REVIEW ARTICLE

Control of eukaryotic protein synthesis by upstream open reading frames in the 5'-untranslated region of an mRNA

Hedda A. MEIJER¹ and Adri A. M. THOMAS

Department of Developmental Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

Control of gene expression is achieved at various levels. Translational control becomes crucial in the absence of transcription, such as occurs in early developmental stages. One of the initiating events in translation is that the 40 S subunit of the ribosome binds the mRNA at the 5'-cap structure and scans the 5'-untranslated region (5'-UTR) for AUG initiation codons. AUG codons upstream of the main open reading frame can induce formation of a translation-competent ribosome that may translate and (i) terminate and re-initiate, (ii) terminate and leave the mRNA, resulting in down-regulation of translation of the main open reading frame, or (iii) synthesize an N-terminally extended protein. In the present review we discuss how upstream

AUGs can control the expression of the main open reading frame, and a comparison is made with other elements in the 5'-UTR that control mRNA translation, such as hairpins and internal ribosome entry sites. Recent data indicate the flexibility of controlling translation initiation, and how the mode of ribosome entry on the mRNA as well as the elements in the 5'-UTR can accurately regulate the amount of protein synthesized from a specific mRNA.

Key words: translational control, translation initiation, uAUG, uORF, 5'-UTR.

INTRODUCTION

Control of gene expression is achieved at various levels, such as transcription, post-transcriptional processing, mRNA stability, translation, post-translational modifications and protein degradation. Transcriptional control is one of the major checkpoints. However, there is no active transcription in early stages of *Xenopus laevis* embryonic development, and thus other controls are required. Many papers have described how mRNAs are activated for protein synthesis during development, as reviewed recently [1,2]. The embryo is dependent on maternal mRNAs in these early stages. Translational control of maternal mRNAs is dependent on the selection of the mRNA to be translated, on covalent modifications of the mRNA such as capping and polyadenylation, on binding of repressor and activator proteins – mostly studied for the 3'-end of the mRNA – and on localization of the mRNA. Once the mRNA is activated for translation, several elements in the mRNA control the efficiency of protein synthesis. Multiple proteins can be synthesized from one mRNA, not only by alternative splicing but also by the choice of a translation initiation codon. This choice, as well as the efficiency of translation, is controlled by mRNA-specific elements such as the GC content of the 5'-untranslated region (5'-UTR), the size of the 5'-UTR, and the location of potentially active upstream initiation codons (uAUGs).

An mRNA consists, in the 5' → 3' direction, of the m⁷G (7-methylguanosine) cap, the 5'-UTR, the open reading frame (ORF), the 3'-UTR and a poly(A) tail. All elements contribute

significantly to translation efficiency of an mRNA, although the ORF itself is in general not involved. When a 5'-UTR sequence is analysed, certain features can suggest translational control of the messenger. In most cases, 5'-UTRs that enable efficient translation are short, have a low GC content, are relatively unstructured and do not contain uAUG codons [3].

The mechanism of how ribosomes most frequently select the initiation codon on an mRNA is the ribosomal scanning process [4]. The scanning process, as well as the role of eukaryotic initiation factors (eIFs), have been recently reviewed [5]. Two other mechanisms are internal entry on an internal ribosome entry site (IRES) and shunting of ribosomes (reviewed in [6]), processes that will be discussed below. The first step of the scanning mechanism is the binding of eIF4E to the cap and to eIF4G (Figure 1). eIF4E is in resting cells associated with eIF4E-binding protein (4E-BP) and is released after phosphorylation of 4E-BP [7]. eIF4G has a binding site for eIF4E and the poly(A)-binding protein (PABP), which in turn is bound to the poly(A) tail, resulting in circularization of the mRNA [2,8]. The ATP-dependent helicase eIF4A binds to eIF4G, together with eIF4B and eIF4H, which stimulate the helicase activity of eIF4A. The unwinding of the 5'-UTR next to the cap enables binding of the 40 S ribosomal subunit. The 40 S subunit is withdrawn from the equilibrium with 60 S and 80 S subunits by the association of eIF1A and eIF3 to the 40 S subunit. Both proteins facilitate the binding of the ternary complex eIF2-GTP-Met-tRNA_i [9]. The resulting 43 S preinitiation complex can land next to the cap and move in a 5' → 3' direction (scanning) until it

Abbreviations used: AAP, arginine attenuator peptide; AdoMetDC, S-adenosylmethionine decarboxylase; Apaf-1, apoptotic protease activating factor; ATF4, activating transcription factor 4; CaMV, cauliflower mosaic virus; C/EBP, CCAAT/enhancer binding protein; CNP, 2',3'-cyclic nucleotide 3-phosphodiesterase; CUGBP1, CUG repeat binding protein 1; eALAS, erythroid δ-aminolaevulinic synthase; eIF, eukaryotic initiation factor; 4E-BP, eIF4E-binding protein; FGF, fibroblast growth factor; gpUL4, human cytomegalovirus early glycoprotein; IGF, insulin-like growth factor; IRE, iron-responsive element; IRES, internal ribosome entry site; IRP, iron-regulatory protein; NMD, nonsense-mediated decay; ODC, ornithine decarboxylase; ORF, open reading frame; PABP, poly(A)-binding protein; PDGF, platelet-derived growth factor; RAR/β2, retinoic acid receptor β2; sAUG, start AUG; TfR, transferrin receptor; TGF, transforming growth factor; uAUG, upstream AUG; uORF, upstream open reading frame; UTR, untranslated region; VEGF, vascular endothelial growth factor; XIAP, X-linked inhibitor of apoptosis protein.

