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Review

Initiation of protein synthesis in eukaryotic cells

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It is becoming increasingly apparent that translational control plays an important role in the regulation of gene expression in eukaryotic cells. Most of the known physiological effects on translation are exerted at the level of polypeptide chain initiation. Research on initiation of translation over the past five years has yielded much new information, which can be divided into three main areas: (a) structure and function of initiation factors (including identification by sequencing studies of consensus domains and motifs) and investigation of protein–protein and protein–RNA interactions during initiation; (b) physiological regulation of initiation factor activities and (c) identification of features in the 5′ and 3′ untranslated regions of messenger RNA molecules that regulate the selection of these mRNAs for translation. This review aims to assess recent progress in these three areas and to explore their interrelationships.

Keywords: translation; initiation; mRNA; review, regulation.

In 1986 I published a review describing the mechanism and regulation of initiation of translation in mammalian cells [2]. By that time most of the polypeptide initiation factors catalysing this process had been extensively purified and their individual activities studied in various *in vitro* systems. Several of them had been shown to be phosphoproteins and, in one case, eukaryotic initiation factor-2 (eIF-2), the effects of phosphorylation had been elucidated and two physiological kinases had been identified. There seemed to be a feeling in some circles that the most interesting problems in protein synthesis had been solved, and that only a few rather boring nuts and bolts awaited discovery.

Over the intervening ten years there has been an explosion of research activity in this area, largely fuelled by information yielded by molecular biology and genetic techniques. Cloning of cDNAs encoding initiation factors has revealed domain structures indicative of function and potential regulatory mechanisms. Experiments exploiting the ability to elucidate and manipulate mRNA sequences have demonstrated that translational control contributes to changes in patterns of gene expression during growth, differentiation and development to an extent that would have seemed inconceivable in 1985. Such experiments have, in particular, revealed important roles for structural features in the 5′ and 3′ untranslated regions (UTRs) of mRNA molecules in the regulation of mRNA utilization. Experiments employing techniques of gene transfection have also implicated translation in the control of cell proliferation. Moreover, a completely novel mechanism of initiation has been defined for the translation of a class of viral RNAs, and there are now indications that such a mechanism may also be utilized by some cellular mRNAs.

In this ten-years-after review, I have first attempted to summarize recent major developments in our understanding of translational mechanisms. In a longer section on regulation I have then tried to draw together two related aspects that often tend to be considered separately, i.e. the control of initiation factor activity and the influence of structural features of mRNA molecules. Clearly in such a wide area I have had to make subjective selections in the material covered and, particularly, in literature citation. I apologise especially to younger workers whose original contributions have been consolidated into citation of reviews by their laboratory head! Readers are directed to two recent general reviews on initiation of translation [3, 4], to a number of others which, though less all-embracing, discuss multiple topics in this area [1, 5–16] and to a new review which, for the first time, covers comprehensively the initiation of protein synthesis in plants [16a].

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Abbreviations. eIF, eukaryotic initiation factor, with suffix denoting individual factors as recently revised by IUB working party (see [1]); UTR, untranslated region (in mRNA); RRM, RNA recognition motif; ORF, open reading frame; EMCV, encephalomyocarditis virus; FMDV, foot-and-mouth disease virus; IRES, internal ribosome entry segment; PTB, polypyrimidine tract binding protein; HCR, heme-controlled repressor; PKR, protein kinase activated by RNA; dsRNA, double-stranded RNA; $p70^{S6K}$ and $p90^{rsk}$, 70 kDa and 90–92 kDa ribosomal protein S6 kinases; GSK, glycogen synthase kinase; IRE, iron-regulatory element; IRP, iron regulatory protein; PABP, poly(A) binding protein; AMV, alfalfa mosaic virus; CPE, cytoplasmic polyadenylation element (also known as ACE, adenylation control element); MAP, mitogen-activated protein.

MECHANISM OF INITIATION

Initiation of protein synthesis involves the sequential binding of first the 40S and then the 60S ribosomal subunit to a messenger RNA molecule. The process in eukaryotes can be divided into three stages (Fig. 1): (1) association of initiator tRNA ($Met\text{-}tRNA_i$) and several initiation factors with the 40S ribosomal subunit to form the 43S preinitiation complex; (2) the binding of this complex to mRNA, followed by its migration to the correct AUG initiation codon and (3) the addition of the 60S ribosomal subunit to assemble an 80S ribosome at the initiation codon, ready to commence translation of the coding sequence. This last step requires the prior release of the initiation factors bound

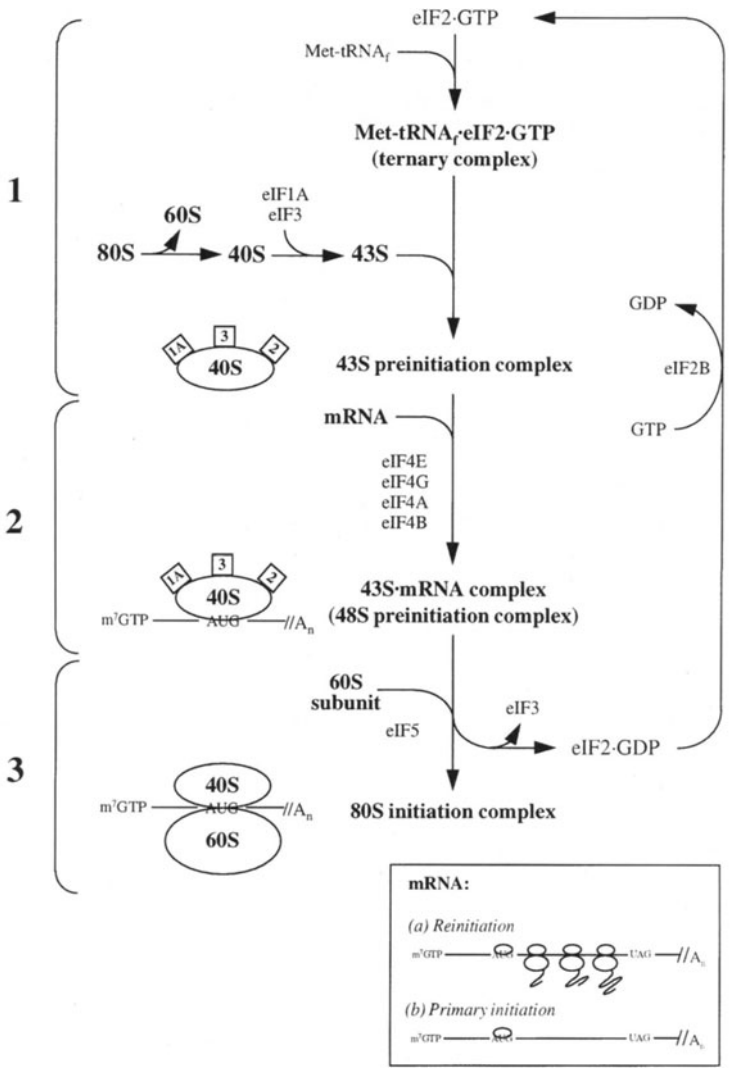

Fig. 1. Mechanism of initiation of protein synthesis. Stage 1, 80S ribosomes dissociate, and 40S subunits are captured for initiation by binding eIF1A and eIF3; the size of the latter causes the particle to sediment at 43S. Initiator tRNA (Met-tRNA$_f$) binds, in the form of a ternary complex with eIF2 and GTP, to give the 43S preinitiation complex. Stage 2, the 43S preinitiation complex binds to mRNA at the 5′ terminal m^{7}GTP cap structure, and then migrates along the mRNA towards the AUG initiation codon. The initial binding involves the factors eIF4E, eIF4G and eIF4A, which assemble at the 5′-end of mRNA, creating conditions that allow the melting of intramolecular secondary structures within the mRNA that would otherwise prevent the binding of the 43S preinitiation complex. In the vast majority of cases the most 5′ AUG codon is utilized for initiation. The term 48S preinitiation complex is frequently used, and refers to the 43S · globin-mRNA complex formed in the reticulocyte lysate (but see inset, below). Stage 3, when the 43S preinitiation complex stops at the initiation codon, the GTP molecule introduced as part of the eIF2 complex is hydrolysed to GDP, and this powers the ejection of the initiation factors bound to the 40S ribosomal subunit. The initiation factor eIF5 is involved in this process. The release of these factors permits the association of a native 60S ribosomal subunit, to reconstitute an 80S ribosome at the initiation codon poised to commence the elongation stage of translation. The continuity of initiation events requires the recycling of initiation factor molecules. eIF2 is released as a binary complex with GDP and requires a guanine nucleotide exchange factor, eIF2B, to catalyse the regeneration of the eIF2. GTP complex required to recruit the next Met-tRNA$_f$ molecule. Inset: (a) Most initiation events *in vivo* involve binding of the 43S preinitiation complex to mRNAs engaged in pre-existing polysomes (sometimes called reinitiation; note, however, that this term is also used to define a separate mechanism involving two initiation events on the same mRNA, as described later in this review). (b) primary initiation on to an mRNA molecule vacant of ribosomes is relatively rare *in vivo*, but is required for utilization of newly synthesized transcripts or for recruitment of mRNAs from an untranslated pool in response to a growth or differentiation signal.

to the 40S ribosomal subunit during the earlier stages; these factors are then recycled to catalyse further initiation events.

Formation of the 43S preinitiation complex

eIF2. This factor is a complex of three polypeptide chains, α, β and γ, which appear to remain associated throughout the initiation cycle. For all three polypeptides, cDNAs have been cloned and sequenced from both mammalian cells and *Saccharomyces cerevisiae* (Table 1); the α and β subunits have also been cloned from *Drosophila*. The most well-defined function of eIF2 is to recruit the initiator tRNA and conduct it as a Met-tRNA$_f$ · eIF2 · GTP ternary complex to the 43S ribosomal subunit (reviewed previously [2] and recently [4, 17]). Met-tRNA$_f$

Table 1. Cloning of initiation factor cDNAs. This table is updated from [8]. Accession numbers refer to GenBank and SwissProt databases. DM = *Drosophila melanogaster.*

Factor	Vertebrates				*S. cerevisiae*			Other species	
	former name(s)	species	mass	accession no.	name	mass	accession no.	species	accession no.
			kDa			kDa			
eIF1A	eIF-4C	human	16.5	L18960	TIF11	17.4	U11585	wheat	L08060
eIF2α		human	36.2	JO2645	SUI2	34.7	M25552	DM	L19196
		rat		36.1	JO2646				
eIF2β		human	38.4	M29536	SUI3	31.6	M21813	DM	L19197
		rabbit	38.3	X73836					
eIF2γ		human	51.8	L19161	GCD11	57.9	L04268		
eIF2Bα		rat	33.7	U05821	GCN3	34.0	M23356		
β		rabbit	39.0	Z48222	GCD7	42.6	L07116		
		rat		U31880					
γ		–			GCD1	65.7	X07846		
δ		rabbit	57.6	X75451	GCD2	70.9	X15658		
		rat	57.8	Z48225					
ε		rabbit	80.2	U23037	GCD6	81.2	L07115		
		rat	80.2	U19151					
eIF3					SUI1	12.3	M77514		
							S31245		
					GCD10	54.4	X83511		
					PRT1	88.1	J02674		
eIF4AI	eIF-4β	mouse	46.3	X14421	TIF1	45.0	X12813	DM	X69045
eIF4AII		human		D30655	TIF2	44.6	X12814	wheat	Z21510
		mouse	46.4	X12507				rice	D12627
								maize	U17979
eIF4B		human	69.2	S12566	TIF3	48.5	X71996		
eIF4E	CBP1, eIF-4α	human	25.1	M15353	CDC33	24.3	M29251	wheat p26	Z12616
		mouse	25.0	A34295				p28	M95818
		rabbit	25.1	X61939				DM	L37034
eIF4G	p220	human	154	D12686	TIF4631	107	L16923	wheat	
					TIF4632	104	L16924	p82	M95747
	eIF-4γ	rabbit	154	L22090					
eIF5		rat	49.0	L11651	TIF5	45.2	L10840		

can only bind to a binary complex of eIF2 and GTP. Cross-linking studies originally suggested interaction of GTP with both β and γ subunits [4, 17], but both the human [18] and yeast [19] γ subunits are now known to contain all three consensus GTP-binding elements with the correct spacing and to show similarity to other GTP-binding proteins. The human β subunit also possesses two of these elements, but none of them occurs in this polypeptide from yeast [19, 20] or *Drosophila* [21]. The binding site for Met-tRNA$_f$ is still not fully identified [4]. Cross-linking studies again show close proximity of this ligand to the N-terminus of the γ subunit and the C-terminus of the β subunit [18]. Sequences in the C-terminal domain of the β subunit show similarity between the human, yeast and *Drosophila* proteins [21], and include a single zinc-finger-like motif [20–22], although active eIF2 is reported not to contain zinc [4]. Much attention has been given to the possibility that binding of eIF2 to mRNA, readily demonstrated *in vitro*, may have functional relevance (reviewed [17, 23]). This binding activity has been localized to the C-terminal domain of the β subunit [24], but appears to be relatively non-specific with respect to RNA. Potential interactions involving eIF2 could be with Met-tRNA$_f$ or with rRNA within the 40S ribosomal subunit as well as with mRNA. However, in yeast, mutations in each of the three subunits of eIF2 have been found to influence the fidelity of the interaction of the Met-tRNA$_f$ anticodon with codons serving as initiation codons [20, 25, 26]. The primary sequence of the α subunit of eIF2 reveals no consensus motifs connected with ligand binding; the most significant feature of this polypeptide is a conserved phosphorylation site (Ser51 in mammalian cells), which is the target for a family of protein kinases important in the regulation of protein synthesis (see below).

eIF3 and eIF1A. Under intracellular conditions the equilibrium between free, or native, ribosomal subunits and 80S couples is strongly weighted towards the latter. The factors eIF3 and eIF1A are thought to bind to newly dissociated 40S ribosomal subunits and to delay reassociation with 60S subunits for long enough to permit their recruitment for initiation. Sequence analysis of eIF1A from human, rabbit and wheat [27] and from *S. cerevisiae* [27a] identified no obvious ligand-binding motifs but revealed a dipolar molecule, with a basic N-terminus and an acidic C-terminus. This may be conducive to a role for the factor

as a bridge between other protein factors or between an initiation factor and the ribosome [27]. In subsequent work Northwestern blotting analysis demonstrated strong interaction between recombinant eIF1A and RNA [28] but the specificity of this has yet to be examined.

