REVIEW

Protein synthesis in eukaryotes: The growing biological relevance of cap-independent translation initiation

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ABSTRACT

Ribosome recruitment to eukaryotic mRNAs is generally thought to occur by a scanning mechanism, whereby the 40S ribosomal subunit binds in the vicinity of the 5'cap structure of the mRNA and scans until an AUG codon is encountered in an appropriate sequence context. Study of the picornaviruses allowed the characterization of an alternative mechanism of translation initiation. Picornaviruses can initiate translation *via* an internal ribosome entry segment (IRES), an RNA structure that directly recruits the 40S ribosomal subunits in a cap and 5' end independent fashion. Since its discovery, the notion of IRESs has extended to a number of different virus families and cellular RNAs. This review summarizes features of both cap-dependent and IRES-dependent mechanisms of translation initiation and discusses the role of *cis*-acting elements, which include the 5'cap, the 5'-untranslated region (UTR) and the poly(A) tail as well as the possible roles of IRESs as part of a cellular stress response mechanism and in the virus replication cycle.

Key terms: protein synthesis, translation initiation, internal ribosome entry segment.

MECHANISMS THAT MEDIATE TRANSLATION INITIATION

Following transcription, processing and nucleo-cytoplasmic export, eukaryotic mRNAs are competent for translation. The regulation of translation rates – the frequency with which a given mRNA is translated – plays a critical role in many fundamental biological processes, including cell growth, development and the response to biological cues or environmental stresses (172). Deregulation of translation may also be an important component in the transformation of cells (38, 166). Indeed, modulation of mRNA translation exerts a profound effect on global gene expression (172). In effect, even though two transcripts are present in the cytoplasm in identical quantities, they may be translated at very

different rates (172). This phenomenon is due, in part, to the fact that the ribosome does not bind to mRNA directly but must be recruited to the mRNA by the concerted action of a large number of eukaryotic translation initiation factors (eIFs) (78, 222). This recruitment step, also referred to as the initiation phase, can be defined as the process in which a special initiator tRNA, Met-tRNA_i, is positioned in the P site of a ribosome located at the correct initiation codon (98). When the initiation stage is complete, the 80S ribosome is capable of dipeptide formation (Fig. 1).

Translation initiation of eukaryotic mRNAs in general occurs by a scanning mechanism. Key features of this model include the recognition of the 5' terminus of the mRNA and its cap structure (m⁷GpppN), followed by binding of the 40S

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Figure 1. Schematic diagram of translation initiation in eukaryotes. Translation of mRNA into protein begins after assembly of initiator tRNA, mRNA and both ribosomal subunits. The complex initiation process that leads to 80S ribosome formation consists of several linked stages that are mediated by eukaryotic initiation factors. See text for details. The 40S ribosomal subunit is captured for initiation via complex arrays of protein-RNA and protein-protein interactions. In the cap-dependant mechanism, the pre-initiation complex binds to the mRNA at the 5' terminal cap structure with help of the eIF4F protein complex and then migrates along the mRNA until it encounters the initiation codon where the 80S ribosome is reconstituted. Upon release, the eIF are recycled. This simplified model has been adapted from Pain (190). Updates include the ribosomal joining model proposed by Unbehauen et al. (263).

ribosomal subunit and scanning downstream to the initiation codon (98, 200). A consequence of cap recognition is that eukaryotic mRNAs are monocistronic, since an mRNA contains only a single 5' terminus. On the other hand, cap-dependency allows the cell to control gene expression by modulating the assembly and activity of the cap-binding complex components. Translational control thereby allows the cell to fine-tune gene expression by stimulating or repressing the translation of specific mRNAs, usually through the reversible phosphorylation of translation factors (79, 221). The study of the picornaviruses allowed the characterization of an alternative mechanism of translation initiation. Picornaviruses can initiate translation *via* an internal ribosome entry segment (IRES), an RNA structure that directly recruits the 40S ribosomal subunits in a cap and 5'-end independent fashion. Therefore, in general and depending on how the 40S ribosomal subunit is recruited to the mRNA, translation initiation can take place by a cap-dependent or a cap-independent fashion.