¹ Present address and address for correspondence: School of Biomedical Sciences, Queens Medical Centre, University of Nottingham, Nottingham NG7 2UH, U.K. (e-mail hedda.meijer@nottingham.ac.uk).

Figure 1 Schematic presentation of the initiation of protein synthesis

Protein synthesis requires the assembly of an 80 S initiation complex on the mRNA. This complex consists of the two ribosomal subunits, the mRNA, and the ternary complex (eIF2-GTP-Met-tRNA_i). According to the ribosomal scanning model, the 40 S ribosomal subunit lands next to the 5' cap structure and scans the 5'-UTR until an initiation codon is recognized via eIF1, eIF2, and base-pairing with Met-tRNA_i; then the 60 S subunit can join. See the text for further details.

recognizes an AUG codon [10], mediated by eIF1, eIF2 and base-pairing with Met-tRNA_i [11]. Upon recognition of the start codon, eIF5B stimulates GTP hydrolysis, resulting in the release of eIF2-GDP and probably of other 40 S-bound initiation factors. eIF5 catalyses the joining of the 60 S subunit to form an 80 S ribosome, and elongation can start. When the ribosome encounters a termination codon, the string of coupled amino acid residues is released, and the ribosome will dissociate into its subunits and disconnect from the mRNA. A subsequent initiation event will require GDP ↔ GTP exchange on eIF2, which is catalysed by eIF2B (reviewed in [5]). The location of initiation factors on the different complexes is unknown in many cases. Figure 1 is therefore a schematic presentation.

In this review the role of uAUGs, as well as upstream open reading frames (uORFs), in the 5'-UTR will be discussed. The effects of these initiation-controlling AUGs will be compared to the effects of a hairpin or an IRES. The effects of hairpins and IRESs will be briefly reviewed and are used for comparison with the effects of uAUGs. The reader is referred to recent reviews on translational control by IRESs and secondary structure [6,10,12–14].

CONTROL OF TRANSLATION INITIATION BY THE 5'-UTR REGION OF THE mRNA

The presence of uAUGs in the 5'-UTR in general decreases the initiation efficiency on the AUG preceding the main ORF [start AUG (sAUG)] [15]. Analyses of translationally relevant features in the 5'-UTR have different outcomes, depending on the database used (Table 1). The average 5'-UTR length varies between different searches from 90 to 210 nt for vertebrate mRNAs. From 11 to 42% of the vertebrate mRNAs and from 20 to 48% of the human mRNAs contain at least one uAUG [16–20]. These large variations can only partially be explained. Kozak analysed 699 vertebrate mRNAs that probably under-

represented very rare messengers [16]. More recent analyses are based on more sensitive methods, so relatively more rare messengers should be present. The group of rare messengers probably contains a relatively high percentage of inefficiently translated mRNAs, which will contain more uAUGs and have more complex 5'-UTRs. It is important to recognize the reliability of all data; for example the transcription start site was not determined for all mRNAs, and increases in 5'-UTR length can lead to more uAUGs. Besides, a fraction of the mRNAs may represent immature mRNAs, still containing introns, and therefore not represent functional 5'-UTRs [21].

The analyses of the nucleotides neighbouring the AUG codons were more consistent (Table 2). The context around an initiation codon optimal for translation initiation was analysed by Kozak [22–24]. The ideal context in higher eukaryotes is CCRCAUGG. The most conserved nucleotides are the **R** (A or G) at –3 and the **G** at +4 (the A of the AUG codon is designated as +1). A strong consensus sequence contains both of these important nucleotides, whereas an adequate sequence contains only one of them. The AUG codons initiating the main ORF of a messenger have a good or an adequate sequence context in 95–97% of the cases. This percentage is lower for the uAUGs (43–63%). However, recognition of an AUG is not only dependent on its context. AUG recognition was stimulated by a hairpin approx. 14 nt downstream of the AUG, slowing the scanning 43 S ribosomal complexes and increasing the efficiency of initiation [25]. The relatively frequent presence of a uAUG within a good or adequate context suggests that the translational machinery can deal with these sequence elements in 5'-UTRs and that the uAUGs might be involved in the translational control of mRNAs. It should be borne in mind that not all proteins will be synthesized at high rates and, therefore, the main ORFs will not always be translated efficiently.

Two modifications of the ribosomal-scanning model were postulated to explain the translation of the main ORF downstream of one or more uAUGs or uORFs. When uAUG codons are not recognized by 100% of the scanning initiation complexes, some ribosomes will pass and initiate at the sAUG: the 'leaky scanning model' [4]. A rarer mechanism is re-initiation [10]. In this case, the 40 S ribosomal subunit remains connected to the mRNA after termination at the uORF and resumes scanning. In general, this is a very inefficient mechanism that is only possible after translation of short uORFs. It is thought that the relatively short time between initiation and termination can induce the rebinding of initiation factors to the 40 S subunit. To be able to recognize the next AUG codon, the 40 S subunit has to be reloaded with eIF2-GTP-Met-tRNA_i. This explains why increasing the distance between two ORFs increases the time available for reloading and therefore enhances re-initiation efficiency. Re-initiation can be stimulated by the sequence around the termination codon [26].