eIF3 is a multimeric complex of total molecular mass 500–750 kDa, consisting of at least eight polypeptide chains in mammalian cells and ten polypeptide chains in wheat germ (reviewed [1, 3, 4]). Two recent reports describe purification of this factor from *S. cerevisiae*, identifying five [29] and eight [30] polypeptide chains, respectively. Recent analysis of complete or partial cDNA clones [31] (and Asano, K., Naranda, T. and Hershey, J. W. B., personal communication) has led to the identification of the yeast polypeptides p90, p62, p39 and p16 as PRT1, GCD10, p36 and SUI1, respectively; the yeast p39 subunit is homologous to the p36 subunit of human eIF3. p16/SUI1 has been identified as an initiation factor previously listed as eIF1 [32]. The best characterized polypeptide of eIF3 is yeast PRT1 [29, 30]. The N-terminal domain contains an RNA recognition motif and is probably involved in ribosome binding [33]. eIF3 has long been known to stabilize 43S preinitiation complexes *in vitro*, and also to be essential for the binding of these complexes to mRNA. The molecular basis of its role in mRNA binding is still not clear, but potentially important interactions have been observed between eIF3 and other initiation factors involved in this step [3, 34]. In addition the yeast eIF3 complex binds directly to RNA [29, 30], probably via the 62-kDa subunit [30]; surprisingly, this is not a homologue of the 66-kDa polypeptide in mammalian eIF3, which has recently been shown to bind to an mRNA transcript in a Northwestern blot assay [28] (Asano, K., Naranda, T. and Hershey, J. W. B., personal communication). Chemical cross-linking experiments on mammalian 48S preinitiation complexes trapped by blocking the 60S joining stage of initiation showed the 66-kDa eIF3 polypeptide to cross-link to globin mRNA within these particles and also to 18S ribosomal RNA of the 40S subunit [35]. Together with immunoelectron microscopy, these studies gave rise to models depicting the alignment of eIF3 and eIF2 on the 40S subunit, and identified relationships between these factors, 18S rRNA and surface domains of specific ribosomal structural proteins [35].

Guanine nucleotide exchange on eIF2: the recycling factor eIF2B. eIF2B, a complex of five polypeptide chains in both mammalian cells and yeast, catalyses the guanine nucleotide exchange reaction required to recycle the eIF2 released from initiation complexes as an eIF-2 · GDP complex to the eIF2 · GTP form capable of recruiting a new molecule of initiator tRNA (Fig. 1). The mechanism and regulation of this reaction are surveyed in an excellent review by Price and Proud [36], and it is clear that there is still a lot to be learned. Two opposing mechanisms have been proposed, one a substituted enzyme, or ping-pong, mechanism similar to that utilized by the elongation factors Tu/Ts and the other involving an intermediate quaternary complex GTP · eIF2B · eIF2 · GDP (see [36]). Dholakia et al. [37] presented evidence in support of the latter mechanism by demonstrating labelling of one of the polypeptide subunits of eIF2B with 8-azido analogues of GTP. However, with complete sequence information now available on all five polypeptide subunits in yeast (see Table 1 and references in [36]) and on four of the mammalian subunits [38-41], no conserved GTP binding elements have been identified. The complexity of both mammalian and yeast eIF2B relative to other guanine nucleotide exchange proteins may reflect the multiple mechanisms involved in the regulation of this step in protein synthesis (see below). The mammalian ε subunit may be responsible for the guanine nucleotide exchange activity, which can be blocked by monoclonal antibodies recognizing this polypeptide [42].

mRNA binding to ribosomes

The binding of the 40S subunit to mRNA involves several initiation factors and has potential for controlling both the overall rate of translation and the relative rates of utilization of different mRNA molecules in response to physiological signals. For most eukaryotic mRNAs, the initiating 40S ribosomal subunit binds at the 5′ end of the message and then migrates in a 5′-3′ direction towards the initiation codon. Sequence analysis of mRNA molecules indicates considerable potential for the formation of hairpin loops and other intramolecular secondary structures. Regions of stable secondary structure within the 5′ untranslated region (5′ UTR) of mRNA impede initiation of protein synthesis, particularly if located near to the 5′ end, where the initial binding of the 43S preinitiation complex takes place [43, 44]. The initiation factors catalysing this mRNA binding step are believed to act in concert to (a) locate the 5′ end of the mRNA, (b) unwind any secondary structure that would impede ribosome binding, (c) direct the binding of the 43S preinitiation complex and (d) melt any further secondary structure that might inhibit migration to the initiation codon. Step (a) is achieved by the specific interaction of eIF4E with the mRNA cap and step (b) by eIF4A and eIF4B. The binding and placement of the 43S preinitiation complex (step c) is most likely achieved by the ability of the factor eIF4G to form a bridge between the cap-bound eIF4E and the incoming 43S complex; this factor may also facilitate step (b) by linking eIF4A into the complex. Subsequent melting of downstream secondary structure (step d) may be achieved by the recruitment of additional eIF4A and eIF4B molecules. I will first outline the properties of the individual initiation factors and some of the evidence for this general mechanism, and then present some further speculation on possible models. Early work on this stage in initiation was reviewed previously [2] and useful reviews of more recent work are [1, 3, 4, 6, 8, 45–49].

eIF4E. The amino acid sequences are now known for this protein in mammalian cells, *S. cerevisiae*, wheat and *Drosophila* (Table 1, see [50]). Its most characteristic function is recognition of the 5′-terminal m^7GTP cap on mRNA; analysis of yeast mutants suggests that at least some of the eight highly conserved tryptophan residues are important in this interaction [51, 52], consistent with biophysical studies on the human protein (see [4]). The eIF4E gene is essential for viability in yeast; a cDNA encoding the mammalian protein, though only 35% conserved at the amino acid level, can substitute for the yeast gene *in vivo* [53]. The yeast cell cycle division mutation cdc33, which induces a G1 block at the non-permissive temperature, has been localized to a single amino acid substitution in eIF4E, close to one of the conserved tryptophan residues; the mutated protein shows reduced cap recognition activity [51]. The phosphorylation of eIF4E in mammalian cells has been a major focus of investigation and is reviewed below.

eIF-4A. Biochemical characterization of this factor showed it to bind ATP and to exhibit RNA-dependent ATPase and ATP-dependent RNA duplex unwinding activity (reviewed previously [2, 4]), leading to the conclusion that its function is to melt secondary structures in mRNA. This idea was strengthened by data from cDNA sequencing, which revealed a series of motifs conserved not just between eIF4A molecules from different organisms but between eIF4A and an extended family of around 70 RNA and (mainly) DNA helicases [45, 49]. Work in the

laboratories of Sonenberg, Linder and Trachsel (reviewed in [45]) examined the functions of some of these conserved sequences by introducing mutations into recombinant yeast and mammalian eIF4A proteins, and led to a model in which the binding and hydrolysis of ATP permitted the binding of eIF4A to RNA and hence the unwinding activity. The ATPase A motif (AXXXXGKT), near the N-terminus, is required for ATP binding, and mutations abrogating this function also abolished the RNA binding and helicase activities of eIF4A. The ATPase B, or D-E-A-D, motif, appears to be involved in ATP hydrolysis. A C-terminal, arginine-rich, motif (HRIGRXXR) is important in RNA binding and is required for helicase activity. A fourth motif (SAT) is needed for helicase activity, but mutations in this area do not inactivate ATP binding, ATPase activity or RNA binding.

eIF-4B. *In vitro* assays of the RNA-dependent ATPase and ATP-dependent RNA unwinding functions of eIF4A show strong dependence of these activities on the factor eIF4B. The human cDNA encodes a 68-kDa protein containing an RNA recognition motif (RRM) near the N terminus and a central region rich in aspartate, arginine, tyrosine and glycine (the DRYG domain) [54, 55]. The polar C-terminal region bears several potential phosphorylation sites, but is not essential for RNA binding [55] and has no equivalent in the yeast homologue [56, 57]. The eIF4B gene is not essential for viability in yeast, but its disruption results in slow growth, and extracts derived from the defective cells show poor ability to translate mRNAs with structured 5′ UTRs [57]. Studies on mutant forms of the human protein indicate that the N-terminal RRM alone is not sufficient for RNA binding, and identify an arginine-rich sequence close to the DRYG region as important for both the RNA binding activity [55] and for the ability of eIF4B to stimulate the helicase activity of eIF4A [58]. The N-terminal RRM may function in an interaction of eIF4B with ribosomes [58, 58a].

eIF4G (eIF-4γ, p220) and the eIF4F complex. Part of the eIF4E in mammalian cells [4], yeast [59], plants [60] and *Drosophila* [61] is found in the form of high-molecular-mass complexes. In mammalian cells these complexes were originally characterized as having three components, eIF4E, eIF4A and eIF4G, and were referred to as eIF4F or eIF4 (reviewed [2, 4, 46]). However, some workers have isolated eIF4E · eIF4G complexes [4, 62], and the absence of an eIF4A polypeptide is also common to cap-binding complexes from yeast [59], plants [60] and *Drosophila* [61]. Emerging evidence now indicates that eIF4G functions to bring together, in the correct orientation and in close proximity to the cap, the components necessary to unwind secondary structure in the mRNA and place a 40S ribosomal subunit at the 5′ end. The N-terminal half of the molecule contains a binding site for eIF4E [34, 63], while the C-terminal half binds to ribosomes, possibly via interaction with ribosome-associated eIF3, and also has affinity for eIF4A [34]. Sequencing of a cDNA encoding human eIF4G revealed a 154-kDa polypeptide (Table 1). In *S. cerevisiae* two genes (*TIF4631* and *TIF4632*) were identified as encoding the largest polypeptide of an eIF4F complex that included eIF4E and a 20-kDa protein of unknown function [59]. The TIF4631 and 4632 proteins were 53% identical overall, but with 80% identity at the C-terminus. Both contained sequences resembling the RRM in other RNA binding proteins, but these were less clearly apparent in the sequence of the human homologue [64]. In plant cells the situation appears to be more complicated. Two distinct cap-binding complexes have been isolated; one form, known as eIF-4F contains polypeptides of 26 kDa and 220 kDa; the other, known as eIF-*iso*4F, contains 28-kDa and 82-kDa proteins [60]. The 26-kDa and 28-kDa proteins have cap-binding activity and show some sequence similarity to human and yeast eIF4E while, of the larger polypeptides, only the 82-kDa protein of eIF-*iso*4F has been sequenced and shows a small degree of similarity to the human and yeast forms of eIF4G [60].

Alternative models for the mRNA binding step. Detailed consideration of the mechanism of this step reveals uncertainty on the exact order of binding and dissociation events involved. Some possible models are shown in Fig. 2. A key question is whether the factors eIF4E, eIF4G and eIF4A associate to form the eIF4F complex before interacting with mRNA [model (a) in Fig. 2] or whether eIF4E first binds alone to the mRNA cap, with the subsequent addition of the other factors to assemble the eIF4F complex *in situ* at the 5′ end of the mRNA [model (b)] (for various views on this see [3, 4, 8, 46, 47]). A further variant of the latter model envisages eIF4G as part of the 43S preinitiation complex [47, 65] [model (c)]. The interaction of this molecule with the mRNA-bound eIF4E would then become a major determining event in the placing of the 43S preinitiation complex on the mRNA molecule. Evidence for this model has been presented by Rhoads' laboratory, who added radiolabelled eIF4E and eIF4G to reticulocyte lysate translation systems and found that the latter, but not the former, was incorporated into 43S preinitiation complexes [47, 65]. In contrast, others favour models involving the prior formation of an eIF4F complex, since *in vitro* assays with purified factors and mRNA indicate that the cap-binding activity of eIF4E and the RNA helicase activity of eIF4A are greater when the factors are in an eIF4F complex than when they are assayed as individual proteins [66, 67]. However, these assays tend to be performed in the absence of other components, such as ribosomal subunits and eIF3, which may themselves play influential roles *in vivo*. The function of eIF4B is still not clear. Even more uncertain is the timing of subsequent dissociation events (Fig. 2). Some models depict the release of eIF4E and eIF4G from the mRNA immediately after 43S binding [68], and a recent extension of this scheme proposes that these factors rapidly reassociate with cytosolic eIF4A (to form new eIF4F complexes) and then cycle back on to the mRNA in order to present additional eIF4A molecules required during scanning [46, 69]. However, work with radiolabelled factors indicates that both eIF4E and eIF4G are present on the 48S complexes that accumulate in the reticulocyte lysate in the presence of inhibitors of 60S subunit joining when, presumably, the 40S subunits have reached the initiation codon [65]. We have no evidence at present, however, on whether these factors are associated with the 40S subunit itself, or whether they remain bound to the mRNA cap (see Fig. 2).

Migration of the 43S ribosomal complex to the initiation codon

For over 95% of vertebrate and yeast mRNAs analysed, the most 5′ AUG codon is utilized as the initiation codon [6, 70, 71]. The migration or scanning process itself has so far proved difficult to examine. Scanning can be impeded by very stable secondary structures, but is less sensitive to inhibition by moderately stable hairpin loops than is the initial association between the 40S ribosomal subunit and mRNA [44]. This may indicate that the 40S subunit, once bound to mRNA, contributes to the unwinding of downstream regions of secondary structure [3, 4]. Most current models suggest that scanning involves further utilization of the ATP-dependent helicase activity of eIF4A, probably stimulated by eIF4B [1, 3, 4, 46, 57], although some data indicate that most of the ATP needed during initiation is utilized by the unwinding associated with the initial, cap-dependent mRNA binding stage [72].