CAP-DEPENDENT TRANSLATION INITIATION

All eukaryotic mRNAs present a 5' terminal nuclear modification, the cap structure. This structure integrates several important functions and affects RNA splicing, transport, stabilization and translation. In translation, the cap structure serves as a "molecular tag" that marks the spot where the 40S ribosomal subunit is to be recruited. Important in this recruitment process is the eIF4F complex (78). EIF4F is a 3-subunit complex composed of eIF4E, eIF4A and eIF4G. EIF4E is the cap-binding protein and is therefore obligatory for the start of cap-dependent translation initiation. EIF4A is a member of the DEA(D/H)-box RNA helicase family, a diverse group of proteins that couples ATP hydrolysis to RNA binding and duplex separation (227). EIF4A participates in the initiation of translation by unwinding secondary structure in the 5'-untranslated region of mRNAs and facilitating scanning by the

40S ribosomal subunit for the initiation codon. EIF4A alone has only weak ATPase and helicase activities, but these are stimulated by eIF4G and eIF4B (227). EIF4B, an RNA-binding protein, stimulates eIF4A helicase activity and promotes the recruitment of ribosomes to the mRNA by interacting with the 18S ribosomal RNA (rRNA) to guide the 40S ribosomal subunit to the single-stranded region of the mRNA (98). EIF4GI and eIF4GII (here generically referred to as eIF4G) serve as a scaffold for the coordinated assembly of the translation initiation complex, leading to the attachment of the template mRNA to the translation machinery at the ribosome. EIF4G brings together eIF4F, as it has two binding sites for eIF4A and one binding site for eIF4E, but more importantly, it bridges the mRNA cap (via eIF4E) and the 40S ribosomal subunit (via eIF3) (86, 97, 215) (Fig. 2). EIF4F is recognized as the key factor in selecting mRNA for translation, it is understood that the binding of eIF4F to an m⁷G cap commits the translational apparatus to the translation of that mRNA. The 40S ribosomal subunit is recruited to the mRNA as part of the 43S initiation complex, composed of the subunit bound to eIF2-GTP/Met-tRNA_i, eIF1A and eIF3 (98, 204, 222). EIF1A and eIF1 are required for binding to the mRNA and migration of the 43S complex in a 5' to 3' direction towards the initiation codon (199). The 5' to 3' migration of the 43S complex towards the initiation codon (ribosome scanning) is a process that consumes energy in the form of ATP. EIF1A enhances eIF4F-mediated binding of the 43S complexes to mRNA, while eIF1 promotes formation of the 48S complex in which the initiator codon is base paired to the anticodon of the initiator tRNA (199). These proteins act synergistically to mediate assembly of ribosomal initiation complexes at the initiation codon and dissociate aberrant complexes from the mRNA (199). EIF1 also participates in ensuring the fidelity of initiation by acting as an inhibitor of eIF5-induced GTP hydrolysis (discussed below) (263). The ribosome stops when it binds stably at the initiation codon to form the 48S initiation complex, primarily through

the RNA-RNA interaction of the AUG (mRNA), and the CAU anticodon of the bound Met-tRNA_i (associated to the 40S subunit via eIF2). The initiation codon is usually the first AUG triplet in an appropriate sequence context (G/AXXAUGG, where X is any nucleotide (nt), downstream of the 5'cap (140). Once positioned on the initiation codon the eIFs bound to the 40S ribosomal subunit are displaced (98, 204). Thus, the first step in ribosomal subunit joining is hydrolysis of eIF2-bound GTP and release of eIF2-GDP from 48S complexes (49). EIF5 induces hydrolysis of eIF2-bound GTP, leading to displacement of eIF2-GDP; the inactive eIF2-GDP is recycled to the activated eIF2-GTP by eIF2B, a guanine nucleotide exchange factor (98). In the absence of eIF1, eIF5 induces rapid hydrolysis of eIF2-bound GTP in 43S complexes. However, the presence of eIF1 in 43S complexes inhibits eIF5-induced GTP hydrolysis. Interestingly, the establishment of codon-anticodon base pairing, in the 48S complexes, relieves eIF1-associated

inhibition of eIF5-induced GTP hydrolysis. Thus, hydrolysis of eIF2-bound GTP in 48S complexes, assembled with eIF1, takes place (263). Therefore, eIF1 plays the role of a negative regulator, which inhibits premature GTP hydrolysis and links codon-anticodon base pairing with hydrolysis of eIF2-bound GTP. Hydrolysis of eIF2-bound GTP and release of eIF2 leads to release of eIF3 from 48S complexes assembled on AUG triplets (263). Finally, eIF5B mediates joining of a 60S subunit to the 40S subunit, resulting in formation of a protein synthesis-competent 80S ribosome in which initiator Met-tRNA is positioned in the ribosomal P site (149, 206) (Fig. 1).

The canonical scanning mechanism rules initiation of most mRNAs, but three non-classical cap-dependent initiation mechanisms have been described: leaky scanning, ribosomal shunting and termination-reinitiation (Fig. 3). These alternative means of cap-dependent translation initiation are expected to allow the scanning complex to overcome a variety of limitations imposed by the 5'UTR.