Table 1 5'-UTR characteristics of vertebrate and human mRNAs

n, number of analysed 5'-UTRs; n.d., not determined.

Species	Reference(s)	5'-UTR length		Initiation			
		<i>n</i>	nt	<i>n</i>	uAUG	uORF	IRES
Vertebrates	[16]	346	90	699	11%	n.d.	n.d.
	[17,18]	1173	198	27 469	42%	n.d.	6%
Human	[17,18]	421	210	10 913	48%	28%	7%
	[19]	954	125	954	29%	11%	n.d.
	[20]	n.d.	n.d.	2312	20%	15%	n.d.

Table 2 AUG context of main and upstream initiation codons

Species	Reference	<i>n</i> *	Context of sAUG† (%)			Context of uAUG† (%)		
			Strong	Adequate	Weak	Strong	Adequate	Weak
Vertebrates	[16]	699	44	53	3	11	32	57
Human	[19]	954	46	49	5	18	45	37

* *n*, number of analysed 5'-UTRs.

† The Kozak consensus sequence is CCRCAUGG (R = purine, A/G); the most important nucleotides (in **bold**) are the A or G at –3 and the G at +4 (with respect to the A of the AUG codon); a strong context means that both important nucleotides are present, an adequate sequence means that only one of these nucleotides is present, whereas a weak sequence lacks both.

EXAMPLES OF uAUG-MEDIATED TRANSLATIONAL CONTROL

(A) Stress-mediated control of initiation

The best-investigated example of translational control via re-initiation is the GCN4 mRNA of *Saccharomyces cerevisiae* (reviewed by Hinnebusch [27]). GCN4 is a transcription factor of a large group of genes involved in amino acid biosynthesis. The 5'-UTR of GCN4 contains 590 nt and four uORFs (Figure 2). An mRNA constructed so as to be devoid of the four uORFs has high translation efficiency. Inclusion of each of the four uORFs inhibits translation. uORF1 decreases translation a modest twofold, whereas uORF4 has the strongest effect and abolishes almost all translation. The 5'-UTR containing only uORF1 and uORF4 is regulated similarly to the wild-type 5'-UTR. Therefore the effects of uORF2 and uORF3 are not considered here. When the cell is starved of amino acids or glucose, or is stressed in some other way, general protein synthesis is down-regulated by phosphorylation of the α -subunit of eIF2 by the GCN2 kinase. Phosphorylation of eIF2 α prohibits the GDP $\leftrightarrow$ GTP exchange and therefore inhibits general protein synthesis. Translation of GCN4 is enabled by a surprisingly sophisticated re-initiation-dependent mechanism that stimulates GCN4 translation when general protein synthesis is inhibited. Almost all scanning ribosomal complexes initiate at uAUG1. Although re-initiation is a very inefficient mechanism in general, the sequences around the termination codon of uORF1 enable 50% of the ribosomes translating uORF1 to resume scanning after termination. In order to be able to re-initiate, these 40 S ribosomal subunits have to rebind eIF2-GTP-Met-tRNA_i. Under normal conditions, most of the ribosomal subunits are capable of re-initiating upon reaching uAUG4. After translation of uORF4, ribosomes will terminate, leave the mRNA, and therefore not translate the GCN4 ORF. Both the rare proline codon (the last codon of uORF4) and the 10 nt 3' to uORF4 are especially efficient in preventing reinitiation after translation of this uORF [26]. When general protein synthesis is inhibited, only half of the ribosomal complexes can initiate and terminate at uORF4. The rest of the complexes will not be loaded with eIF2-GTP-Met-tRNA_i in time for initiation at uORF4, and will be reloaded during the scanning of the remaining 150 nt of the 5'-UTR, enabling translation of the GCN4 ORF.

The only comparably regulated mammalian gene known so far is activating transcription factor 4 (ATF4) [28]. The mouse ATF4 5'-UTR (272 nt) contains two uORFs, of which the second one overlaps with the ATF4 ORF (Figure 2). Translation of ATF4 increases after stress upon eIF2 phosphorylation by PKR-like ER kinase ('PERK'), whereas translation in general is inhibited under these conditions. Stress induces a threefold increase in ATF4 translation when both uORFs are present. Mutation of both uORFs causes a strong increase in ATF4 translation: deletion of uORF1 a 100-fold increase, and mutation of uAUG2 is responsible for a 500-fold increase (compared with the wild-type 5'-UTR under repressed conditions). Whereas mutation of uAUG1 of GCN4 leads to a severe inhibition of translation, mutation of uAUG1 of ATF4 mRNA has a stimulatory effect. uORF1 of ATF4 is less effective than the uORF1 of GCN4 in overcoming the inhibitory effect of the second uORF. This shows that translational control of ATF4 is not identical with the translational control of GCN4, although similar in the response to eIF2 phosphorylation.