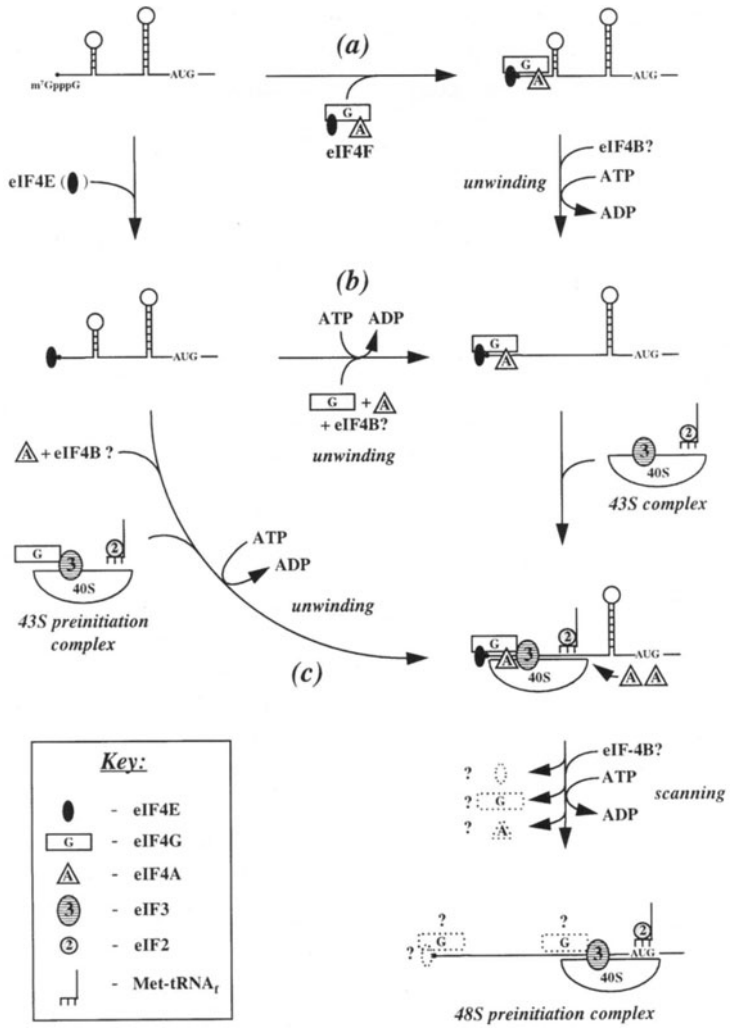

Fig. 2. Alternative pathways for binding the 43S preinitiation complex to mRNA. (a) An eIF4F complex between eIF4E, eIF4G and eIF4A is preformed in the cytosol and then binds to mRNA through the cap recognition function of eIF4E. Nearby secondary structure in the mRNA is unwound by the helicase activity of eIF4A, possibly stimulated by eIF4B, permitting the binding of the 40S ribosomal subunit and its subsequent migration to the AUG initiation codon. (b) eIF4E binds alone to the 5′ cap structure, followed by eIF4G and eIF4A to assemble the eIF4F complex on the mRNA itself. Subsequent steps are then as described for (a). (c) eIF4E binds alone to the 5′ cap, while eIF4G binds to the 43S preinitiation complex, probably by association with eIF3 already bound. After preliminary unwinding of the mRNA by eIF4A, the 43S preinitiation complex is directed to bind by virtue of protein–protein interaction between eIF4E and eIF4G. Symbols depicting initiation factors are listed in the key. Symbols with dotted outlines indicate uncertainty concerning the binding relationships between factors in the 48S preinitiation complex and the timing of factor dissociation from the initiation complex. Note that the 40S subunit covers about 30 nucleotides of mRNA.

Initiation at the 5′ proximal AUG codon. The next problem to consider is the mechanism that arrests the scanning 40S subunit at the AUG initiation codon. An obvious means of identifying an AUG codon is by codon-anticodon recognition involving the initiator tRNA, but this is clearly not the whole story. Kozak investigated the role of surrounding nucleotides in influencing the selection of AUG codons for initiation (reviewed in [43, 70]), using two complementary approaches: (a) analysis of sequences around the initiation codons of 699 vertebrate mRNAs and (b) examining the effects of sequence manipulation in the 5′ UTR on expression of a transfected gene. The sequence analysis revealed a consensus around the initiation codon: GCCGCCA/GCCAUGG, while the expression experiments showed that mutation to pyrimidines of the purine at −3 (relative to the first nucleotide of the initiation codon) or the G at +4 greatly decreased the chance of an AUG being recognized. Codons lacking these conserved, neighbouring nucleotides were described as being in a poor or weak context. Mutations in nucleotides immediately upstream, though less influential, were able to modulate the utilization of AUG codons in a weak context. Further experiments demonstrated the ability of an additional AUG codon in good context, inserted upstream, to intercept scanning 40S subunits. This provided a further assay for context effects, since changes in the sequence surrounding the inserted AUG codon could be assessed for their influence on its ability to intercept. Yeast mRNAs also tend to have a purine nucleotide, usually A, in the −3 position relative to the initiation codon, but they show weaker consensus at other positions [6, 73]. In plant mRNAs a G residue at the +4 position appears to be important [9].

CUG, ACG or GUG are occasionally used as initiation codons (see references in [74]); their recognition is again dependent on context. Studies with two viral RNAs identified the nucleotide in the +5 position (C or, preferably, A) to be as important as that in the +4 position in facilitating selection of non-AUG initiation codons [74, 75]. This position, not examined previously, may also be important for recognition of AUG codons in cellular mRNAs under some conditions, since reexamination of the sequences of the 699 mRNAs analysed by Kozak indicated a high frequency of A or C at position +5 [74]. Initiation at AUG codons in poor context, or at non-AUG codons, can be enhanced by base-paired structures around 14 nucleotides downstream [43]. Since the leading edge of a 40S ribosomal subunit poised over the AUG initiation codon would protrude about 12–15 nucleotides into the coding sequence, a hairpin structure at this position could serve to arrest or slow down the scanning particle at the appropriate position to allow more time for codon recognition [43, 71]. Under normal conditions, a strong surrounding context may in some way function similarly to slow down scanning in the region of the initiation codon. Finally, it should be noted that, in addition to codon–anticodon interaction between Met-tRNA$_f$ and mRNA, the factor eIF2 may also play a role in codon recognition (see above).

Initiation at downstream AUG codons. Although most initiation events take place at the first AUG codon encountered by a scanning 40S subunit, there are some notable exceptions, many of which have turned out to be of particular interest. These fall into three groups; two of these can be explained in terms of the scanning mechanism, but the third is incompatible with many of its features.

Leaky scanning. This occurs when the first AUG codon is in poor context and is consequently by-passed by many 40S subunits, which then initiate at an AUG codon in stronger context further downstream. This accounts for a number of cases where an RNA (often viral) appears to encode two products [76]. If the first and second AUG codons are in the same open reading frame, with no termination codon in between, the result is two translation products with the longer having an N-terminal extension. If the two AUGs are in different reading frames, two distinct products are translated.

Reinitiation. Some mRNAs have one or more AUG codons upstream of the authentic initiation codon. An upstream AUG in strong context will severely inhibit translation by intercepting scanning 40S subunits. Upstream AUG codons are frequently followed closely by an in-frame termination codon, such that a short peptide is encoded. After translation of some such minicistrons, some of the 40S subunits remain associated with the mRNA, continue scanning and eventually commence initiation at the authentic initiation codon (see [43, 77, 78] for reviews). The criteria that determine whether or not this will occur are not yet clear, but some clues are emerging. (a) Nucleotides around the termination codon of the minicistron or the actual peptide sequence encoded may determine whether or not 40S subunits are released or resume scanning [77, 79, 80]; in addition, some property of the downstream cistron may influence its effectiveness at recruiting reinitiating 40S subunits [81]. (b) The chances of initiation at the downstream AUG codon increase with the distance of this from the upstream open reading frame (ORF). This is thought to reflect the time needed for the 40S subunit to reacquire a Met-tRNA$_f$ · eIF2 · GTP ternary complex, which is needed for recognition of the authentic initiation codon [10, 81]. (c) Successful reinitiation appears to require the upstream minicistron to be relatively short. This may be due to retention of some essential factor for a short time after completion of the primary initiation event [10, 70, 82]. The reinitiation mechanism underlies the well-known translational control system for the yeast transcription factor GCN4 [83], which is discussed below.

Internal initiation. Among mRNAs translated in eukaryotic cells, those of picornaviruses are exceptional; they are uncapped and possess 5′ UTRs that are several hundred nucleotides long and include regions of secondary structure sufficiently stable to prevent 40S subunits scanning from the 5′ end. In each case their translation product is a single polyprotein that undergoes posttranslational cleavage to yield a distinct set of functional viral polypeptides. The 5′ UTRs of these mRNAs also contain AUG codons in good context that would be difficult to by-pass if actually encountered by scanning 40S subunits. In the late 1980s an alternative, cap-independent, initiation mechanism was proposed for picornavirus RNAs. This involves the binding of the 43S preinitiation complex directly to an internal site either very close to the authentic AUG initiation codon or up to 160 nucleotides upstream of it, depending on the species of virus concerned. At first this mechanism was vigorously challenged [10, 11], but the mass of detailed information on the involvement of RNA structure now provides compelling evidence in favour of internal initiation, at least in the case of picornavirus RNAs. Further evidence comes from the demonstration that elements from picornavirus 5′ UTRs can direct ribosomes to translate artificial circular mRNAs [84]. The mechanism of internal initiation has been reviewed in detail [85–91] and will be discussed relatively briefly here.

The basic test for the ability of a 5′ UTR to direct internal initiation is that, when placed between the two cistrons in a bicistronic construct, it can promote active translation of the downstream cistron, even under conditions that prevent cap-dependent translation of the upstream cistron (reviewed [85–87, 90]). Careful consideration of potential pitfalls is required when applying this test to new mRNAs [87]. Such experiments were extended, particularly in the case of poliovirus and encephalomyocarditis virus (EMCV) RNAs, to examine the effects of progressive deletions in the 5′ UTR and thus delineate the minimum region required to direct internal initiation of the downstream cistron. These essential regions, approximately 450 nucleotides long [85], and at first given the evocative name of ribosome landing pads [92], are now, rather more soberly, referred to as internal ribosome entry segments or IRESes. Most studies so far have focussed on two major classes of picornavirus that infect mammalian cells, the cardiovirus/aphthovirus group (including EMCV, Theiler's murine encephalomyelitis virus and foot-and-mouth-disease virus [FMDV]) and the enterovirus/rhinovirus group (including poliovirus, human rhinovirus and Coxsackie virus). The distinguishing features of these classes, and the structures of the 5′ UTRs of their RNAs, are thoroughly described by Jackson and colleagues [85, 86]. For both classes the entry site on the RNA for the incoming 43S preinitiation complex is an AUG codon near the 3′ end of the IRES. For cardiovirus RNAs the AUG at the entry site is utilized for initiation by most 40S subunits, and little or no scanning is required. Initiation on the RNA of the aphthovirus FMDV utilizes two closely placed AUG codons [93, 94]. For enterovirus RNAs the AUG at the entry site is not utilized for initiation, and the normal scanning mechanism is thought to be involved in the passage of the 40S subunit to the next AUG downstream. However, the mechanism may be more complicated [95]. In addition to the main classes of picornavirus mentioned above, internal initiation appears to be responsible for translation of the RNA of the third class, hepatitis A [91], and those of the non-picornaviruses hepatitis C [88] and pestiviruses [96]. However, there is some doubt in the case of plant coronaviruses and potyviruses [87].

In all the picornavirus RNAs examined the IRES regions in the 5′ UTR comprise a complex pattern of stem-loops organized into a series of highly structured domains [85, 86, 97–99]. The one widely conserved region of sequence conservation between the 5′ UTRs of picornavirus RNAs is an oligopyrimidine tract at the 3′ end of the IRES, followed by a spacer region of about 25 nucleotides, immediately upstream of the 40S subunit entry site. However, the role of this is still uncertain [85, 100]. Secondary structure is much more highly conserved, and mutational analysis demonstrates its importance in the function of IRES elements. A role for base-pairing interactions between picornavirus 5′ UTRs and the 3′ end of 18S ribosomal RNA has been suggested (reviewed [101]). Secondary and tertiary structure could facilitate this by providing the correct orientation, but to date this proposal awaits supporting evidence. While the secondary structure of the RNA may itself function sterically to form a ribosome binding site, reports are rapidly accumulating of general and tissue-specific polypeptides that interact with different elements of picornavirus IRESes [85, 89, 91, 97–99, 102, 103]. This binding mainly involves recognition of secondary and tertiary structural motifs, and it seems likely that protein complexes are assembled by virtue of protein–protein, as well as protein–RNA, interactions [91, 97–99]. However, caution may be required in the interpretation of cross-linking experiments involving IRES elements [87]. A recent, intriguing, report suggests that a small RNA, so far isolated only from yeast, may specifically inhibit poliovirus RNA translation by competing for essential IRES-binding proteins [104]. Surprisingly, two proteins found to interact with several IRESes, the 57-kDa polypyrimidine tract binding protein (PTB) and the autoantigen La [91, 102, 103, 105, 106], already have known functions in nuclear events such as RNA transcription and processing. La has been reported to increase in concentration in the cytosol during poliovirus infection, to have RNA unwinding activity *in vitro* [107] and to improve the fidelity of initiation at the correct AUG codon of poliovirus RNA [105, 106], although unphysiologically high concentrations of La are required for the last effect. However, for neither of these proteins has a precise role in internal initiation yet been established. Of equal interest is the potential role of *trans*-acting cellular proteins that may be specific to, or unequally distributed between, different cell types [88, 89]. Translation of poliovirus RNA is notoriously sluggish in the reticulocyte lysate translation system but is stimulated by the addition of crude HeLa cell extract; this may partly reflect differences in concentrations of La or PTB and associated proteins [85, 102], but it seems likely that other factors are involved [88]. The distribution of *trans*-acting proteins may be an important factor in determining relative degrees of virulence of picornaviruses in different cell types [89, 91, 98]. Since it is unlikely that cells and organisms would evolve proteins whose primary function was to facilitate the replication of invading viruses, an important question is whether the phenomenon of internal initiation extends beyond picornavirus RNA translation. Indeed, several cellular mRNAs have now been reported to utilize this mechanism (see below).