Figure 2. Mechanism of cap-dependent translation initiation. Schematic representation of the closed-loop model of translation initiation. In this model, the eIF4F complex interacts with both the 5' end of the mRNA (via eIF4E) and the poly(A) tail (via PABP) and recruits the 40S ribosomal subunit via its interaction with eIF3. For simplicity, other proteins, as well as a second eIF4A molecule known to interact with eIF4G, have been omitted.

Figure 3. Alternative mechanisms to the classical scanning model. Alternative mechanisms to the classical scanning model can be used to avoid inhibitory effects imposed by the 5'UTR. The initiation complex initially recruited in proximity to the 5' cap structure may (A) scan pass an encountered putative initiation codon if this is in a non-optimal context. In the mechanism known as leaky scanning, translation will initiate in a downstream AUG in an optimal context, (B) jump over the secondary structure or upstream initiation codons in a process termed ribosomal shunt, or (C) initiate at an upstream AUG codon and translate the upstream ORF, terminate and then reinitiate at a downstream AUG codon, termination-reinitiation. For diagram simplicity, eIF and other proteins known to participate in these processes have been omitted.

The scanning model predicts that ribosomes should initiate at the first AUG codon encountered by a scanning 40S subunit. In most mRNAs, initiation usually does indeed occur at the AUG triplet that is proximal to the 5' end of an mRNA. However, the first encountered AUG codon can be by-passed if it is present in a poor context. In this case, the 40S subunit will initiate at an AUG in a better context further downstream, in a process known as "leaky scanning" (Fig. 3A) (139). Leaky

scanning is widely used in viruses, where it presumably helps economize on coding space. In HIV-1, for example, the envelope protein (ENV) is translated from an mRNA that contains an upstream ORF encoding an accessory protein Vpu in a different reading frame. To permit Env synthesis, the *vpu* initiation site is in a weak context (235). Similar examples exist for the hepatitis B virus (HBV) (153), the human papillomaviruses (HPV) (250), the rabies virus (36), and the simian virus 40 (238).

The scanning model also postulates that when a scanning 40S ribosomal subunit encounters a hairpin loop in the 5'UTR, it does not skip over the loop but unwinds it (141, 142, 196). Nevertheless, there are some cases when a scanning 40S ribosomal subunit encounters the structures present in the 5'UTR and skips or shunts over a large segment, bypassing intervening segments including AUG codons and strong secondary structures that normally would block the scanning process (Fig. 3B). First characterized in cauliflower mosaic virus (CaMV) 35S RNA (68) and plant-related pararetroviruses, shunting has also been observed in Sendai (147), papillomaviruses (224), and adenovirus late mRNAs (278). In ribosome shunting, ribosomes start scanning at the cap but large portions of the leader are skipped. Thereby, the secondary structure of the shunted region is preserved.

In the reinitiation mechanism, a second ORF located in the same mRNAs can be translated without the 40S subunit becoming disengaged from the mRNA after reaching the first ORF stop codon. If the 5'-proximal AUG triplet in a mammalian mRNA is followed by a short ORF, a significant fraction of ribosomes resume scanning after termination of short ORF translation and reinitiate at a downstream AUG (Fig. 3C). For example, translation of yeast GCN4 mRNA occurs by a reinitiation mechanism that is modulated by amino acid levels in the cell (99). Ribosomes that translate the first of four upstream open reading frames (uORFs) in the mRNA leader resume scanning and can reinitiate downstream. The frequency of reinitiation following uORF1 translation depends on an adequate distance to the next start codon and particular sequences surrounding the uORF1 stop codon (99).

THE mRNA POLY(A) TAILS' INVOLVEMENT IN TRANSLATION INITIATION

Most eukaryotic mRNAs, with the notable exception of histone mRNAs, possess a polyadenylated [poly(A)] tail (50-300 nt in length) at their 3' end. The majority of the eukaryotic transcripts are post-

transcriptionally polyadenylated in the nucleus (273). The poly(A) tail interacts synergistically with the 5'cap in stimulating translation (22, 70, 178, 181, 214). The poly(A) tail of most transcripts is coated with multiple copies of the poly(A)-binding protein (PABP), a 70-kDa protein with four highly conserved RNA recognition motifs (55, 82, 125). PABP is a ubiquitous, essential factor with well-characterized roles in translational initiation and mRNA turnover (82, 87, 267). Both yeast and human PABPs interact with the translation initiation factor eIF4G, thereby causing circularization of the mRNA via bridging of its 5' and 3' termini (eIF4E/eIF4G/PABP) (Fig. 2) (110, 125, 256). This circular mRNA complex has been reconstituted *in vitro* using purified components and visualized by atomic force microscopy (271).