(B) Synthesis of nested proteins

Another regulatory function of uAUGs is the possibility of initiation at different AUG codons, resulting in the synthesis of

different proteins. Transcription factors of the CCAAT/enhancer binding protein (C/EBP) family are important for differentiation and cell proliferation. C/EBP α and C/EBP β mRNAs have a common structure (Figure 2). Both mRNAs contain one uORF in a reading frame different from the C/EBP reading frame (initiation at site D). Four (C/EBP α) or three (C/EBP β) potential translation initiation sites are present in the same reading frame, enabling the production of four or three proteins with a different N-terminal extension and an identical C-terminal DNA-binding domain. The shorter isoforms lack different parts of the N-terminus of the full-length protein and do not contain the activation domain. Therefore the isoform ratio is important for the activity of these proteins. Translational control determines the isoform ratio and is dependent on the presence of the out-of-frame uORF D. Initiation on A and B1 is not dependent on initiation at AUG codon D, but initiation on B2 and C is dependent on re-initiation after uORF D translation [29]. The CUG repeats 5' to the uORF and the CCG repeats in the uORF of C/EBP β are binding sites for CUGBP1 (CUG repeat binding protein 1). Binding of CUGBP1 stimulates the translation of the truncated C/EBP isoforms, and therefore CUGBP1 inhibits C/EBP-dependent transcription of target genes [30]. The isoform ratio is not only modulated by uORFs, but by translation-factor activity as well. Both high eIF2 activity and eIF4E overexpression resulted in an increase of the truncated isoforms and a decrease of the full-length protein. This is due to stimulated initiation at site D, reducing initiation at site B1 and increasing re-initiation at site C. Therefore, uORF D is crucial for the regulation of the isoform ratio and consequently for the transcriptional regulation of C/EBP target genes [29].

The correct expression of the proto-oncogene *fli-1* is important for differentiation and cell migration during early embryonic development. Two different Fli-1 protein isoforms of 51 and 48 kDa are synthesized from the same transcript by using two in-frame initiation codons [31]. The synthesis of these isoforms is dependent on two uORFs that both overlap the initiation codon of the 51 kDa isoform (Figure 2). Translation termination at the stop codon of the uORFs stimulates the synthesis of both Fli-1 isoforms. Initiation at the 48 kDa isoform AUG codon is stimulated by enabling re-initiation after translation of the uORFs. The synthesis of the 51 kDa isoform is stimulated by the block caused by the terminating ribosomes, which cause the upstream scanning ribosomal subunits to pile up and therefore increase upstream initiation [31].

One of the mRNAs encoding 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP2) directs the synthesis of two protein isoforms: CNP1 and CNP2 [32]. CNP1 translation is initiated at an internal AUG codon (Figure 2). The CNP2 isoform is initiated at AUG1 and has a 20-amino-acid extension at the N-terminus. Although several tissues contain the CNP2 transcript, the CNP2 polypeptide is only present in myelinating cells in the adult rat brain, whereas all tissues produce the shorter isoform, CNP1. Initiation on the upstream codon was tissue-specific, a phenomenon not studied further, but suggested a transactivating inducer or repressor of upstream initiation.

(C) Stalling of ribosomes at uORFs

Repression of translation of the human cytomegalovirus early glycoprotein (gpUL4) is dependent on the amino acid sequence of the second uORF [33] (Figure 2). Several amino acids in the 22-amino-acid peptide are essential for repression. The synthesis of the 22-amino-acid peptide itself has been shown, as well as the existence of a 20 kDa product consisting of the peptide covalently linked to tRNA^{Pro}, which is the tRNA decoding the last codon

Figure 2 Schematic representation of uORF-containing 5'-UTRs

Summary of the 5'-UTRs that are discussed in the text and that are under uORF-mediated control. Main ORFs are depicted by grey boxes, whereas uORFs are indicated by white boxes. When more than one upstream initiation site is present, they are numbered at the left upper corner of the initiation site in the same way as in the reference. The numbers under the uORFs indicate the number of amino acids in the encoded peptides. Ribosome stalling sites are indicated with upward-pointing arrows (↑). The double lines under the 5'-UTR of C/EBPβ mark the CUGBP1 binding sites. The dotted line under the 5'-UTR of CaMV 35S ORF VII indicates the region involved in the formation of the stable secondary structure, and the single underlining marks the ribosome landing site.

of uORF2 [34]. After translation of uORF2, the ultimate tRNA is not released and the ribosomal complex, including peptidyl-tRNA, forms a barrier for upstream scanning 40 S ribosomal subunits. Although uAUG2 is recognized inefficiently, once the uORF is translated the scanning subunits are stalled and translation of the gpUL4 ORF is strongly inhibited [35]. The peptide is supposed to interact with the ribosome and/or ribosome-associated translation factors.