Joining of the 60S ribosomal subunit to the preinitiation complex

It would seem reasonable that the reassociation of a 60S subunit with the 40S particle bound at the initiation codon would require the prior release of the factors that reinforced dissociation of the ribosomal subunits earlier in the pathway. This was indeed found in early work using translation systems reconstituted from purified initiation factors (reviewed previously [2, 4]). The release of initiation factors is dependent on the hydrolysis of the GTP molecule bound to the eIF2 within the preinitiation complex, and this is catalysed by the factor eIF5. The amino acid sequences of both mammalian [108] and yeast [109] eIF5 are now known to include motifs typical of the GTPase superfamily. However, the factor only promotes GTP hydrolysis in the presence of 40S subunits. Both the mammalian and yeast factors are monomers (49 and 45 kDa respectively [108, 109]), but in earlier studies eIF5 activity from ribosome salt-wash preparations always behaved as a complex of around 150 kDa [110, 111]. Recent work suggests that this is due to a highly specific association of eIF5 with eIF2 [112]. Thus it seems likely that these two factors interact on the surface of the 40S subunit when the initiation complex is aligned over the initiation codon, resulting in activation of GTPase, hydrolysis of the eIF2-bound GTP molecule and ejection of eIF2 · GDP, eIF3 and probably other factors from the initiation complex. Presumably there is a mechanism to prevent this occurring prematurely on the 43S preinitiation complex or during mRNA binding or scanning. Very little attention has been given to the regulation of the 60S subunit joining step or its potential as a control point in translation. However, there are a few provocative observations. Firstly, eIF5 is a phosphoprotein, with at least two sites that can become metabolically labelled with [^{32}P]phosphate in mammalian cells [110]. Secondly, at least in the reticulocyte lysate, the joining of the 60S subunit seems to be a slow step relative to mRNA binding to the 40S subunit [113]. Finally, again in the reticulocyte lysate, this is the step at which the possession of a poly(A) tail seems to confer kinetic advantage on recruitment of mRNAs into polysomes [114] (see below).

REGULATION OF TRANSLATIONAL INITIATION

Translation is now recognized as an important site of regulation of gene expression, with the initiation stage as the most commonly observed target for physiological control. Modulation of initiation can influence both the overall, global, rate of protein synthesis (quantitative regulation) and the relative rates of synthesis of different proteins (qualitative regulation); frequently, controls at these two levels are superimposed. Control of the overall rate of protein synthesis is potentially important in achieving cell growth during the G1 phase of the cell cycle, while the concentrations of an increasing number of specific proteins involved in the control of cell proliferation or differentiation are now thought to be modulated at least in part at the translational level. Two particular steps of the initiation pathway appear to be hot spots for physiological regulation, the binding of Met-tRNA$_f$ to the 40S ribosomal subunit, mediated by eIF2, and the initial binding of the 43S preinitiation complex to the 5′ end of mRNA, mediated by eIF4E and associated factors. The first of these, which precedes mRNA involvement, is mainly, but not exclusively, relevant to quantitative regulation, whereas the mRNA binding step can, in addition, exert preferential effects on the translation of different mRNAs. Two recurrent themes repeatedly surface during investigation of translational regulation, namely phosphorylation of initiation factors and the influence of structural features in the 5′ and 3′ untranslated regions of mRNA molecules. Links between these two themes may be forged where features of an mRNA molecule may render its translation particularly sensitive to modulation of the activity of particular initiation factors. Several of the initiation factors are phosphoproteins, but the clearest links between phosphorylation and the regulation of translation concern the factors eIF2 and eIF4E. Regulatory features in mRNA molecules include structures that may act directly (for example by impeding 40S subunit binding or scanning) or indirectly, by providing a binding site for a *trans*-acting protein.

Table 2. Phosphorylation of initiation factors.

Factor	Site	Protein kinases	Physiological conditions	Effect on activity
eIF2α	Ser51	HCR	iron (heme) deficiency (reticulocytes)	increased affinity for eIF2B, which becomes sequestered in eIF2B · eIF2 · GDP complexes
		PKR	virus infection/interferon treatment; depletion of calcium stores	
		GCN2	amino acid starvation (*S. cerevisiae*)	
		?	heat shock	
		?	amino acid or serum starvation (mammalian cells)	
eIF4E	Ser209	not known (Protein kinase C or protamine kinase *in vitro*)	phosphorylation increased by: mitogenic stimulation of quiescent mammalian cells; overexpression of *src* or *ras* oncogenes; meiotic maturation of *Xenopus* oocytes phosphorylation decreased by: heat shock; entry into mitosis; infection by adenovirus or influenza virus.	? increased affinity for mRNA cap ? increased association with eIF4G

Regulation of eIF2 activity

Many physiological conditions that inhibit initiation of protein synthesis have been shown to decrease the activity of eIF2, and, consequently, to impair the formation of 43S initiation complexes (reviewed [2, 4, 7, 8, 17, 23, 115–121]). To date, the site of control of eIF2 activity has always been identified as the recycling step involving the guanine nucleotide exchange factor, eIF2B. Two different physiological mechanisms appear to regulate this step. The first involves phosphorylation of the α subunit of eIF2 at a single serine residue, Ser51 (Table 2); this results in increased affinity of eIF2 for eIF2B, but the complex formed fails to carry out guanine nucleotide exchange. The effect of this is to decrease the concentration of eIF2B available to recycle even the remaining non-phosphorylated eIF2 · GDP. This mechanism is rendered particularly effective by the low molar ratio of eIF2B/eIF2 in all mammalian tissues examined [42]. The second mechanism, discovered more recently, involves direct regulation of eIF2B activity, independent of changes in eIF2 phosphorylation.

Regulation of eIF2 phosphorylation. Three protein kinases that specifically phosphorylate eIF2α at Ser51 have been cloned and sequenced (Table 2; see reviews [116, 122–125] for comparisons of domains). These each possess 11 catalytic domains widely conserved between protein kinases, but show little sequence similarity elsewhere. Another common feature is the presence of an insertion sequence separating groups of kinase domains, though only a small portion of the insert shows significant similarity between the three kinases. Regions within each of the individual kinases have been suggested to have regulatory functions associated with their responses to different physiological signals.

The heme-controlled repressor (HCR; Table 2) [122, 123, 125–128] is a 70 kDa protein which behaves as a 90-kDa polypeptide on SDS/PAGE. Its physiological function is to prevent protein synthesis in erythroid cells (over 90% of which is devoted to the production of globin chains) in the absence of the heme prosthetic group. Its ability to phosphorylate eIF2α closely correlates with autophosphorylation at multiple sites. Interaction with heme results in loss of both autophosphorylation and kinase activity towards eIF2α, and is probably associated with inability to bind ATP. Heme-mediated inactivation is thought to involve the formation of disulfide links between HCR subunits (reviewed [122, 123, 125, 126]). However, interactions between HCR and heat shock proteins, particularly hsp90 and hsp70, have also been demonstrated [129–131] (reviewed [123, 125]). In addition a 67-kDa protein in the reticulocyte lysate that can inhibit the phosphorylation of eIF2α by activated HCR has been proposed to play a physiological role [132]. Chen and coworkers find that, in keeping with its proposed physiological function, expression of HCR at both the protein and the mRNA level is restricted to erythroid cells [123, 126, 133]. However, Mellor et al. [134] have succeeded in cloning a rat brain cDNA encoding an eIF2α kinase with over 80% similarity at the amino acid level with rabbit reticulocyte HCR. They also detected low levels of mRNA recognized by this cDNA in a number of other tissues. This raises the possibility that HCR-like kinases may exist in non-erythroid cells to mediate effects of one or more of the other physiological signals that result in increased phosphorylation of eIF2α, such as heat shock or nutrient starvation [23, 117, 118, 121] (Table 2).

The second eIF2α kinase, PKR (Protein Kinase activated by double-stranded RNA), is important in the defence of mammalian cell populations against viral invasion. It is markedly induced by transcriptional activation in response to interferons α or β released by neighbouring cells. Upon subsequent viral infection, the kinase is activated and severely inhibits translation by increasing eIF2α phosphorylation and blocking the recycling activity of eIF2B. This, while clearly deleterious to the individual cell, prevents the utilization of its translational apparatus for the production of viral proteins and hence restricts viral replication within the cell population as a whole. The structural features of PKR are described in recent reviews [116, 124, 125, 128, 135]. In addition to the catalytic domains common to protein kinases, it possesses two N-terminal domains involved in binding its activator, double-stranded RNA, of which the first is more critical for activation [116, 124, 125, 128, 135–140]. Activation is closely associated with autophosphorylation at multiple (but unidentified) sites and appears to involve dimerization. A popular model attributes the autophosphorylation to mutual phosphorylation between two PKR molecules brought into close proximity by binding to a single molecule of dsRNA. This model appears to be consistent with many observations [128, 135, 137, 138, 140a], but it seems likely that protein–protein interactions are also involved; recent data suggest that alternative mechanisms bringing about dimerization without the mediation of dsRNA may also exist [136, 139]. The nature and origin of the dsRNA molecules responsible for the physiological activation of PKR are not clear in all cases, although such molecules are produced as part of the replication cycle of many viruses.

Many viruses have evolved strategies to subvert the host defence mechanism mediated by PKR (see Table 3 and reviews [10, 128, 135, 141–146a]). These involve a variety of virally encoded molecules, including small RNAs with extensive sec-

Table 3. Strategies employed by viruses to ensure efficient translation of their own products during viral infection.

Strategy	Virus	Mechanism
Production or activation of agents that challenge host defences mediated by interferon-induced PKR	adenovirus	production of virus-associated RNA (VA-1) which interacts with PKR to prevent activation by double-stranded RNA
	influenza virus	activation of cellular protein (p58) which binds PKR and prevents activation
	poliovirus	degradation of PKR protein
	vaccinia virus	encodes two proteins: (i) E3L, which binds double-stranded RNA and prevents it activating PKR; (ii) K3L, which resembles part of eIF2 and probably acts as a decoy
	herpes simplex virus	encodes protein that inhibits PKR by unknown mechanism
Production of viral RNAs with high translational efficiency	alfalfa mosaic virus (AMV); adenovirus	viral mRNAs have unstructured 5′ UTRs, thought to exhibit lower initiation factor requirements than host mRNAs
	influenza virus	5′ UTRs of viral RNAs confer high translational efficiency in infected cells (unknown mechanism)
Inactivation of mRNA-binding initiation factors, conferring advantage on viral RNAs which exhibit low factor requirements	adenovirus	decreased phosphorylation of eIF4E in infected cells; may have relatively low impact on translation of viral RNAs with unstructured 5′ UTR
	influenza virus	
	poliovirus	Cellular eIF4G cleaved in infected cells, probably by virally encoded proteases; translation of uncapped picornavirus RNAs remains operative when eIF4G is cleaved.
	human rhinovirus	
	Coxsackie virus	
	foot-and-mouth disease virus	

ondary structures that bind PKR, proteins that bind and sequester dsRNA, a protease that degrades PKR in poliovirus-infected cells and a protein that resembles a truncated version of the eIF2α substrate of PKR. Infection by influenza virus activates a cellular inhibitor of PKR, p58, by somehow effecting its release from a complex with another cellular protein, I-p58, which normally holds it in an inactive form [146, 147].

While the importance of PKR in the battle between host cells and invading viruses is clearly evident, much excitement has been generated recently by observations implicating this protein kinase in regulation in *uninfected* cells. This would require activation either by cellular RNA molecules or by alternative mechanisms. Two reports implicate PKR in the increased eIF2α phosphorylation in mammalian cells treated with calcium-mobilizing agents [148, 149]. A role for this kinase in growth regulation was suggested by observations of increased PKR activity in 3T3 fibroblasts as they approached stationary phase [150]; the cloning of a cDNA encoding human PKR subsequently permitted more direct approaches. Overexpression of PKR in yeast cells was found to result in severe inhibition of growth, concomitant with increased eIF2α phosphorylation [151]. This experiment has not proved feasible for higher eukaryotic cells, probably because high levels of PKR are so inhibitory that one cannot obtain enough cells to examine. However, it has been possible to do the converse experiment, i.e. to suppress endogenous PKR activity, because several different mutations in PKR result in molecules that have *trans*-dominant inhibitory properties both *in vivo* and *in vitro*. These include a point mutation [152, 153] and a deletion [154] within the catalytic (kinase) domains and the deletion of the more critical of the two dsRNA binding domains [139, 140]. Mechanisms by which these mutant molecules might down-regulate endogenous PKR activity are reviewed in [135]. Expression of each of these mutant forms in NIH3T3 cells has been shown to result in malignant transformation [139, 152, 154]; similar results were obtained in cells where PKR was down-regulated by over-expression of the inhibitory protein, p58 [155] (see Table 3). The simplest conclusion from these experiments is that wild-type PKR in normal cells may be involved in restraint of cell growth. An important question is whether this potential tumour-suppressor role involves regulation of phosphorylation of eIF2α. Most of these *trans*-dominant mutants reduced the phosphorylation of endogenous eIF2α [139, 156], and this was also the case in cells overexpressing p58 [155]. If eIF2α phosphorylation does mediate a role for PKR in growth control, one might expect that overexpression of the non-phosphorylatable mutant of this factor, Ser51→Ala, would itself be tumourigenic. However, conflicting results have been reported [149, 156]. In any case, it now seems likely that PKR has other, possibly unrelated, roles in cellular regulation; recent data link this kinase to signal transduction pathways involving a number of growth factors and cytokines [136, 157, 158] and it can phosphorylate another target, IκB, which is involved in transcriptional control [136, 159, 160]. Moreover, a further challenge to a simple role for PKR as a tumour suppressor comes from the recent report that mice with the PKR gene inactivated failed to develop spontaneous tumours [161].

The third well-characterized eIF2α kinase, GCN2, is a *Saccharomyces cerevisiae* protein of 182 kDa, which is activated during chronic amino acid starvation. Expression of a constitutively active mutant of this enzyme in yeast results in inhibition of overall protein synthesis in a manner similar to that resulting from activation of HCR or PKR in mammalian cells. However, the physiological control mediated by GCN2 is more subtle: overall protein synthesis is scarcely inhibited when yeast is starved for amino acids, yet the perturbation of eIF2 function is able to switch on the translation of the mRNA encoding a specific protein, GCN4, which is not produced at all in fed cells. The physiological significance of this is that GCN4 is a transcription factor that promotes expression of genes encoding a number of enzymes involved in *de novo* amino acid synthesis; thus the ability to switch on its synthesis provides a mechanism for the yeast cells to compensate for their nutritional deficiencies.