An ongoing debate exists regarding the mechanism by which PABP-induced circularization of the mRNA stimulates translation. The current closed-loop model suggest that circularization of mRNA improves translation efficiency by facilitating the utilization or recycling of 40S ribosomes (117, 125). This notion has been reinforced by reports that establish a clear link between translation initiation and termination. Eukaryotic release factor 3 (eRF3) is a 628 amino acid protein implicated in translation termination (281). eRF3 interacts with eRF1, which recognizes the stop codon of the mRNA, and together they release the newly synthesized polypeptide (135). Recently, eRF3 was shown to interact with the C-terminal domain of PABP (262). The large distance between the translation termination codon and the poly(A) tail in mRNAs with long 3' UTRs has served as an argument against the role of the poly(A) tail in ribosome recycling. However, the interaction between eRF3 and PABP may provide a physical link between translation-terminating ribosomes and the 3' poly(A) tail for recruitment to the 5' end of the mRNA (262). Long 3'UTRs can be "looped-out", bringing terminating ribosomes and the 5' translation initiation complex into close

proximity, in such a way that would allow these ribosomes to re-initiate a round of translation. Alternatively, it can be that circularization allows for efficient translation of only intact mRNAs, thus diminishing the possibility of generating potentially dominant-negative forms of proteins from nicked mRNAs. A third hypothesis posits that PABP promotes 60S ribosomal subunit joining at the start codon (237). Finally, PABP binding to eIF4G may engender conformational changes that promote eIF4F activity. A recent report might have shed light on this issue by showing that in mammals PABP exerts its stimulatory effects at multiple stages of translation initiation (126). The work of Kahvejian and colleagues suggests that PABP would regulate both initiation factor binding to the mRNA (thus controlling 40S recruitment) and 60S joining (thus 80S assembly) (126).

Even though the mechanism by which PABP enhances translation initiation is not fully defined, experimental evidence does support the need of mRNA circularization for efficient protein synthesis. Further attestation for this model comes from the study of viral RNAs that lack a 3'poly(A) tail (Fig.4). Rotavirus, a member of the *Reoviridae*, contains eleven double-

stranded RNA segments (191). All segments are transcribed into mRNAs that possess a 5'cap structure but lack 3'poly(A) tails. Instead, the 3' end sequences contain a tetranucleotide motif that is recognized by the virus-encoded protein NSP3. NSP3 protein binds specifically to the conserved viral 3' end sequences and to eIF4G (84, 210, 211, 265). Because eIF4G has a higher affinity for NSP3 than PABP, the interaction between PABP and eIF4G is disrupted in rotavirus-infected cells (56, 178, 211, 265). The two consequences of NSP3 expression, then, are reduced efficiency of host mRNA translation and circularization-mediated translational enhancement of rotavirus mRNAs (Fig. 4).

Two PABP-binding partners have been identified: PABP interacting protein 1 and 2 (Paip1 and Paip2) (45, 132). Paip1 is a 54-kD protein that shares homology with the central region of eIF4G, stimulates translation *in vivo*, interacts with eIF4A, and is involved in mRNA turnover (45, 87). Paip2 inhibits 80S ribosome complex formation thus inhibiting translation (132). Paip2 competes with Paip1 for binding to PABP. Moreover, the binding site for Paip2 and eIF4G on PABP overlap, suggesting that they compete for binding to PABP. Paip2 is also capable of displacing PABP

Figure 4. Translation initiation in rotavirus, an alternative to the closed loop model. The rotavirus NSP3 protein binds to the 3' end of viral mRNAs, can displace PABP from eIF4G and assure rotavirus mRNA circularization for translation.

from the poly(A) tail (132). Consequently, Paip2 strongly interdicts the interaction of PABP with both the poly(A) tail and eIF4G, thus inhibiting translation by disrupting circularization of mRNAs. These observations further bolster the idea that mRNA circularization is a key step in translation and represents a target for translational control. Thus, it can be concluded that the 5' and 3' termini of mRNAs communicate to facilitate efficient translation initiation through interaction of PABP with the translation initiation factor eIF4G. Interestingly, the functional relevance of this interaction is not limited to translation initiation. During the process of mRNA decay, the poly(A)-specific ribonuclease (PARN) interacts with the 5' cap in a manner that increases the processivity of poly(A) tail shortening (53). In serum-starved culture conditions, PARN phosphorylation is increased, thus increasing its affinity for the 5' cap, whereas the phosphorylation of both eIF4E and the 4E-binding protein, 4E-BP1, is diminished (236). The eIF4E-eIF4G interaction is of central importance for cap-dependent initiation and can be blocked by small regulatory proteins that bind to eIF4E, known as 4E binding proteins (4E-BPs). Mammalian 4E-BPs inhibit cap-dependent protein synthesis by binding to eIF4E. Three members of the 4E-BPs have been described. 4E-BP1 and 4E-BP2 share 56% identity, while 4E-BP3 shares 57% and 59% identity with 4E-BP1 and 4E-BP2, respectively (193, 213). 4E-BPs act as molecular mimics of eIF4Gs (90, 163, 167). EIF4Gs and the 4E-BPs occupy mutually exclusive binding sites on the surface of eIF4E. The interaction of 4E-BPs with eIF4E is modulated by the extent of 4E-BP phosphorylation (76, 77). The 4E-BPs strongly interact with eIF4E when in their hypophosphorylated state and dissociate from eIF4E upon hyperphosphorylation. Therefore, under serum-starved conditions eIF4E-4EBP1 interactions prevail granting PARN access to the 5'cap-structure (236). Therefore, it appears that for both mRNA translation initiation and degradation, the 5' and 3' termini of mRNA must be brought into