S-Adenosylmethionine decarboxylase (AdoMetDC) is a key enzyme in the pathway of polyamine biosynthesis. The uORF in the 5'-UTR encodes a hexapeptide with the sequence MAGDIS. The amino acid sequence, especially amino acids 4 (aspartic acid, D) and 5 (isoleucine, I), is crucial for translational control [36]. In the presence of polyamines, the uORF functions as a negative regulatory element; inhibition is partially relieved under low polyamine levels [37]. Polyamines stabilize an intermediate in the termination process, comparable with the peptidyl-tRNA-ribosome complex found for gpUL4, which causes stalling of the translating ribosome [38]. The reduced rate of uORF translation can therefore cause a block for the upstream ribosomal subunits [39]. The extent of translational repression of AdoMetDC by the uORF is dependent on the cell type [40]. Initiation at the uAUG, which is located only 14 nt from the cap, is relatively infrequent in non-lymphoid cells, and the translation efficiency of AdoMetDC in these cells is therefore relatively efficient [41]. Whether lymphoid cells have a more stringent control over polyamine synthesis remains to be shown. The suggested association of polyamines with the MAGDIS peptide is reminiscent of the influence of arginine on translation of arg-2 mRNA. The *Neurospora crassa* arg-2 mRNA contains an evolutionarily conserved uORF encoding a 24-amino-acid peptide, the arginine attenuator peptide (AAP; Figure 2). Other AAP-encoding uORFs are found in the arg-2 5'-UTRs of *Magnaporthe grisea*, *Trichoderma virens* and *Aspergillus nidulans*, as well as in the CPA-1 5'-UTR of *S. cerevisiae*. Translation of the uORF causes stalling of elongating/terminating ribosomes in the presence of arginine. Stalling on the uORF is reversible; inhibition of arg-2 translation is released when arginine is depleted [42]. Regulation is dependent on the peptide sequence – the mRNA sequence is not important. Only amino acids 2–4 can be omitted [43]. Regulation of arg-2 translation is not modulated by the charge status of arginyl-tRNAs [44]. Toeprint assays showed that ribosomes are stalled around the termination codon. However, mutation of the termination codon still causes stalling a few codons downstream of the (eliminated) stop codon, showing that termination itself is not necessary for arg-2 regulation. Introduction of a rare leucine codon (UUA) instead of a common leucine codon (CUC) causes extra stalling on the rare codon, but, surprisingly, this did not result in decreased reporter-gene expression [43].

Translation of *Xenopus laevis* connexin41 mRNA is controlled by three overlapping uORFs (Figure 2). Each of the three uORFs contributes to down-regulation of connexin41 expression. The presence of uORF1 has the largest impact on connexin41 synthesis [45]. Preliminary results indicate that, despite the large impact of uAUG1 on downstream translation, uORF1 is very inefficiently translated, suggesting a stalling-dependent mechanism, as described above for gpUL4, AdoMetDC and arg-2.

In these examples, stalling of scanning 43 S ribosomal complexes is due to slow elongation or termination of ribosomes translating a uORF. Stalling is not necessarily dependent on the presence of uORFs, since 43 S complexes can be stalled on an adenine-rich element in the 5'-UTR of PABP1 mRNA [46]. Also, the translational inhibition of HAC1 (a transcription factor that controls the unfolded protein response) by ribosomes trans-

lating the main ORF is caused by stalling due to interaction of the 5'- and 3'-ends of the mRNA. This example shows that secondary structure can be responsible for stalling as well [47].

(D) Some other examples of uORF-containing mRNAs

The long 5'-UTR of mouse retinoic acid receptor β 2 (RAR β 2) mRNA (461 nt) contains five uORFs (Figure 2) involved in the regulation of translation efficiency as well in localization-dependent translation [48,49]. The stimulatory roles of uORFs 2 and 5 suggest an eIF2-dependent control, similar to GCN4. uORF4 encodes a peptide that inhibits expression specifically in heart and brain. The amino acid sequence, but not the nucleotide sequence, is critical for its regulatory function. uORFs 1 and 5 might additionally stimulate translation in heart and brain. The mechanism responsible for the regulation of initiation at the different uAUGs is not known so far, nor is it known how the putative peptides control tissue-specific expression.

Translation of two uORFs in the *S. cerevisiae* transcription-factor YAP2 5'-UTR stimulates decay of the YAP2 mRNA (termination-dependent decay) [50]. The destabilizing effect is dependent upon the efficiency of release of terminating ribosomes from the mRNA. G/C-rich sequences and secondary structure downstream of the termination codon stimulate efficient termination and therefore YAP2 mRNA decay. Nonsense-mediated decay (NMD) can be responsible for degradation of mRNAs harbouring uORFs in their 5'-UTRs [51]. The influence of termination codons on NMD has been reviewed recently [52].

uORFs play an essential role in the translation of cauliflower mosaic virus (CaMV) 35 S ORF VII. Translation of uORFs is one of the essential elements for non-linear ribosomal scanning, or shunting. This is a cap-dependent form of translation initiation in which the 40 S ribosomal subunit skips part of the 35 S 5'-UTR (612 nt and seven uORFs; Figure 2). Two sequence elements in this 5'-UTR are crucial for translation: the first uORF (uORF-A) and a stable hairpin that is located immediately downstream [53,54]. Both uORF-A and the hairpin inhibit translation in the absence of the other element. After translation of uORF-A the ribosomal subunit is shunted to a site downstream of the hairpin and resumes scanning. Shunting on 35 S RNA is a special case of re-initiation. Nevertheless, shunting is possible without preceding translation of a short uORF as well. Shunting in adenovirus and heat-shock protein 70 mRNAs is facilitated by sequences in the 5'-UTR complementary to 18 S rRNA sequences [55], suggesting that the re-binding of the 40 S subunit occurs via base-pairing between rRNA and mRNA.