The mechanism by which eIF2α phosphorylation, normally associated with inhibition of overall protein synthesis, can actually enhance the translation of GCN4 mRNA is summarized in several recent short reviews [83, 117, 124, 128] and, in more detail, with recent experimental evidence in [162]. The studies leading to the elucidation of this mechanism, in the laboratory of Hinnebusch and colleagues, illustrate particularly well the

power of applying complementary genetic and biochemical approaches to translational regulation; Wek [124] provides a clear introduction to this aspect. At the heart of the mechanism is the location of the GCN4 coding sequence downstream of four short open reading frames (ORF 1–4), such that its translation is dependent on a reinitiation mechanism as described above. The most 5′ of these minicistrons has properties that permit about 50% of 40S ribosomal subunits to remain on the mRNA and resume scanning. Under fed conditions virtually all these reacquire a new Met-tRNA$_f$ · eIF2 · GTP ternary complex in time to translate the third or fourth ORF, both of which have properties leading to complete release of the ribosomes that translate them. Thus access of ribosomes to the GCN4 coding sequence is completely precluded. During amino acid starvation, the activation of GCN2 leads to an increase in eIF2α phosphorylation which, by impairing eIF2B activity, reduces the availability of ternary complexes. The reinitiating ribosomes therefore take longer to recapture a ternary complex, and some of them become competent to translate only after scanning past the inhibitory ORFs. These are able to reach and recognize the initiation codon of the GCN4 coding sequence, which is then translated.

An important consequence of the study of this system was the characterization of yeast eIF2γ and all the polypeptide subunits of yeast eIF2B, which were originally identified as products of genes involved in the regulation of GCN4 translation [36, 83, 117, 124, 128, 162]. This greatly assisted the characterization of mammalian eIF2B, and is beginning to shed light on the interactions of eIF2B with phosphorylated eIF2, which is also important for the understanding of regulation by HCR or PKR. The yeast homologue of the smallest subunit of eIF2B, eIF2Bα, is the product of the *GCN3* gene; genetic analysis predicts this to be non-essential for basic eIF2B function but important in the response to activation of GCN2. Recent work now indicates that two further subunits of eIF2B, GCD2 and GCD7 (corresponding to mammalian eIF2Bβ and δ), while essential for guanine nucleotide exchange activity, also play a similar regulatory role in recognizing the phosphorylated form of eIF2 [162]. Interestingly, regions of these three subunits exhibit some sequence similarity with each other in both yeast and mammalian eIF2B [36].

Two important questions concerning GCN4 regulation are still not fully answered. Firstly, the phosphorylation of eIF2α during amino acid starvation seems to delay the capture of ternary complexes by reinitiating 40S subunits under conditions where overall rates of initiation, including primary initiation at ORF1 of GCN4 mRNA, are hardly affected. Thus the main role of ORF1 seems to be to ensure that initiation at ORF3 or ORF4, which precludes GCN4 translation, is extra sensitive to a subtle change in eIF2 phosphorylation. But why is reinitiation more sensitive? One possibility, suggested by Hinnebusch [162], is that ternary complex binding is rate-limiting for reinitiation but not necessarily for primary initiation, which may instead be controlled at the mRNA binding step. The second question concerns the complex area of how GCN2 activation is triggered by amino acid starvation. Current ideas on this result from structural and mutational analysis of two domains in the C-terminal half of the kinase molecule that are essential for activation [124, 162]. One of these is a region of 530 amino acids that shows 22% identity and 45% similarity with yeast histidyl-tRNA synthetase, and evidence is beginning to appear for recognition by this domain of uncharged tRNA, thought to accumulate during amino acid starvation [163]. The other is a region of 120 amino acids at the C-terminus, required for association of GCN2 with ribosomes, which appears to be necessary for activation. Such association may bring GCN2 into closer contact with its activator (uncharged tRNA bound to the ribosomal A-site) and/or allow the kinase activity to be targeted specifically at ribosome-bound eIF2. Additional genes, *GCN1* and *GCN20*, recently identified as involved in the activation, are currently under investigation [162, 164]. A type-I protein phosphatase, GLC7, appears to reverse the effect of GCN2 by dephosphorylating eIF2α [165].

Direct regulation of eIF2B activity. Over recent years several examples have emerged where the guanine nucleotide exchange activity of eIF2B appears to be regulated in the absence of a change in eIF2α phosphorylation (reviewed [36, 117, 119, 120, 125]. A particularly well characterized example concerns regulation by insulin (reviewed [36, 120, 166, 167]). Treatment of fibroblasts, or Chinese hamster ovary cells overexpressing the insulin receptor, with the hormone increases the eIF2B activity detectable in crude cell extracts, apparently by down-regulating an inhibitory activity [168, 169]. Studies on the purification behaviour of this inhibitor led to the suggestion that it may be glycogen synthase kinase-3 (GSK-3), an insulin-regulated enzyme known to phosphorylate a number of other targets. Indeed, purified GSK-3 does phosphorylate the largest (ε) subunit of mammalian eIF2B, and also inhibits guanine nucleotide exchange activity when added to crude cell extracts [36, 169]. This evidence, together with other data on insulin regulation of GSK-3 activity, has led to the proposal of the pathway shown in Fig. 3 for the regulation of eIF2B activity by insulin. The ε subunit of eIF2B also contains consensus phosphorylation sites for casein kinase II, although reports differ on whether or not phosphorylation by this enzyme enhances eIF2B activity (reviewed [17]). The physiological importance of allosteric regulation of eIF2B by NADP/NADPH, polyamines and glucose 6-phosphate is also unclear at present [17].

Regulation of initiation factors involved in binding the 43S initiation complex to mRNA

Regulation of eIF4E activity. eIF4E has long been regarded as important in the regulation of initiation. Apart from the reasoning that a regulatory role would be expected for a factor that performs the initial step in mRNA recruitment, this belief is based on two lines of evidence. First, this factor undergoes regulated phosphorylation in response to a very wide range of physiological stimuli. Second, early work indicated that, in HeLa cells [170] and reticulocytes [171], eIF4E was present at very low molar concentrations relative to ribosomes and other initiation factors. However, even after much investigation in several laboratories of the potential regulatory roles of eIF4E phosphorylation and availability, many important questions remain unanswered, and it is worth subjecting each of these possibilities to critical scrutiny.

eIF4E phosphorylation. Increases in eIF4E phosphorylation are frequently observed when quiescent or dormant cells are stimulated by appropriate hormones, growth factors or mitogens; conversely, decreased phosphorylation is seen in some states where translation is inhibited (see Table 2 and more extensive lists in [8, 48, 67, 120, 172]). It is widely assumed that such changes in phosphorylation mediate changes in both the overall rate of protein synthesis and the pattern of recruitment of individual mRNAs, which are thought to differ in their dependence on the activity state of eIF4E by virtue of features such as 5′ secondary structure (see below). However, changes in overall translation rate are often relatively modest in cells undergoing growth stimulation, and in some of the better characterized conditions, e.g. insulin treatment [120, 166] and heat shock [118, 121, 173], regulation at the level of eIF2/2B activity also makes a major contribution. Most evidence indicates that the major site of phosphorylation of eIF4E is a single serine

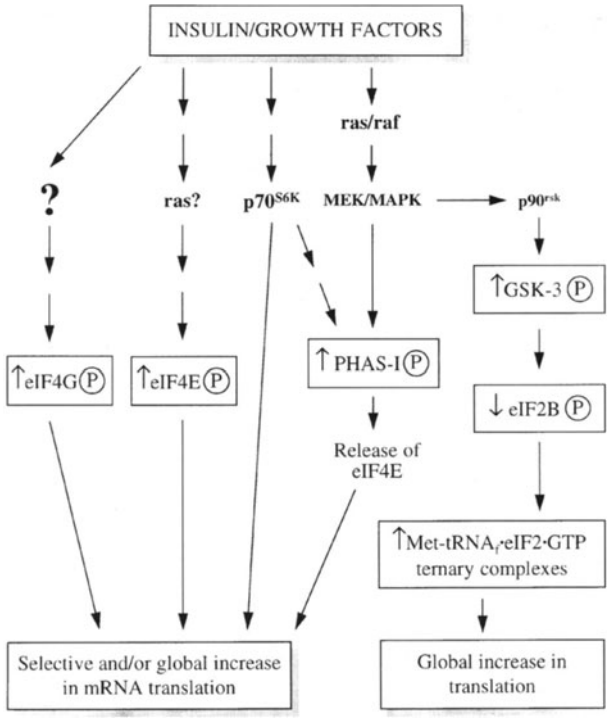

Fig. 3. Potential signalling pathways involved in the regulation of translation in mammalian cells by insulin and growth factors. Adapted from [48, 166]. Hormones and growth factors stimulate phosphorylation of eIF4E [8, 67, 120], eIF4G [176, 184] and ribosomal protein S6 [361] in a variety of cell types. In at least some cases, stimulation of eIF4E phosphorylation has been linked to activation of *ras* [67, 362]. Phosphorylation of PHAS-I is believed to result in the release of eIF4E sequestered in eIF4E. PHAS-I complexes. Both the MAP kinase [208] and $p70^{S6K}$ [209] pathways have been implicated in PHAS-I phosphorylation following insulin treatment of cells. Stimulation by insulin of eIF2B activity has been attributed to down-regulation of an inhibitory kinase, with some evidence identifying this kinase as GSK-3 [36, 169]. GSK-3 can be inactivated by phosphorylation via the MAP kinase pathway, as shown here and discussed in [167], but others have suggested that $p70^{S6K}$ can mediate regulation of this enzyme by insulin [363].

residue, but the search for physiological kinase(s) was hampered for several years by mis-identification of this site as Ser53; recently the phosphorylation site has been identified as Ser209 by labelling studies in the reticulocyte lysate [174], and this has been confirmed by analysis of eIF4E labelled in cultured cells stimulated with serum [175]. The nature of the signalling pathways regulating eIF4E phosphorylation is still unclear. There is evidence both for and against the involvement of protein kinase C [48, 172]. One might expect the mitogen-activated protein (MAP) kinase pathway to be involved in the elevation of eIF4E phosphorylation in cells overexpressing the *src* or *ras* oncoproteins [8, 67, 120, 172], and in *Xenopus* oocytes the time of onset of eIF4E phosphorylation coincides closely with that of MAP kinase activation during hormone-induced meiotic maturation [176]. However, attempts to phosphorylate the factor *in vitro* with MAP kinase, other cell-cycle-related kinases and, indeed, protein kinase C, have generally given poor results [172], suggesting that the physiological kinase has yet to be identified. An insulin-stimulated protamine kinase has recently been shown to phosphorylate eIF4E at Ser209 [177], but further evidence is needed on the general physiological relevance of this.

While the physiological correlations strongly suggest a regulatory role for eIF4E phosphorylation, it is by no means clear how this modification affects the activity of the factor in molecular terms. The factor participates in two key recognition events: the binding to the mRNA cap and the interaction with eIF4G and associated factors. In the case of cap recognition, there is clearly no all-or-nothing effect, since both phosphorylated and unphosphorylated eIF4E bind to m^7GTP-Sepharose. However, more subtle effects on the affinity of the factor for capped oligonucleotides were observed by Minich et al. [178], who separated the phosphorylated and unphosphorylated forms of the factor by chromatography on RNA-cellulose. In the case of complex formation with eIF4G and other factors, there have been several observations in intact cells of parallel changes in the extent of phosphorylation of eIF4E and its degree of engagement in high-molecular-mass complexes that include eIF4G [62, 176, 179–181]. Isolation of the fraction of eIF4E in mammalian cells present in high-molecular-mass eIF4F complexes, or associated with ribosomes, reveals a higher degree of phosphorylation than is seen for the free factor [62, 182, 182a], suggesting that phosphorylation may regulate complex formation. However, physiological stimulation of eIF4E phosphorylation tends to occur in parallel with increased phosphorylation of other factors, such as eIF4B, p120 of eIF3 and eIF4G [176, 180, 183–185]. This may also enhance the mRNA binding step in initiation; in the case of eIF4G there is direct *in vitro* evidence for this [186].

Direct comparison of the distribution of phosphorylated and unphosphorylated eIF4E within the reticulocyte lysate reveals that ribosome-bound eIF4E includes a substantial amount of unphosphorylated factor, and, conversely, at least half the phosphorylated eIF4E exists in low-molecular-mass form [182a]. Hence, while eIF4E phosphorylation may enhance its function, it is neither necessary nor sufficient for involvement of the factor in initiation complexes. A more fundamental challenge to an essential role for eIF4E phosphorylation, at least at Ser209, comes from studies on eIF4E from non-vertebrate sources. No phosphorylation site equivalent to Ser209 is seen in eIF4E from wheat or *S. cerevisiae* (see [50]). Labelling studies do suggest phosphorylation of the yeast factor, but the sites are at the N-terminus and do not appear to play a regulatory role [186a]. *Drosophila* eIF4E does have a serine residue at an equivalent location [50], although with differing surrounding sequence, but labelling studies suggest very low levels of phosphorylation of the factor *in vivo* [187].

It is difficult to assess the significance of this in terms of evaluating the role of eIF4E phosphorylation in the regulation of overall protein synthesis, since few studies of physiological regulation have been made in these invertebrate systems. However, mechanisms mediating the global shut-off of translation during moderate and severe heat shock have been extensively studied in both mammalian and *Drosophila* cells (reviewed [118, 121, 173]). Although eIF2α phosphorylation clearly plays an important role in this effect, it has been shown in both the *Drosophila* [188] and mammalian [62] systems that defective translation in extracts from heat-shocked cells is most effectively rescued by addition of eIF4E in the form of complexes with eIF4G. In both cell types, there is evidence for a fall in the proportion of eIF4E in eIF4E · eIF4G complexes during heat shock [170, 179, 188]; although this effect may be less pronounced in *Drosophila* cells [187], the observations suggest that such effects are not specific to cells clearly exhibiting regulation of eIF4E phosphorylation. However, the absence of the key phosphorylation site in at least some invertebrates seems much less of a challenge to the concept that eIF4E phosphorylation is important in the qualitative regulation of translation. Data available so far from sequence analysis suggest that 5′ UTRs of vertebrate mRNAs tend

to have a higher G+C content than those of *S. cerevisiae*, *Drosophila* and plants [9, 73, 189], indicating that 5′-secondary structure is more likely to impede the mechanisms mediating mRNA binding in vertebrate cells. Thus there may be more potential for selective regulation at the level of mRNA unwinding in vertebrate cells than in *S. cerevisiae*, insects and plants, and an additional level of regulation involving eIF4E phosphorylation may be an advantage in providing a means of linking mRNA selection to cell signalling events.