close proximity to effectively perform either process. This suggests that mRNA circularization may be a pivotal control point that determines the fate and regulates translation rates of a given mRNA.

IRES-MEDIATED TRANSLATION INITIATION

In 1988, it was discovered that translation of the uncapped picornaviral mRNA is mediated by an RNA structure which allows assembly of the translational machinery at a position close to or directly at the initiation codon, the internal ribosome entry segment (IRES) (Fig. 5) (121, 195). This finding broke one of the cardinal rules of translation initiation, that is, that eukaryotic ribosomes can bind to mRNA only at the 5' end. Functionally, the IRES was identified by inserting the poliovirus (PV) 5' UTR into the intercistronic spacer of a bicistronic construct coding two proteins (Fig. 6) (195). Expression of the second cistron documented the ability of the inserted sequence to promote internal ribosome binding and translation independent of the first cistron. In general, IRES-mediated translation is independent of the nature of the extreme 5' end of the RNA as it does not require a cap structure (115). In the artificial bicistronic mRNA model, translation of the downstream cistron occurs even when translation of the upstream cistron is abolished (115). As an alternative to bicistronic constructs, circular mRNA can be used to identify IRESs (34, 35). The principle behind this strategy relies on the observation that in cell free systems, eukaryotic ribosomes are unable to bind to small circular RNAs, 25-110 nucleotides in length, suggesting that eukaryotic ribosomes can only bind RNAs via a free 5'end. However, by *in vitro* translation of a circular mRNA, Chen and Sarnow (34) showed that spatial constraints imposed by circularization of IRES-containing RNA molecules do not interfere with IRES function, confirming that IRESs allow recruitment of the 40S ribosomal subunit totally independently from the 5' and 3' ends of the mRNA.

Figure 5. Schematic representation of internal ribosome entry site (IRES)-mediated translation initiation. Internal initiation is an alternative mechanism to cap-dependent translation initiation which allows loading of the 40S ribosomal subunit on the mRNA in a 5' end- and cap-independent fashion. Among the different IRESs canonical initiation factor requirements are variable. However, most IRESs require specific cellular proteins, IRES *trans-acting* factors (ITAFs), to be functional. See the text for details. For diagram simplicity, other proteins, as well as a second eIF4A molecule known to interact with eIF4G, have been omitted.

Figure 6. Bicistronic mRNAs. In bicistronic constructs, the first cistron is cap-dependent while the second cistron will be translated only if the intercistronic sequences can function as an IRES since ribosome recruitment to the intercistronic spacer is independent from the 5' cap structure. For diagram simplicity, eIF and other proteins known to participate in these processes have been omitted.

At present, IRESs are defined solely by functional criteria and cannot yet be predicted by the presence of characteristic RNA sequences or structural motifs. For cellular IRES elements, Le and Maizel have predicted that a Y-shaped, double-hairpin structure followed by a small hairpin constitute an RNA motif that can be found upstream of the start-site codon in a variety of cellular IRESs (148). However, currently there is no experimental evidence to support this prediction. Despite these apparent restraints, since the initial characterization of picornavirus IRESs, other RNA and DNA viruses have been shown to initiate translation internally. These include members of the *Flaviviridae* (212, 225, 242, 261), the *Retroviridae* (10, 13, 14, 26, 30, 52, 157, 184), and the *Herpesviridae* (15, 88, 111). IRESs also have been found in insect and in plant viruses (tobacco etch virus and the tobamovirus) (71, 114, 275) and have been described in insect and rodent retrotransposons (14, 158, 174, 228). As a general rule, there are no significant similarities between individual IRESs (unless they are from related viruses). The mechanism of internal initiation is not restricted to viruses, and IRESs have been increasingly recognized in cellular mRNAs (reviewed in 94). Although capped, some cellular mRNAs – including those encoding translation initiation factors, transcription factors, oncogenes, growth factors, homeotic genes and survival proteins – contain IRES elements in their 5'UTR sequences that may allow them to be translated under conditions when cap-dependent synthesis of proteins is impaired. For an extensive list of cellular IRESs, we direct the reader's attention to the IRES database: <http://ifr31w3.toulouse.inserm.fr/IRESdatabase/>.