TRANSLATIONAL CONTROL VIA INTERNAL RIBOSOME ENTRY

IRES-mediated translation is a cap-independent mechanism. The ribosome lands close to the initiator AUG codon on the mRNA. IRES activity is dependent on secondary and tertiary structure [56], on transacting factors [57], and on sequence complementarity to 18 S rRNA [58]. Canonical eIFs are necessary for proper IRES function, except for the cap-binding eIF4E. However, IRES-mediated initiation on certain viral RNAs is possible without some of the initiation factors [59]. Cricket-paralysis-virus RNA can bind 40 S subunits without any assisting proteins [60]. Initially, IRESs were described in picornavirus mRNA, and many viral IRESs followed. Some IRESs are more active in the presence of additional cellular proteins like La autoantigen, polypyrimidine-tract-binding protein ('PTB') and poly(rC)-binding protein ('PCBP'). It is suggested that these cellular proteins function as RNA chaperones and that their cell-specific distribution may account for the cell-specific IRES activity of some mRNAs [57]. Besides the extensively studied

Table 3 Cellular mRNAs containing a regulated IRES element

mRNA	Conditions of IRES-dependent translation	Reference(s)
p58 ^{PITSLRE}	G2/M phase of cell cycle	[62]
ODC	G2/M phase of cell cycle	[63]
FGF-2	Growth control	[71]
PDGF2/C-SIS	Growth control	[72]
MYT2	Growth control	[73]
VEGF	Hypoxia	[69,70]
cat-1	Cellular stress	[74–76]
Apaf-1	Apoptosis	[67]
XIAP	Apoptosis	[68]
<i>c-myc</i>	Apoptosis, tissue specific translation	[64,66]
Ubx	Tissue specific translation	[81]
Antp	Tissue specific translation	[81]

viral IRESs, some cellular IRESs have been described, although they are relatively rare (Table 1). An overview of some regulated cellular IRESs is given in Table 3. It is noteworthy that most IRES containing messengers are activated when general protein synthesis is inhibited.

Under certain conditions, like the G2/M phase of the cell cycle, cap-dependent protein synthesis is inhibited [61]. However, p58^{PITSLRE} (a protein kinase) and ornithine decarboxylase (ODC, a rate-controlling enzyme in polyamine biosynthesis) are translated efficiently at this stage of the cell cycle [62,63]. Both mRNAs contain an IRES that functions exclusively during G2/M phase. When cap-dependent translation is not inhibited, both mRNAs are translated via cap-dependent initiation. This results, for the PITSLRE transcript, in a different isoform, namely p110^{PITSLRE}, initiated at an AUG codon 1014 nt upstream of the p58^{PITSLRE} initiation codon.

The mRNA of *c-myc* (a proto-oncogene involved in proliferation and apoptosis) has a long complex 5'-UTR that contains an IRES. The *c-myc* IRES activity is dependent on the cell type, and expression of *c-myc* during embryogenesis was found to be restricted to certain tissues [64]. During apoptosis, cap-dependent translation is decreased, probably owing to eIF4G cleavage, although other translation factors are also cleaved [65]. Several genes involved in apoptosis, e.g. *c-myc* [66], apoptotic protease activating factor (Apaf-1) [67] and X-linked inhibitor of apoptosis protein (XIAP) [68], contain an IRES in their 5'-UTR [12]. Overall protein synthesis is compromised in hypoxic cells as well. Under these circumstances vascular endothelial growth factor (VEGF) is translated via internal ribosome entry [69]. The VEGF 5'-UTR contains two IRESs, each followed by at least one initiation codon [70]. The synthesis of several other proteins involved in growth control appears to be IRES-dependent: fibroblast growth factor 2 (FGF-2) [71], platelet-derived growth factor 2 (PDGF2/*c-sis*) [72] and MYT2 [73].

Amino acid starvation [74], glucose limitation [75] and the presence of double-stranded RNA [76] in mammalian cells result in an increase in translation of the arginine/lysine transporter cat-1 mRNA. Under these conditions, general protein synthesis is inhibited, owing to phosphorylation of eIF2. The cat-1 5'-UTR contains an IRES element that stimulates translation under stressful conditions. This requires the translation of a 48-amino-acid uORF [74] and is indirectly dependent on eIF2 phosphorylation. The existence of an IRES element regulated by eIF2 phosphorylation bridges two seemingly different ways of translational control: by IRES control and by eIF2 phosphorylation.

In general, the above-mentioned mRNAs with an IRES are translated when cap-dependent translation is inhibited by stress, cell-cycle stage or apoptosis. They have in common that cellular conditions can disfavour cap-dependent translation, inducing a switch to IRES-dependent translation. Whether non-initiation factors or transactivating proteins are assisting this switch to IRES-dependent translation is an intensely studied field [12].