Availability of eIF4E. As stated above, cellular concentrations of eIF4E have been estimated to be low relative to those of other components of the translational machinery, and the factor is widely regarded as potentially rate-limiting in amount [4, 67, 120, 170]. Recently, however, we have detected in reticulocyte lysates substantially higher concentrations of this factor than previously reported [182a], and, moreover, a large proportion of the eIF4E can be removed by treatment with m^7GTP affinity resin with little detriment to translational activity. However, some of the most exciting studies on translational factors in recent years have shown that gross manipulation of cellular eIF4E levels can have dramatic effects on growth control and protein synthesis. Initially, work in the laboratories of Sonenberg [190] and Rhoads [191] demonstrated that overexpression of this factor in several types of cultured cells variously led to changes in cell morphology, abrogation of growth control and acquisition of the ability to induce tumour formation in nude mice. A study using cells in primary culture indicated co-operativity between eIF4E overexpression and *v-myc* or E1A in the induction of tumourigenesis [192]. The effects of eIF4E appeared to be exerted via a *ras*-mediated pathway, since they were blocked by overexpression of the negative regulator of *ras*, GAP [193]. The generally favoured mechanism for this effect of eIF4E overexpression is based on the observation that mRNAs encoding proteins involved in growth or growth regulation often possess stable secondary structure in the 5′ UTR to an extent that would lead to a prediction of extremely low translational efficiency [194]. Translation of such mRNAs might be expected to be particularly dependent on the unwinding activity of initiation factors, and thus overexpression of eIF4E may lead to inappropriately high rates of translation [67]. Although evidence in favour of this model was provided by experiments with reporter constructs bearing highly structured 5′ UTRs [195], there are still relatively few convincing demonstrations of natural mRNAs that behave according to this model (see below). The best probably concerns the mRNA encoding ornithine decarboxylase [196], the rate-limiting enzyme in polyamine biosynthesis, which itself can induce cell transformation when overexpressed [197]. However, it is important to note that part of the eIF4E in both mammalian [198] and yeast [199] cells is located in the nucleus, indicating a possible role in other processes that may contribute to growth control.

In contrast to the effects of eIF4E overexpression, Rhoads' group have engineered severe depletion of the factor in HeLa cells by introducing anti-sense RNA [200–202]. This reduced protein synthesis to very low levels, with a pattern of products very similar to that in severely heat-shocked cells. This is easier to relate to the expectation that the dependence of mRNA on eIF4E for translation reflects its degree of 5′-secondary structure, since mRNAs encoding heat-shock proteins have relatively unstructured 5′ UTRs (see below). However, a surprising, and perhaps important, aspect of these anti-sense experiments is that the cellular level of eIF4G was also profoundly depleted [200].

The studies discussed above concern the effects of artificial manipulation of eIF4E levels, but it is now clear that effective concentrations of this factor can be physiologically modulated. First, the expression of mRNA encoding eIF4E (and also that encoding eIF2α) is enhanced in cells transformed with c-*myc* [203] and during T cell activation [204]. Elevated levels of eIF4E mRNA and protein have also been observed in a variety of transformed cell lines and tumours [205, 206], although it is not clear whether this effect is specific to eIF4E or part of a general increase in the translational apparatus. More recently, however, a mechanism for regulating the availability of eIF4E on an acute basis has been revealed, and it seems likely that this can play an important role in translational regulation. Sonenberg and collaborators identified cDNAs encoding two novel eIF4E-binding proteins, which shared a degree of sequence similarity. One of these, which they called eIF4E-BP1, was found to share extensive sequence similarity with a protein called PHAS-I, which had previously been identified on the basis of its rapid phosphorylation in adipose cells following insulin treatment [207, 208]. Evidence rapidly accumulated that insulin could regulate the binding between PHAS-I and eIF4E in responsive cells, and a model emerged in which association with PHAS-I serves to restrain eIF4E from participating in protein synthesis [63, 207, 208] (reviewed [48, 166, 167]). Sonenberg and co-workers have now demonstrated competition between PHAS-I and eIF4G for eIF4E binding [208a], and identified sequence similarity between a region of PHAS-I and the eIF4E binding site on eIF4G [63]. Insulin treatment appears to remove this block on eIF4E by promoting the phosphorylation of PHAS-I, which triggers the release of the associated eIF4E (Fig. 3). Initially this phosphorylation was proposed to be mediated by a signalling pathway involving MAP kinase, which directly phosphorylates PHAS-I on a serine residue, Ser64, very close to the eIF4E binding site [208]. However, recent studies with 3T3-L1 adipocytes treated with the inhibitors rapamycin and wortmannin suggest that multiple signal transduction pathways are involved, including the $p70^{S6K}$ pathway [209].

Regulation of initiation factors involved in mRNA binding during viral infection. As in the case of PKR, some viruses have evolved cunning ways to modify the eIF4 initiation factors in infected cells so as to favour translation of their own products. Cells infected by either adenovirus [210] or influenza virus (see Table 3) [211] show reduced phosphorylation of eIF4E. In each case, translation of the virally encoded mRNAs is likely to compete well with host cell mRNAs under these conditions. Late adenovirus messages bear a particularly unstructured 5′ UTR (see below) and show low dependence on eIF4E for translation [212, 213]. The 5′ UTR of influenza virus mRNAs also confers high efficiency of translation in infected cells; the structural basis of this is not yet clear [146, 214], but interaction with virally encoded proteins may be involved [215].

The other major example of viral interference with cap-dependent initiation mechanisms is the proteolytic degradation of eIF4G during infection with picornaviruses (reviewed previously [2, 141]). This process was functionally linked to the virus-encoded 2A protease, which is primarily responsible for the cleavage of the viral polyprotein translation product into multiple polypeptides [89, 216]. Later the same phenomenon was observed, and again linked to the 2A protease, in cells infected by other members of the enterovirus group, but it does not occur in cells infected by cardioviruses such as EMCV [217]. Cleavage of eIF4G is also seen in cells infected by the aphthovirus, FMDV, but in this case the leader (L) protease is responsible [218]. Original work with the poliovirus system suggested that the 2A proteinase did not act directly on eIF4G, but instead initiated an activation cascade of cellular proteases [89, 216, 219]. However, it is now clear that purified, recombinant 2A and L proteases can cleave eIF4G directly, each initially at a single site (between Arg486 and Gly487 in the case of 2A prote-

ase and Gly479 and Arg480 in the case of L protease) [220, 221].

These observations suggested a viral strategy whereby the cleavage of eIF4G would shut off the translation of capped cellular mRNAs, but permit the continued translation of picornavirus RNAs, which are uncapped and utilize the internal initiation mechanism (reviewed [89]). Moreover, the viral RNA translation would benefit from the removal of the competition of host protein synthesis for common initiation factors. However, this rather neat model may be rather simplistic, since there have been reports that at early stages in poliovirus infection, or in the presence of some inhibitors of viral replication, it is possible to observe comprehensive proteolysis of eIF4G without commensurate inhibition of host cell mRNA translation [222, 223]. Effects of 2A protease on other processes in infected cells [224, 225] have also been reported. Furthermore it seems increasingly likely that proteolysis of eIF4G may benefit picornavirus RNA translation in a more positive way than by merely removing the competition from host cell mRNAs, since evidence is accumulating for an active role of the cleavage products [89, 226, 227]. This is reflected in *in vitro* translation assays, where addition of the 2A or FMDV L proteases, which inhibit translation of capped transcripts, has little effect on that driven by IRES elements from cardioviruses or FMDV [228–231], and actually enhances translation driven by IRES elements from enteroviruses [231], or of uncapped transcripts encoding cellular proteins [230]. These data appear at variance with earlier reports of a role for the eIF4F complex even in internal initiation [232–234]. However, recent results from our laboratory indicate that the eIF4F requirement for IRES-driven internal initiation can be fulfilled by the C-terminal L proteinase cleavage product of eIF4G in the absence of either eIF4E or intact eIF4G [234a]. This C-terminal product is also responsible for the stimulatory activity towards the translation of uncapped cellular mRNA transcripts mentioned above [234a]; it lacks the eIF4E binding site, but remains bound to ribosomes in the reticulocyte lysate, and is thought to include the domains in eIF4G responsible for interactions with eIF3 and eIF4A [34].

STRUCTURAL FEATURES IN THE UNTRANSLATED REGIONS OF mRNAs THAT INFLUENCE TRANSLATIONAL EFFICIENCY

Structures in the 5′ untranslated region (5′ UTR) associated with inefficient translation

Secondary structure. Experimental studies with both *in vivo* and *in vitro* systems clearly demonstrate that mRNAs with a high potential to form stable secondary structure in the 5′ UTR tend to be translated inefficiently [43, 76]. This effect is position-dependent, suggesting that the initial binding of initiation factors or 40S ribosomal subunits to mRNA is more sensitive to impairment than subsequent scanning [14, 43, 44, 76, 235]. *In vitro* evidence has linked secondary structure close to the cap with poor cap-binding activity of eIF4E and eIF4B [236]. The group of natural mRNAs possessing highly structured 5′ UTRs includes a disproportionately high number of examples encoding proteins that take part in or regulate processes involved in cell proliferation; their translational inefficiency may play a crucial role in the maintenance of correct restraints on cell growth [76]. The significance of this may be linked with the frequent expression by tumour cells of alternative transcripts lacking the inhibitory regions [237, 238]. Mechanisms permitting the transient release of this translational repression during growth stimulation are under active investigation. An attractive possibility is the activation of general initiation factors involved in mRNA binding (e.g. by phosphorylation [8, 48, 67, 120, 172]). Alternatively, activation of the MAP kinase or $p70^{S6K}$ signalling pathways could lead to phosphorylation of the eIF4E binding protein, PHAS-I, resulting in a transient increase in the availability of eIF4E for initiation [48, 166, 167, 208, 209] (see above). If eIF4E (or eIF4F complex) were rate-limiting for translation, these effects could be expected to confer a selective advantage on mRNAs that normally competed poorly [68]. Such mechanisms seem likely in the case of ornithine decarboxylase mRNA; increased translation of this message has been correlated with phosphorylation of eIF4E and eIF4B in insulin-treated cells [183] and, moreover, is observed in eIF4E-overexpressing cells [196]. There is also some evidence for enhanced translation of ornithine aminotransferase [239], the growth-related protein P23 [240] and the cell-cycle-regulating protein cyclin D1 [241] in eIF4E-overexpressing cells, though in the last case the effect may not be primarily exerted at the level of translation [242]. In contrast, eIF4E overexpression failed to promote translation of mRNA constructs with structured 5′ UTRs in *S. cerevisiae* [199] or that of the highly structured *c-sis*/PDGF2 mRNA in NIH3T3 cells [243]. However, the most inhibitory element in *c-sis* mRNA was localized to a position a long way downstream of the cap [243], making a mechanism involving regulation of eIF4E activity less likely. An interesting report recently demonstrated that microinjection of eIF4E into early embryos of *Xenopus laevis* promoted mesoderm induction and specifically increased the translation of microinjected activin mRNA [244]. However, this seems difficult to relate to 5′ secondary structure in this mRNA, since, in the transcript employed, the activin coding sequence was inserted downstream of the 5′UTR of *Xenopus* β-globin mRNA, which is associated with high translational efficiency. Thus the jury is still out on the general importance of regulation of the eIF4 group of general initiation factors in the selective regulation of translation of growth- or differentiation-related mRNAs with structured 5′ UTRs, and further studies with different mRNAs and systems are needed.

It is increasingly clear, however, that translation of a number of mRNAs with structured 5′ UTRs is highly responsive to the cellular environment. Translation of coding sequences downstream of the 5′ UTR of *c-myc* is relatively efficient following transfection into immortalized cell lines, or in extracts prepared from HeLa cells, but elements within this 5′ UTR strongly inhibit translation in wheat germ extracts and reticulocyte lysates [245]. The inhibitory effect of the *c-myc* mRNA 5′ UTR seen in unstimulated *Xenopus* oocytes is relieved in mature or fertilized eggs [246], and, in another study, fertilization of *Xenopus* eggs increased translation of structured mRNA constructs [247]. Some of these observations would be consistent with negative regulation of PHAS-I by activated MAP kinase or $p70^{S6K}$, but no evidence is available as yet and other helix-unwinding activities could well play a role in such regulation [14, 45, 248]. In a different system the La autoantigen, which exhibits RNA helicase activity *in vitro* [107], has been proposed to contribute to alleviating translational repression by the highly structured 5′ TAR element in HIV-1 mRNAs [249]. Finally, several observations now suggest that, in addition to general initiation or unwinding factors, more specific interactions between proteins and 5′ UTRs may play a role in regulating translation of growth factor mRNAs [250–252].

Upstream AUG codons. In many cases these are followed by a termination codon to produce short open reading frames (see above). They are often encountered in mRNAs encoding proteins involved in growth control of mammalian cells [76], and occur at a strikingly high frequency in *Drosophila* mRNAs (see [78]), where their significance has been little studied as yet. Again, tissue specificity may play a regulatory role (see reviews

[77, 78]). For example, one group reported that an upstream AUG near the 5′ end of the mRNA encoding *S*-adenosylmethionine decarboxylase is ignored in all mammalian cell types except T-lymphocytes, where it exerts translational repression [79, 253]. However, other workers identified the same upstream open reading frame as a major contributor to poor translational performance of this mRNA in non-lymphoid cells and suggested that it may be important in regulation of synthesis of the enzyme by polyamines [254].