IRES-mediated translation initiation is strictly dependent on the structural integrity of the IRES. Small deletions or insertions, and even substitution of single nucleotides in the IRES elements, can severely reduce or enhance their activity (64, 121, 169, 171, 195, 279). The tertiary structure of the IRES is supported by both RNA-protein (discussed in the next section) and long-

range RNA-RNA interactions between functional domains (144, 168). The latter interactions are strand specific and, *in vitro*, dependent on RNA concentration, ionic conditions and temperature, suggesting that IRES folding is a dynamic process. It is likely that the structural dynamism shown by IRESs plays an important role in their biological function, that is, IRESs may adopt specific structures, showing differential translational activities, depending upon the specific environmental conditions (168-170).

In vitro reconstitution of the translation initiation event using the encephalomyocarditis virus (EMCV) IRES showed that formation of 48S complexes is ATP-dependent and requires almost the same factors as the cap-dependent initiation mechanism except for eIF4E (203, 208). Specifically, the cap-binding protein complex eIF4F can be replaced by a complex of eIF4A and the central domain of eIF4G (lacking the eIF4E binding domain, see below) (155, 186, 187). Both eIF4A and the function of the central domain of eIF4G are essential for 48S complex formation, exemplified by the profound inhibition of EMCV IRES-mediated translation by dominant negative mutant forms of eIF4A, which sequester the eIF4A/4G complex in an inactive form (194). IRES requirement of eIFs is not a general feature. Biochemical reconstitution of the initiation process on the hepatitis C virus (HCV) and cricket paralysis virus (CrPV) IRESs revealed that in these particular cases, formation of the 40S/IRES complex is eIF-independent. For the former, studies show that the 40S ribosomal subunit binds specifically to the HCV IRES without any requirement for initiation factors, in such a way that the ribosomal P site is placed in the immediate proximity of the initiation codon (123, 137, 189, 207). Subsequent addition of the eIF2-GTP/Met-tRNAⁱ complex to the 40S/IRES complex is necessary and sufficient for formation of the 48S complexes. Although eIF3 is not needed for 48S complex formation, it binds specifically to the HCV IRES and is likely to be a constituent of the 40S/IRES complexes *in vivo* (123, 189, 243).

Significantly, initiation on the HCV IRES has no requirement for ATP or any factor associated with ATP hydrolysis and, as would be expected, is resistant to inhibition by dominant negative eIF4A mutants. For further insights into the mechanism of HCV translation initiation, we direct the reader to the 1999 review by Hellen and Pestova (93). The case of the CrPV IRES is even more astounding as this element cannot only recruit the 40S ribosomal subunit in a eIF-independent fashion but does not require eIF2, initiator tRNA, eIF5B, or GTP hydrolysis to form an 80S/IRES complex (119, 202, 205, 247). Most interesting is the fact that for CrPV the first encoded amino acid is not methionine and that initiation does not occur at a cognate AUG codon or even a weak cognate codon such as CUG or GUG (120). The N-terminal residue of the CrPV capsid protein precursor is either alanine (encoded by GCU or GCA) or glutamine (encoded by CAA) (120). Studies aimed at understanding the molecular basis of this mechanism revealed that for the CrPV, IRES translation initiates from the triplet positioned in the A site of the ribosome (274).

Supplementary support for the notion of cap-independent translation initiation came from studies conducted to examine the mechanism by which picornaviruses inhibit translation of capped cellular mRNAs (60, 154). Infection of cells by PV, rhinoviruses and aphthoviruses results in a rapid inhibition of host cell protein synthesis. During infection eIF4Gs are cleaved by viral proteases 2A of PV, coxsackievirus (CV) and human rhinovirus (HRV) or the leader (L) protease of foot and mouth disease virus (FMDV) into an amino-terminal fragment, which contains the eIF4E-binding site, and a carboxy-terminal fragment (p100), which contains the binding site for eIF3 and eIF4A (91, 145, 187). Consequently, cleavage of eIF4Gs following viral infection results in the inactivation of the eIF4F complex with respect to its ability to recognize capped mRNAs and hence in a severe inhibition of cap-dependent translation initiation (21, 145, 187). Yet, while p100 supports cap-independent translation initiation, its interaction with the IRES requires host

factors (11, 21, 145, 187). Therefore, upon infection with these viruses, host protein synthesis is blocked, and the viral genome is translated without competition from cellular mRNAs for the required host components. Inhibition of cap-dependent translation initiation by cleavage of eIF4G by virus-encoded proteases is a strategy that has been recently extended to other IRES-containing viruses. In fact, the retrovirus-encoded protease, a protein responsible for virus maturation, cleaves eIF4G, affecting translation initiation (8, 185, 197, 266). Not all *Picornaviridae* use this cleavage strategy to inhibit cap-dependent translation initiation, however. The cardioviruses inhibit host cell protein synthesis by inducing dephosphorylation of 4E-BPs (80).