TRANSLATIONAL CONTROL VIA SECONDARY STRUCTURE

Translational control of mRNAs involved in iron homeostasis is dependent on the binding of iron regulatory proteins (IRP1 and IRP2) to an iron-responsive element (IRE) (reviewed by Mikulits et al. [77]; Figure 3A). An IRE consists of a small hairpin with a terminal hexaloop region and a bulged cytidine residue 5 nt 5' to the loop. A single IRE is located close to the cap in the 5'-UTR of, for example, ferritin and erythroid δ -aminolaevulinate synthase (eALAS). These proteins are involved in iron storage and utilization respectively and are not required under low iron levels. Low intracellular iron levels increase the mRNA binding activity of IRP1 and the stability of IRP2. Binding of IRP to the IRE prevents landing of the 43 S ribosomal complex next to the cap and therefore strongly inhibits ferritin and eALAS translation (Figure 3A). Under high-iron conditions, IRP will not longer bind to the IRE and this therefore enables the 43 S complex to bind to the mRNA (Figure 3B). IRES are also found in the 3'-UTR of transferrin receptor (TfR) mRNA. Binding of IRP to this 3'-located IRE prevents degradation of the TfR mRNA and therefore stimulates iron uptake. The translational control of ferritin, eALAS and TfR is responsible for maintaining iron homeostasis via the IRESs present in 5'- or 3'-UTRs [77].

The 5'-UTRs of IGF2 (insulin-like growth factor-2) leader 1, transforming growth factor- β 5 (TGF β 5) and PDGF-A are highly structured. Translation of these messengers in young *Xenopus* embryos is strongly inhibited. After the onset of embryonic transcription this inhibition is alleviated, suggesting that a transacting factor facilitates translation of these complex messengers [78,79].

TRANSLATION INITIATION IS CONTROLLED BY VARIOUS ASPECTS

As described above, translation initiation can be controlled via different elements: secondary structure, IRE and protein-binding sites, uORFs, and IRESs. An interesting question is why translation is controlled by these diverse elements. In general, the elements mentioned down-regulate translation, and the difference between these elements is expected to be related to the response of the cell to extracellular changes. A breakthrough was reached by analysing how the cell cycle controls initiation of the two IRES-containing mRNAs p58^{PITSLRE} and ODC [62,63]. These two mRNAs escape the temporal defects in translation by virtue of their cap-independent initiation. The induction of translation of some mRNAs during mitosis, apoptosis or hypoxia is counter-intuitive: eIF4E is dephosphorylated, suggesting inactivation; 4E-BP1 is hypophosphorylated, sequestering eIF4E [62,63,66–69] and preventing mRNA recruitment and translation. Therefore cap-dependent translation is blocked, allowing a switch to IRES-dependent initiation. The IRES is thus required for translation under conditions when normal, cap-dependent translation is made impossible. This adaptive switch seems also possible for mRNAs with uORFs or uAUGs. The effect of uAUGs or uORFs can vary with changing cellular conditions, such as shown for AdoMetDC (polyamines) [40] and GCN4

Figure 3 Inhibition of 43 S complex binding to the mRNA by IRE or uORF

(A) Under low-iron conditions, IRP1 or IRP2 will bind to the IRE, thereby preventing the landing of 43 S ribosomal complexes (40 S plus attached Met-tRNA) next to the cap. This results in the inhibition of translation. (B) Under high-iron conditions, IRP will bind to iron ions instead of to the IRE and therefore create enough space for the 43 S complex to bind to the mRNA. The helicase activity of eIF4A, eIF4B and eIF4H results in the unwinding of the IRE and consequently translational activation. (C) During the translation of a uORF located relatively close to the cap, no new 43 S ribosomal complexes can bind to the mRNA. If the uAUG is recognized efficiently, or when elongation and/or termination proceed very slowly, this causes a strong translational inhibition of the main ORF. (D) When a close to the cap located uAUG is not recognized by the 43 S complex, or when the 43 S complex re-initiates on the sAUG, the main ORF will be translated and a new 43 S complex can start scanning of the 5'-UTR.

(nitrogen starvation) [27]. The uAUG is not a static hindrance, but a modulator of translation; this aspect will be further discussed below.

An adaptive mechanism for initiation may be operative during apoptosis. Translation of several genes involved in apoptosis – *c-myc*, Apaf-1 and XIAP – is IRES-mediated [66–68]. Translation of messengers under stress conditions that inhibit cap-dependent translation can be IRES-dependent as well, e.g. FGF2, VEGF and eIF4G [69,71,80]. Furthermore, changes in cell metabolism can be controlled by IRESs. Many IRES-containing mRNAs are especially active when phosphorylation and activity of eIF4E is diminished. In contrast, some uORF-containing mRNAs, such as GCN4, are regulated via the phosphorylation status of eIF2. An extraordinary example of an IRES-containing mRNA is the *cat-1* mRNA. The function of this IRES is not only dependent on eIF2 phosphorylation, but on the presence of a uORF as well [74–76]. It is expected that in many more mRNAs uORFs or uAUGs do not just function as a static translational block, but that the recognition efficiency of the uAUGs is regulated by initiation factors, most likely eIF2, or transacting factors. An example is the C/EBP mRNA described above: differential recognition of the AUGs is mediated by CUGBP [29]. As far as we are aware, no study has been directed towards the phosphorylation status of eIF2 during the cell cycle or during development, but this should prove interesting.

Translation of a group of uORF-containing messengers is controlled by re-initiation: GCN4, ATF4, C/EBP α , C/EBP β , and Fli-1 [27–29,31]. Until now, re-initiation was thought to be a very rare mechanism, and has been shown in only a small percentage of uORF-containing messengers. Re-initiation on downstream initiation codons in the above-mentioned mRNAs – with the possible exception of Fli-1 – is dependent on the phosphorylation state of eIF2. eIF2 is phosphorylated, and thus

inactivated, under several stress conditions like viral infection, amino acid or glucose starvation, that inhibit general protein synthesis. Therefore these mRNAs are activated, especially when translation of most mRNAs is inhibited by eIF2 phosphorylation.