Elements recognized by specific binding proteins. Structural motifs in mRNA molecules can provide sites for the binding of specific proteins. In the case of the 5′ UTR, such binding can impede initiation by stabilizing an element of secondary structure not itself strong enough to be inhibitory. The potential for protein binding to the 5′ UTR to act as a negative regulator of translation is illustrated by work from Hentze's laboratory, in which various specific protein-binding sites, not normally involved in translational control, were inserted into mRNA constructs [255, 256]. The archetypal physiological example of this mechanism concerns translation of the mRNA encoding the iron-binding protein, ferritin. At the 5′ end of this mRNA is a small hairpin loop termed the iron-responsive element (IRE), which is recognized by specific proteins termed iron regulatory proteins (IRPs; formerly known as iron regulatory factors or IRE-binding proteins). The binding of the IRPs to the IRE in ferritin mRNA is responsible for a regulatory mechanism that ensures that ferritin is only synthesized in cells adequately supplied with iron (reviewed [257]). The IRE is only effective in regulating translation when placed at or near its natural position near the 5′ cap, consistent with the observation that association of IRP1 blocks the initial placement of the 43S preinitiation complex on the mRNA [255, 257]. The iron status of the cell regulates the association of the IRPs with the IRE in different ways. In the case of IRP1, iron status regulates its interconversion between high- and low-affinity forms [257], while the more recently identified IRP2 [258, 259] contains a sequence that confers rapid, proteasome-mediated degradation in iron-replete cells [259a]. The mRNA encoding another protein involved in iron metabolism, 5′-aminolevulinate synthase, also contains an IRE and is subject to the same regulation. Further interesting developments on the physiological regulation of this system seem likely in the near future, following reports that the IRE-binding activity of IRP1 can be regulated by the signalling molecule, nitric oxide, in an iron-independent manner [260–262]. Phosphorylation of IRP1, possibly mediated by protein kinase C, has also been reported [263].

Other studies are beginning to reveal that binding of proteins to mRNA 5′ UTRs can mediate autoregulation of translation by the protein product. In the case of thymidylate synthetase mRNA, one of two elements recognizing the cellular enzyme includes the AUG initiation codon, the other being within the coding sequence [264]. As with the IRE/IRP interaction, binding may be regulated by a redox mechanism involving protein -SH groups [265]. The poly(A)-binding protein (PABP) represses its own translation by binding to an oligo(A) sequence found in the 5′ UTR of all PABP mRNAs so far examined [266]. This repression can be relieved *in vitro* by addition of exogenous poly(A), which competes the binding protein off the 5′ UTR. It is thought that physiologically the sequence in the 5′ UTR competes unfavourably for the PABP with the poly(A) tails of cellular mRNAs, such that its synthesis is only repressed when PABP accumulates.

Oligopyrimidine tracts. Both prokaryotic and eukaryotic cells have evolved multiple mechanisms to ensure balanced production of the protein and RNA components of ribosomes. In vertebrate cells translational control contributes to the co-ordinate regulation of synthesis of ribosomal proteins. All known mRNAs encoding these proteins, and some encoding other polypeptides involved in translation, possess at the extreme 5′ end a short (5–14 nucleotides) oligopyrimidine tract that is predicted to assume a hairpin structure (reviewed [267]). Possession of this element confers a distinctive pattern of translational utilization: (a) relative underutilization of the mRNA, with a high proportion in the untranslated mRNP pool even in growing cells; (b) bimodal distribution across the polyribosome profile in which mRNA is either untranslated or utilized in full-size polyribosomes, suggesting specific translational control; (c) a selective shift into polysomes during growth stimulation [268]. Translation of these mRNAs may be cell-cycle-regulated [267]. The regulation of translation of ribosomal protein mRNAs has been linked with eIF4F function [269] or eIF4E phosphorylation [270], but overexpression of eIF4E did not prevent the translational repression of ribosomal protein mRNAs in NIH3T3 cells undergoing growth arrest in response to inhibitors of DNA synthesis [271]. An exciting, recent, observation is that the selective enhancement of translation of several of these mRNAs in growth-stimulated cells is impaired by the immunosuppressive drug, rapamycin [272, 273]. This drug blocks the signalling pathway involving the $p70^{S6K}$, which is responsible for the phosphorylation of ribosomal protein S6 in response to a variety of hormones and growth factors [274]. Thus the translation of mRNAs bearing 5′-oligopyrimidine tracts may be regulated by this pathway, perhaps by a phosphorylation/dephosphorylation mechanism involving a specific binding protein. Alternatively, phosphorylation of S6 itself, a protein located in the mRNA binding region of the 40S ribosomal subunit [35], may regulate the recruitment of this class of mRNA.

Potential internal ribosome entry segments (IRESes) in the 5′ UTR

The acceptance and increasing understanding of the cap-independent, internal initiation mechanism for the translation of picornavirus RNAs (see above) has been accompanied by growing support for the more radical proposal that such a mechanism may be utilized by a wider range of cellular and viral mRNAs. As with other examples of translational regulation by elements in the 5′ UTR, the candidate cellular mRNAs for an internal initiation mechanism encode an interesting selection of proteins involved in cell regulation. mRNAs reported as possessing IRESes that pass the test of conferring cap-independent translation behaviour on the downstream cistron of a bicistronic construct (see above) include those encoding the immunoglobulin heavy chain binding protein (BiP) [275], the growth factor FGF2 [276] (Vagner, S., Touriol, C., Gensac, M.-C., Amalric, F., Bayard, F., Prats, H. and Prats, A.-C., unpublished results) the *gag* precursor proteins of murine leukemia viruses [277, 278], the product of the *Drosophila* homeotic gene, *Antennapedia* [279], and two yeast transcription factors, TFIID and HAP4 [280]. However, the 5′ UTRs of these mRNAs do not resemble those of picornaviruses. In some of these cases the regulated utilization of the IRES-mediated mechanism is suggested to promote usage of alternative initiation codons and thus modulate the balance of synthesis of products or isoforms with different biological activities [276, 278] (Vagner et al., unpublished results). Transacting protein factors, which may be regulated in response to physiological conditions, have been implicated, but have yet to be fully characterized [276, 278] (Vagner et al., unpublished results).

5′ UTRs conferring high translational efficiency

Some mRNAs are translated very efficiently and show strong resistance to inhibition of translation by cellular stresses

that impair overall protein synthesis. One might expect such mRNAs to possess 5′ UTRs relatively free of secondary structure, upstream ORFs and other impeding features. Indeed, Kozak [281] improved the translational efficiency of mRNA constructs simply by inserting repeats that extended the 5′ UTR without introducing secondary structure. The improved performance was accompanied by decreased cap dependence *in vitro*, which presumably indicated a lower requirement for eIF4E or eIF4F complex, although these may not necessarily be synonymous [9, 68]. In a later study, Kozak concluded that greater length, together with a lower potential to form secondary structure near the cap, formed the basis of the translational advantage of β-globin mRNA over α-globin mRNA [282].

The possession of a 5′ UTR conferring high translational efficiency can be of particular advantage to invading viruses, whose mRNAs need to commandeer the host cell translational machinery. Examples are the 5′ leaders of the plant viral mRNA encoding alfalfa mosaic virus coat protein 4 (AMV 4) [9] and the first segment of the 5′ UTR of late adenovirus mRNAs (the so-called adenovirus tripartite leader [213]). The efficiency of mRNAs encoding heat shock proteins is also conferred by their 5′ UTRs. These mRNAs can be translated actively under conditions where overall protein synthesis in severely down-regulated by cellular effects on both eIF2 and eIF4F, and earlier reports that heat shock, adenovirus and AMV 4 mRNAs can be translated in poliovirus-infected cells, or in extracts derived from them [283–285], suggested that they may utilize a cap-independent mechanism. However, more recent studies have challenged this view [87]. Expression of the FMDV L protease, which cleaves eIF4G, inhibited *in vitro* translation of mRNAs bearing the adenovirus tripartite leader [228] and substantially impaired that of *Drosophila* hsp70 mRNA [286]. In addition, mRNA electroporation experiments indicated that possession of a methylated cap was necessary for efficient translation of hsp70 mRNA in either normal or heat-shocked *Drosophila* cells [286]. Re-evaluation of the translation of AMV4 mRNA in extracts from poliovirus-infected cells also indicated that efficient translation was cap-dependent [287].

In the case of heat-shock-protein mRNAs, as with late adenovirus mRNAs, it is an attractive hypothesis to link the efficiency conferred by their relatively unstructured 5′ UTRs to their selective translation under conditions where eIF4E is underphosphorylated. However some holes are beginning to appear in this argument. Although selective survival of heat-shock mRNA translation was observed in mammalian cells in which eIF4E and eIF4G had been drastically down-regulated by antisense technology [200–202], preferential translation under heat shock conditions is much more pronounced in *Drosophila* cells [121], in which eIF4E phosphorylation seems less likely to play a major regulatory role [187]. Studies on the 5′ UTRs of *Drosophila* heat shock mRNAs suggest that a low degree of secondary structure is necessary, but not sufficient, to permit translation under heat shock conditions [288]. Specific regions in the 5′ UTR, including one near the cap, seem to be important, but attempts to identify a role for precise primary sequences or for hsp-mRNA-specific binding proteins have so far been unsuccessful [118, 121, 289]. A recent survey, however, has challenged the idea that the 5′ UTRs of heat shock mRNAs are significantly less burdened with secondary structure [189]; although many more samples of normal mRNAs are needed to substantiate this, it is clear that, for all the examples of insect mRNAs quoted, the proportion of G+C bases in the 5′ UTRs is very low by vertebrate standards. It may therefore be of doubtful relevance to extrapolate data from mammalian cells on the regulatory role of 5′ secondary structure to organisms like *Drosophila* and *S. cerevisiae*.

Regulation by elements in the 3′ UTR

It is relatively easy to envisage effects of structural features in the 5′ UTR of an mRNA on translational efficiency, since the 40S subunit comes into close contact with this region during initiation. However, in the last few years reports of regulatory elements in the 3′ UTR have constituted an even faster growth area. A few of these concern studies involving mRNAs in somatic cells. For example, cytokine mRNAs frequently contain U+A-rich sequences (often repeated) in the 3′ UTR. Although these tend to be associated with instability, they can also confer inefficient, and probably regulated, translation [290, 291]. Regulatory elements in the 3′, as well as 5′, UTRs of plant viral mRNAs have also been defined [9]. However, 3′-regulatory elements really come into their own in exerting translational control during development, as discussed in the following sections. Finally, the role of the poly(A) tail as a regulator of initiation is emerging as an important topic for all eukaryotic systems.

Translational repression and activation during development

Developmental stages up to and including early embryogenesis are characterized by pronounced changes in the pattern of gene expression in the absence of transcription. Oocytes, eggs and early embryos of a number of organisms have thus provided rich pickings for investigators of translational control. Oocytes and unfertilized eggs are packed with ribosomes and maternal mRNAs that are largely withheld from translation; during meiotic maturation of *Xenopus* oocytes, for example, the proportion of ribosomes engaged in polysomes rises from 1–2% to about 4% [292]. The mechanisms underlying this suppression are multiple and complex. Activation of protein synthesis during early development is a highly co-ordinated process, involving effects on general initiation factors (mainly eIF2/eIF2B and eIF4F [176, 293–295]) together with global mobilization of mRNA from the untranslated pool. A 56–60-kDa mRNP protein thought to be responsible for global repression of mRNA recruitment in *Xenopus* oocytes has been identified as belonging to the Y box group of transcription factors [291, 296–300]. Newly transcribed mRNAs interact with this and related proteins before export from the nucleus, but escape if they possess introns or are microinjected directly into the cytoplasm [301, 302]. The repressor proteins dissociate from the mRNA during progesterone-induced maturation, and their activity is probably regulated by phosphorylation/dephosphorylation [301, 303]. So far no specificity has been identified for this mechanism, either with respect to species of mRNA involved or binding sites within indvidual mRNA molecules [291, 300]. Interestingly, translational repression can be induced in somatic cells by overexpression of the Y box protein FRGY2 [304], and one of the major proteins of mRNP particles in reticulocytes has been identified as a member of this family [305].

Sequence-specific regulation of mRNA translation during development

This topic has been the subject of several excellent, specialized, reviews in recent years [15, 306–315]. In most organisms there are profound changes in translational priorities during meiotic maturation and/or fertilization in favour of a rather specific group of mRNAs encoding proteins required to bring about meiotic maturation and to support the rapid cycles of cell division that characterize early embryogenesis. Each mRNA appears to be programmed for translational activation at an appropriate time and, in many cases, for withdrawal from translation at a

later stage in development. Conversely, mRNAs encoding housekeeping proteins, such as components of the cytoskeleton and ribosomal proteins, are translated in oocytes, but their production is less essential during maturation and early embryogenesis and their translation is then down-regulated. Signals conferring these translational patterns are almost invariably located in the 3′ UTR and appear to be of two main types. First, a wide range of sequence elements exerts negative regulation in *cis*; possession of one or more of these elements prevents translation of the mRNA until a critical developmental stage is reached. Second, a more homogeneous set of sequences confers stage-specific elongation of the poly(A) tail, which shows a strong correlation with translational activation.

Sequence elements conferring negative regulation. Elements in the 3′ UTR are involved in extremely complex patterns of translational control (Table 4). Evidence for interaction with specific proteins has often been obtained by genetic analysis, and detailed knowledge of the regulatory mechanism is confined to very few examples. In addition to repressing translation prior to a developmental signal (temporal control), elements in the 3′ UTR can confer spatial regulation by undergoing interactions that prevent the mRNA being translated until it reaches a specific location. This type of translational regulation is of immense importance in controlling pattern formation in the early embryo (reviewed [311, 312, 314]), and studies on some of the mRNAs listed in Table 4 are beginning to unravel amazing linked networks of regulated expression, particularly in the case of *Drosophila*. Other examples, such as the regulation of lipoxygenase mRNA translation in reticulocytes, indicate that this type of control is not limited to early development.

Sequences regulating poly(A) tail length during early development. A phenomenon first observed in general terms, but now understood in considerable molecular detail, is the strong correlation of translational activation of an mRNA during maturation or early embryogenesis with the elongation of its poly(A) tail from less than 100 residues to 100−200 residues (reviewed [15, 306, 308, 313−317]). Conversely, mRNAs whose translation is switched off during early development undergo parallel deadenyation or poly(A) shortening. Developmentally regulated elongation of the poly(A) tail is conferred by the possession of two sequence elements in the 3′ UTR, the consensus signal required for nuclear polyadenylation, AAUAAA, and, upstream from it, a more variable U-rich element, initially termed a cytoplasmic polyadenylation element, or CPE [315]. *Xenopus* mRNAs with these features include a group (G10, B4, D7) selected on the basis that they moved into polysomes during meiotic maturation [318−320], together with cyclins [321], cdk2 [322] and c-*mos*, whose translational activation is an important enabling step in maturation [321, 323]. Examples in mouse oocytes include the mRNAs encoding tissue-type plasminogen activator (tPA) [313] and, again, c-*mos* [324]. In all these cases deletion of the consensus elements prevents both poly(A) elongation and translational recruitment. Interesting insight into the function of these elements came from studies of the tPA mRNA in mouse oocytes. This mRNA receives a long (>300 nucleotides) poly(A) tail in the nucleus, which is immediately cut to <60 nucleotides upon export into the cytoplasm. The deadenylation, as well as the subsequent readenylation during meiotic maturation, is dependent on the U-rich CPE in the 3′ UTR [325]. This dual function has led to some groups re-naming this sequence as the adenylation control element, or ACE.