Despite being independent of the presence of cap-binding complexes, translation of some IRESs is stimulated by the presence of a poly(A) tail (12, 72, 156, 177, 178, 192, 254). However, particularly in the case of the cellular IRESs found in BiP and c-myc (257), as well as some picornaviruses, the mechanism by which poly(A) enhances IRES-mediated translation is far from clear. In these examples, IRES activity is increased even though eIF4G is cleaved, under which circumstances poly(A) enhancement of IRES activity must be PABP independent as the eIF4G-PABP interaction and eIF4G-IRES recognition domains are cleaved apart. Nonetheless, it is possible that interactions between the IRES and 3' RNA regions can be established by as-yet-undetermined mechanisms. This is exemplified by the RNAs of barley yellow dwarf virus (6), satellite tobacco necrosis virus (152) and HCV, which lack both a 5'cap and a poly(A) tail (138) yet which contain sequences in the 3' UTR that are required to confer efficient IRES-dependent translation initiation (47, 113, 175, 176, 258, 269). These observations suggest that an RNA-RNA or an RNA-protein bridge is established between sequences or factors interacting with specific elements within the 3'UTR and the IRES. There are certainly a number of proteins, unrelated to PABP, capable of interacting with viral 3' UTRs that are also implicated in IRES interaction,

providing potential links between 3' and IRES sequences (81, 112, 159, 248, 249). This notion has not been demonstrated experimentally and thus remains speculative. There is one notable report, however, in which an RNA-RNA interaction between the 5' and 3' UTRs of an uncapped, nonpolyadenylated plant viral mRNA confers translation initiation (89).

IRES TRANS-ACTING FACTORS

The precise molecular mechanism by which the host translation apparatus recognizes IRESs is unknown, but accumulating data strongly suggest the utilization of both canonical initiation utilization of both factors, as well as specific cellular proteins known as IRES *trans-acting* factors (ITAFs), the later are normally not involved in cap-mediated initiation. ITAFs are important in this recognition process (Fig. 5) (11, 226). Initial support for the notion that some IRESs might require additional factors to enable their activity came from the observation that IRESs of the encephalomyocarditis virus (EMCV), foot-and-mouth disease virus (FMDV) and Theiler's murine encephalomyelitis virus (TMEV) were all active in rabbit reticulocyte lysate (RRL), whereas translation mediated by poliovirus (PV) and rhinovirus (hRV) IRESs was inefficient unless the lysate was supplemented with HeLa cell extracts (19, 27, 57). This phenomenon turned out not to be restricted to PV and hRV, as similar evidence was reported for the fibroblast growth factor 2 (FGF-2) and the human immunodeficiency virus type 1 (HIV-1) IRESs (17, 26, 179).

The list of known ITAFs is continually growing. Among the most studied factors are the human La autoantigen (La), the poly(rC) binding protein-2 (PCBP2), upstream of N-ras (Unr) protein, and the polypyrimidine tract binding protein (PTB). La and PTB are important for the IRES activity of some picornaviruses (24, 44, 83, 109, 127, 128, 134, 173, 223, 260), La is required by the HCV IRES (5, 44, 216), while Unr specifically activates the IRES of HRV and PV (25, 108). Functional *in vitro*

assays revealed that some IRES elements require not just one, but a specific combination of two or three ITAFs for efficient translational activity: PTB plus ITAF₄₅ –the latter a cell-cycle-dependent protein homologous to Mpp1 (murine proliferation-associated protein)– for the IRES of FMDV (209); PTB plus PCBP2 for the PV IRES (109); PTB plus Unr plus PCBP2 for the HRV IRES (108); and PCBP1, PCBP2, the heterogeneous nuclear ribonucleoprotein C and K (hnRNP C and hnRNP K) activate the c-myc IRES (62, 133); while La, hnRNP C1 and hnRNP C2 activate the X-linked inhibitor of apoptosis (XIAP) IRES (100, 102).