Re-initiation can play a role in the synthesis of different protein isoforms encoded by the same mRNA, although it is not the only possible mechanism. The isoform ratio of Fli-1, C/EBP α and C/EBP β is dependent on the presence of a uORF. For C/EBP α and C/EBP β , the choice of initiation site is highly controlled by the activity of eIF2 and eIF4E, and by the binding of CUGBP to the CCG and CUG repeats around the uAUG codon of C/EBP β . Since the short isoforms lack the activating domain, the activity of C/EBP-mediated transcription is dependent on the isoform ratio.

Tissue-specific translation can be directed by both uORFs and IRESs. The brain-specific translation of the longest CNP2 isoform is mediated by alternative AUG recognition [32], whereas tissue-specific translation of RAR β 2 is dependent on one of the uORFs [49]. Examples of IRES-mediated tissue-specific expression are Antennapedia, Ultrabithorax and *c-myc* mRNAs [64,81].

The presence of translation-limiting hairpins in mRNA will probably not be a static, inhibitory property: transcriptional control could serve the same purpose of allowing a low expression level. The IRE-binding proteins have until now provided a rather unique example of how protein binding to hairpins in 5'- and 3'-UTRs can influence translation efficiency (Figures 3A and 3B). Although secondary structures seem to be static structures, present because of their ability to down-regulate translation, a few examples have been described when this down-regulation is released. The extensive secondary structures of IGF2, TGF β 5 and PDGF-A effectively block translation in young *Xenopus*

embryos [78,79]. After the onset of embryonic transcription, translation repression is diminished, showing that translational control via secondary structures can be dependent on the condition of the cell, as exemplified by the iron control of IRE-containing mRNAs.

A uORF can function in the same way as an IRE (Figure 3). During translation of a uORF that is located relatively close to the cap, new 43 S ribosomal complexes cannot bind to the mRNA (Figure 3C). Especially, a uORF inefficiently translated due to stalling during elongation and/or termination, will cause a severe translational inhibition. If the uORF is located more downstream, the effect can be similar. The 43 S complexes will be able to bind at the 5'-end of the mRNA, but they will scan very slowly as long as they are held up by the downstream ribosome (H. A. Meijer and A. A. M. Thomas, unpublished work). When the uAUG is not recognized the main ORF will be translated and a new 43 S complex can start scanning the 5'-UTR (Figure 3D).

The different elements in the described 5'-UTRs – IRESs, hairpins, protein binding sites, uAUGs and uORFs – are probably used to enable the translational control of several gene groups by a distinct mechanism. In particular, the activity of eIF2, eIF4E and eIF4G cause the preferential translation of mRNAs under circumstances when general protein synthesis is inhibited. Translation of these messengers facilitates the response of the cell to stress, and is therefore a powerful tool for enabling the cell to survive.

FUTURE RESEARCH

The recent identification of mRNAs that are translated via an IRES only during certain stages of the cell cycle has promoted a search for proteins that mediate this switch. Although it is possible that the mere inhibition of cap-dependent translation induces a latent ability of the IRES to achieve prominence, it is likely that recruitment of the initiation apparatus to internal sequences requires transactivating proteins. These hypothetical proteins may guide initiation factors to the IRES, but may also change the conformation of the mRNA, allowing access to internal sequences. An elegant way of modulating the switch between cap-dependent and IRES-mediated translation would be the activation of a protein that mediates this switch, such as a cell-cycle-dependent phosphorylation. Initiation-factor activity is already known to be modulated during the cell cycle (eIF4E, 4E-BP1). Whether phosphorylation of other important initiation factors, such as eIF2 or eIF2B, is also cell-cycle-dependent is yet to be clarified.

Whereas progress has been made in understanding the function of IRESs during normal and stress conditions, relatively little is understood on the influence of uAUGs and uORFs. The many examples of mRNAs with one or more uAUGs in the 5'-UTR indicate that the translational block serves a regulatory role, not a static, inhibitory role. The low expression of an mRNA coding for the relatively abundant connexin41 protein and the very inefficient translation of the mRNA [45] suggests that inhibition of uORFs can be controlled. Adaptation to changing conditions is well understood for the GCN4 mRNA, but this example is rather unique. Skipping of a normally inhibitory uORF in a 5'-UTR can occur when initiation is blocked until the ribosome is loaded with eIF2 and Met-tRNA. The skipping of the uAUG in AdoMetDC in some cell lines, but not in others, suggests that AUG recognition can be modulated by protein–RNA interaction. Whether the ribosome can be induced to ignore uAUGs will be an interesting matter for future study. Similar arguments can be used that the stalling of the ribosome over termination codons or rare codons can be modulated. In these cases the

simplest explanation would be that translation elongation or termination activity is controlled by cellular conditions and is specific for certain mRNAs.

Transacting factors have been studied for quite some time, especially in respect of picornavirus RNAs [82]. The description of transacting factors modulating cellular IRES function is for the future. It will be intriguing to find out how transacting factors are themselves controlled and how they activate cellular mRNAs with IRESs, hairpins or uORFs.

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