Within the general association between the possession of CPE/ACE elements and the ability to undergo poly(A) tail elongation during early development, the programming conferred by these sequences on an individual level is extremely subtle. Comparison of four such mRNAs in *Xenopus* oocytes during maturation showed each to have a different pattern of behaviour, both in the timing and the extent of polyadenylation during maturation [321]. Other mRNAs only undergo polyadenylation and translational activation during embryogenesis. For two of these, C11 and C12 mRNAs in *Xenopus*, manipulation of the distance between the CPE/ACE and the downstream AAUAAA elements had profound effects on both the timing and extent of polyadenylation [326, 327]. In the same mRNAs a much larger element, encompassing the CPE/ACE, was also found necessary to prevent premature polyadenylation during maturation [308, 326, 327]. A strong correlation between translational activation and poly(A) tail elongation during embryogenesis is also seen in the case of three *Drosophila* mRNAs, *bicoid*, *torso* and *toll*, although specific CPE/ACEs have yet to be delineated [328]. In *Xenopus*, some mRNAs activated during maturation subsequently undergo programmed shortening of their poly(A) tails and concomitant withdrawal from translation. This appears to require additional 3′ UTR sequences that do not overlap with the CPE [322, 329]. Proteins that interact with the CPE/ACE and AAUAAA elements clearly play a major role. The general mechanics of cytoplasmic poly(A) elongation require a poly(A) polymerase and a CPE/ACE binding protein, which are very closely related to enzymes that catalyse polyadenylation in the nucleus [314, 315, 330−332]; Richter [315] has proposed a model for their interaction on the mRNA. In addition. proteins binding CPE elements of specific mRNAs have been identified in *Xenopus* oocyte and egg extracts, and mechanisms regulating their interaction are beginning to be elucidated [314, 315, 333−335].

In contrast to mRNAs possessing CPE/ACE elements, mRNAs translated efficiently in oocytes but down-regulated in mature eggs and early embryos undergo poly(A) tail shortening after germinal vesicle breakdown, which releases a deadenylase enzyme, previously sequestered in the nucleus, and allows it access to cytoplasmic mRNAs (reviewed [308]). Deadenylation appears to be relatively non-specific and to act in default, affecting all mRNAs except those possessing a CPE in the 3′ UTR [314, 336, 337]. This would down-regulate translation of mRNAs whose products are not required during early embryogenesis.

Correlation between poly(A) tail length and translation. The strong correlation between poly(A) tail elongation and translation activation, right across the range of mRNAs and organisms investigated so far, provides powerful, but not conclusive, evidence for a causal relationship [15, 308, 314, 315]. There are, however, differences in detail between some of these examples. In several cases the increased efficiency of translation seems to depend solely on the possession of a longer poly(A) tail; thus if an mRNA transcript with an extended poly(A) tail is microinjected its translation is prematurely activated. This experiment works for a number of CPE/ACE-regulated mRNAs, e.g. B4 in *Xenopus* [338], tPA in mouse [325] and, partially, *bicoid* in *Drosophila* [328]. However, other mRNAs, e.g. G10 [333] and C12 [326] in *Xenopus*, seem to have to undergo the polyadenylation process itself to permit translational activation. The mechanisms underlying this distinction are not clear, but an effect similar to that observed for G10 and C12 mRNAs has now been observed for cellular mRNAs undergoing reactivation in somatic *Drosophila* cells following heat shock [339]. An important question still under investigation concerns the relationship between poly(A)-linked regulation and other processes required for translational activation. Translational activation of several mRNAs *in vivo* involves both liberation from sequence-specific repression and poly(A) tail elongation [328, 340], but in some cases these processes can be dissociated *in vitro* [15]. In other cases, changes in poly(A) tail length do not appear to

Table 4. Messenger RNAs with translational control elements in the 3′ UTR.

Organism/mRNA	3′ UTR element	Binding factor(s)	Function/target	References
Drosophila				
Oskar	not identified	Staufen	during oogenesis, directs movement of *oskar* mRNA to posterior pole, mediated by interaction with microtubules; may play role in preventing premature translational activation	[311, 312, 364]
	Bruno response elements (multiple)	Bruno	represses translation of *oskar* mRNA prior to localization	[311, 365]
Nanos	not identified	unidentified repressor throughout embryo; oskar and vasa, localized to posterior pole	localization of *nanos* mRNA to posterior pole during oogenesis; translational activation at fertilization, restricted to *nanos* mRNA localized to posterior pole, where it is protected from a repressor that prevents its translation elsewhere in the embryo; vasa is a homologue of eIF4A and has RNA helicase activity, but its function is unknown	[311, 314, 342]
Hunchback	Nanos response elements (2) (NREs)	pumilio; unidentified 55-kDa protein	represses translation of mRNA at posterior pole, leading to preferential expression at anterior pole; binding of pumilio and the 55-kDa protein to NREs thought to provide a landing pad for nanos	[311, 314, 366]
Bicoid	three regions predicted to form stem loops; BLE1 localization signal	Staufen	Staufen binds to *bicoid* mRNA to form particles that undergo microtubule-dependent localization to anterior pole after eggs laid; anchors mRNA at anterior pole; two copies of BLE1 element required for localization but not involved in anchoring; poly(A) elongation closely associated with translational activation (see text)	[364]
Cyclin B	two closely located segments of 94 and 87 nucleotides	not identified	required for localization to posterior pole during late oogenesis	[367]
	39-nucleotide region containing an NRE-like sequence	not identified	repression of translation until posterior pole cells commence proliferation during embryogenesis	[367]
C. elegans				
tra-2	two direct repeat elements (DREs)	DRE-binding factor	*tra-2* gene directs feminine development; repression of *tra-2* mRNA translation promotes spermatogenesis at one end of gonad	[314, 368]
fem-3	regulatory element with five critical nucleotides	unidentified binding activity in *C. elegans* extracts	represses masculine development; hence promotes oogenesis in gonad cells	[314]
glp-1	temporal control region within 3′ terminal 125 nucleotides	not identified	translation repressed until 2–4 cell stage	[369]
	61-nucleotide spatial control region containing NRE-like elements	not identified	translation confined to anterior blastomeres	[369]
lin-14	seven conserved sequences	two small, untranslated RNAs, 22 and 61 nucleotides (products of *lin-4*)	RNA products of *lin-4* have anti-sense complementarity to the seven sequences in *lin-14* mRNA; binding represses translation at later larval stages either directly or by creating site that binds (unknown) protein	[314, 370, 371]
Surf clam				
ribonucleotide reductase; cyclin A	masking boxes (≈ 130 nucleotides)	82-kDa binding protein	prevents translation until maturation; probably regulated by phosphorylation	[307, 372]
Mammalian erythroid cells				
lipoxygenase (LOX)	19-nucleotide sequence (10 repeats in rabbit, 4 in mouse and human)	48-kDa protein	represses translation until reticulocyte stage. LOX protein initiates breakdown of mitochondria during terminal differentiation to erythrocytes	[341]

be involved at all [341, 342]. On the other hand, as mentioned above, elongation of poly(A) tracts on some mRNAs is alone sufficient to increase translational activity in *Xenopus* and mouse oocytes. This complex question is considered in more detail in recent reviews [15, 306, 314].

General role of poly(A) and PABP in translation

Even before the studies on developmental regulation discussed above there were many reports in the literature that the poly(A) tail contributed to the translational efficiency of mRNA molecules (reviewed [114, 316]). The effects are often small in *in vitro* translation systems, but more pronounced differences have been seen in experiments where mRNAs were microinjected into *Xenopus* oocytes [343] or electroporated into cells [344] (see, however, [345] which describes rather different behaviour in yeast). In the reticulocyte lysate, a rather small increment in efficiency of recruitment of poly(A)-bearing mRNA was localized to the joining of the 60S ribosomal subunit to the 43S · mRNA complex [346], consistent with the observation that the effects of mutating the yeast poly(A)-binding protein gene were suppressed by a mutation in a structural protein of the 60S subunit [347]. Addition of exogenous poly(A) inhibits the activity of cell-free translation systems, probably by sequestering the PABP away from the poly(A) tails of the mRNAs [306, 316, 348]. Increasing attention is now being given to the idea that there may be interaction between poly(A) tails and mRNA caps or 5′ UTRs. This stemmed from observations of synergistic effects of caps and poly(A) tails on translational efficiency in mRNA-electroporated cells [344] and, later, from studies on mRNA degradation mechanisms in yeast which clearly implicated communication between these two features [310]. Such interactions are likely to be indirect, and could be mediated by PABP, by initiation factors binding mRNA 5′ UTRs or by unknown proteins. One report suggests association between poly(A) and initiation factors of the eIF4 group [349]. Various forms of a closed loop model, bringing the poly(A) tail close to the 5′ end of the mRNA, are considered in a recent review by Jacobson [316].

CONCLUDING REMARKS

I will finish this article with a summary of topics where I feel that interesting developments are imminent or where there are outstanding questions requiring elucidation.

Functions and molecular interactions involving initiation factors. There is still much to be learned here, even concerning relatively well-characterized factors like eIF2. The recent cloning and sequencing of cDNAs encoding the five subunits of eIF2B should facilitate elucidation of the individual roles of these polypeptides. Even more encouraging is the news of progress on the molecular characterization of the multiple subunits of eIF3, since lack of information on the function of this factor, clearly at the heart of the initiation process, has constituted a large hole in our understanding for so many years. For the better-known factors, a remaining outstanding question concerns the functions of the various RNA-binding domains identified in sequencing studies. How does each of these relate to the interactions of the factors with mRNA, rRNA and tRNA during initiation? In many cases RNA-binding studies have revealed little or no sequence specificity. The function of the RNA helicase activity of eIF4A is still not fully clear. This activity has always been regarded as responsible for unwinding secondary structure in the mRNA 5′ UTR. However, extracts prepared from *S. cerevisiae* strains with disrupted eIF4A genes show total dependence on exogenous eIF4A for the translation of any mRNA, whether or not the 5′ untranslated region is highly structured [350]. The enhanced unwinding activity of this factor *in vitro* when presented as part of an eIF4F complex [66] is probably explained by the ability of eIF4G to bring it into proximity to the RNA, either by direct eIF4G · RNA association or by binding to cap-associated eIF4E [34]. The significance of the 3′−5′ RNA duplex unwinding activity of eIF4F, demonstrated *in vitro* with uncapped RNA substrates [66], needs to be elucidated; possible functions could be in internal initiation or in transient melting of structures in exposed regions of rRNA involved in ribosome−mRNA interactions during initiation. For eIF4B, long regarded on the basis of *in vitro* assays as a mere facilitator of eIF4A-catalysed RNA unwinding, a whole new area of interest is opened up by a recent paper demonstrating duplex annealing activity of this factor [351]. It should be remembered, however, that a great deal of the experimental evidence currently available on the function of the individual initiation factors, particularly those of the eIF4 group, is derived from *in vitro* assays performed in the absence of other components, such as ribosomal subunits and eIF3, with which they would be associated in the intact cell; caution is therefore needed in extrapolating these data to the situation *in vivo*. Finally, as illustrated in Fig. 2, there is still much to be learned about the exact sequence of association and dissociation events involving initiation complexes.

Role of initiation factor phosphorylation in regulation. Many factors exhibit regulated phosphorylation, but for only one (eIF2α) has a clear role for this been established. For eIF4E, apart from one study [178], evidence indicating a functional role for phosphorylation remains entirely correlative. Work is needed to assess the relative importance of phosphorylation and PHAS-I-mediated sequestration in the regulation of this factor in response to physiological signals. The absence of an appropriate C-terminal phosphorylation site in *S. cerevisiae* eIF4E raises interesting questions, and information on this factor from other invertebrate species is needed. Does the importance of eIF4E phosphorylation in different organisms relate to the incidence of stable secondary structure in mRNA 5′ UTRs? It should be noted that regulation of eIF4E phosphorylation during early development of marine invertebrates shows strong resemblance to that seen in *Xenopus* [176, 295], though sequence information on phosphorylation sites is not yet available. Returning to yeast, it is perhaps of interest that *S. cerevisiae* homologues of ribosomal protein S6, and possibly eIF4B, also lack potential phosphorylation sites present at the C-termini of the mammalian proteins.

Translational control and growth regulation. Further work is needed to establish whether the apparent roles of eIF4E and PKR in the regulation of tumourigenesis are related to their functions in translation or whether the presence of these proteins in the cell nucleus indicates dual function. In the case of eIF4E one might ask whether one role for the PHAS-I-mediated sequestration mechanism is to regulate the amount of the protein available to enter the nucleus. For PKR, work on the effects of expression of dominant negative mutants could be criticized for having been limited so far to NIH-3T3 cells, and it is now essential to investigate the significance of the recent report that PKR-knockout mice do not exhibit a tumourigenic phenotype [161]. Another important area in which research will continue is the role of both general initiation factors and specific mRNA-binding proteins in controlling the translation of mRNAs encoding growth-regulatory proteins. Recent hints of the involvement of

internal initiation in the translation of such mRNAs [276, 280] may also lead to interesting developments.

Cross-talk between the 5′ and 3′ ends of mRNA and spatial relationships within cells. An exciting aspect of recent work on the translational control of individual mRNAs has been the recognition of the role of regulatory elements at the 3′ end, including the poly(A) tail. Together with related work on the control of mRNA degradation [352], this leads to the inescapable conclusion that the two ends of mRNA interact, either directly or indirectly. Further, studies on mRNA localization during early development clearly implicate interactions of mRNAs and associated regulatory proteins with cytoskeletal components [314, 353, 354]. It is clear that future work on translational control both in embryos and in somatic cells will have to focus more and more on how attachment to cytoskeletal networks [355–359] and involvement in other macromolecular complexes [360] can orientate the interactions between mRNAs, ribosomes, tRNAs and initiation factors within cells.

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