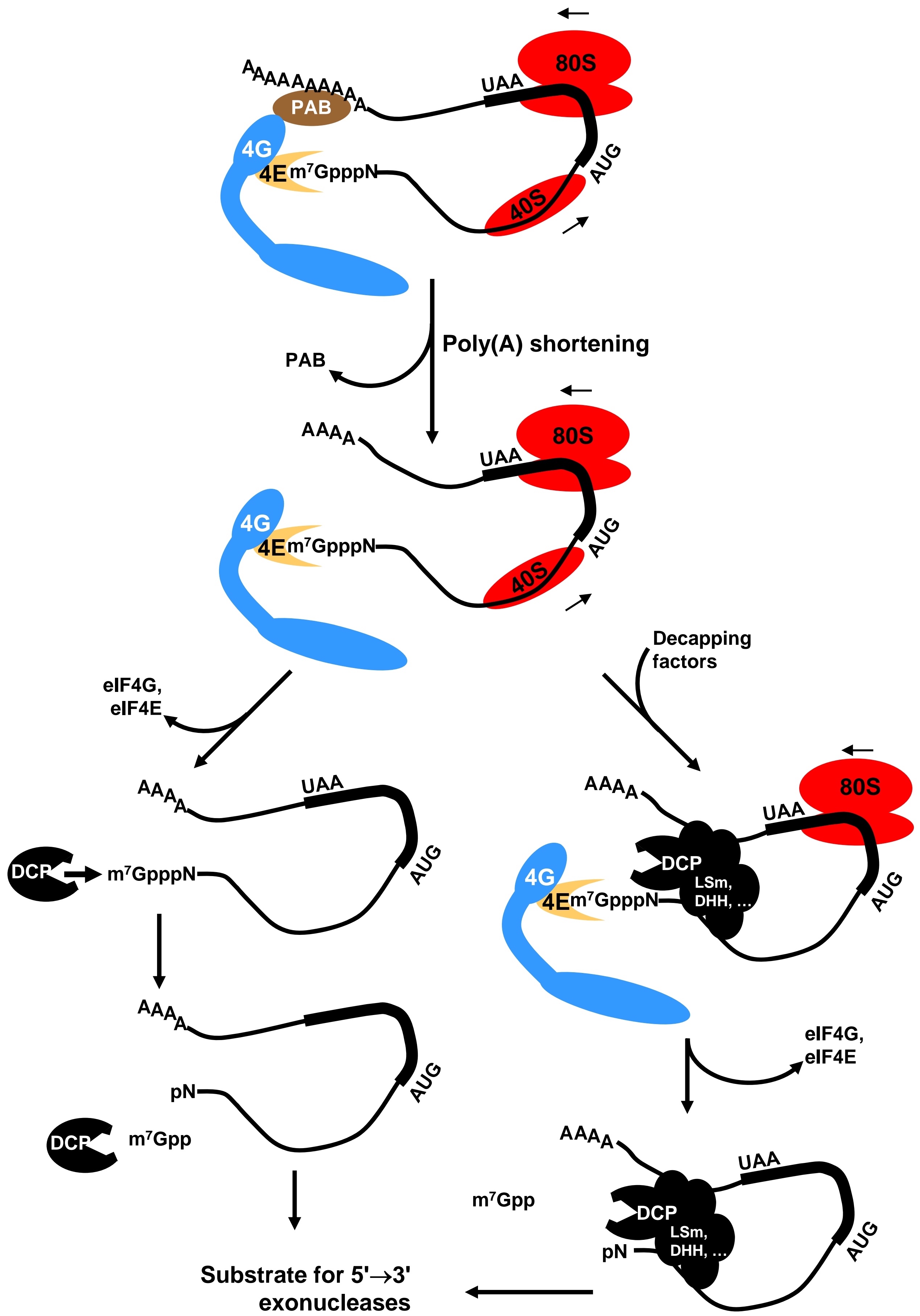


Figure 4