The mechanism by which ITAFs facilitate the recruitment of ribosomal subunits is so far unknown. One hypothesis posits that ITAFs possess chaperone activity and help to fold the IRES into the conformation required for translational activity (94, 116). This hypothesis is based mainly on the structural properties of these RNA-binding proteins. All ITAFs possess multiple-RNA-binding domains, such as cold shock domains in the case of Unr (108, 118), RNA recognition motif (RRM) domains for La (3, 131, 188), and PTB (198) and KH domains for PCBP2 (54, 150, 268). Furthermore, most of these proteins dimerize in solution (46, 198). Accordingly, these proteins may make multiple contacts with the IRES and modulate IRES conformation by a concerted interaction between several RNA binding sites (180).

IRES-MEDIATED TRANSLATION INITIATION AND THE CONTROL OF GENE EXPRESSION

Regulation of translation initiation is a central control point in gene expression (172). Interestingly, several oncogenes, growth factors and proteins involved in the regulation of programmed cell death, cell cycle progression and stress response contain IRES elements in their 5' UTRs. Internal initiation escapes many control mechanisms that regulate cap-dependent translation. Thus, a distinguishing hallmark of IRES-mediated translation is that it allows for enhanced or continued protein

expression under conditions where normal, cap-dependent translation is shut-off or compromised. For instance, IRES elements were found to be active during irradiation (102), hypoxia (146, 251), angiogenesis (2), apoptosis (252) and amino acid starvation (63). Together, these observations suggest that IRES-mediated translation initiation of certain mRNAs represents a regulatory mechanism that helps the cell cope with transient stress. Moreover, IRES activity may also participate in the maintenance of normal physiological processes such as adequate synthesis of some proteins during cell cycle progression (42, 219, 230).

Since 1966, it has been known that translation is inhibited during mitosis in higher eukaryotes (239). In fact, while cap-dependent translation is prevalent in the G₁/S phase, it is inhibited in the G₂/M phase (42, 219). Our current understanding of how translation initiation is inhibited at mitosis surmises that it is the result of multiple events that lead to disruption of the eIF4F complex, thereby by inhibition of cap-dependent translation (219). Two such events are the dephosphorylation of eIF4E and the hypophosphorylation of 4E-BPs at mitosis, which prevent eIF4F function and assembly, respectively (59, 217, 219). In contrast to cap-dependent translation, IRES-mediated translation initiation is independent of the cap and is therefore independent of eIF4F integrity (78). In agreement, the synthesis of some proteins required for the completion of mitosis, such as ornithine decarboxylase (218) and the cdk-like p58^{PITSLRE} (43), is maintained by an IRES-mediated mechanism. Other examples of IRESs that are active during the G₂/M phase of the cell cycle are those harbored by the hepatitis C virus (HCV) (103), some members of the *picornaviridae* (18), HIV-1 (26), the human cysteine-rich61 protein (Cyr61) (220), La (220), nucleosome assembly protein 1-like 1 (NAP1L1) (220), and c-Myc encoding mRNAs (133, 136, 218). These reports all provide important insight into cell cycle-specific modulation of IRES activity and support the notion that unique, IRES-mediated mechanisms of translation initiation are activated during G₂/M to specifically translate IRES-containing

mRNAs (219, 230). Even though highly attractive, this hypothesis may not be adequate, as a recent report showed that not all IRES containing mRNAs are selectively translated during mitosis (220). This suggests that the switch from cap- to IRES-mediated translation initiation is not mediated exclusively through the increased availability of canonical translation initiation factors due to the inhibition of cap-dependent translation. Therefore, it is plausible to propose that the enrichment in the cytoplasm of the specific factor(s), ITAFs, required for certain IRESs to function during the different phases of the cell cycle, would collectively play a role in modulating IRES activity.

Intriguingly, most of the known ITAFs have a role in nuclear RNA metabolism and are therefore preferentially confined to the cell nucleus. However, these factors are expected to diffuse into the cytoplasm during the G₂/M phase of the cell cycle due to nuclear envelope breakdown. This cytoplasmic enrichment of specific ITAFs, in part, may be responsible for the increased activity of some IRESs. Consistent with this possibility, ITAF₄₅ (209), heterogeneous nuclear ribonucleoprotein C (hnRNP C) (133) and Unr (259) are enriched in the cytoplasm during the G₂/M phase of the cell cycle. The IRES of FMDV requires ITAF₄₅ (209), the *c-myc* IRES activity is increased by hnRNP C (133), and the cdk-like p58^{PITSLRE} IRES interacts with the Unr protein (259). Additionally, complementary DNA microarray studies show that a number of other factors known to bind or interact with IRESs such as PCBP2, PTB, hnRNP L, eIF3 and La protein are induced during the S and G₂/M phases (106).

It is also possible that RNA-binding proteins differentially inhibit the activity of some IRESs. Consistent with this possibility, HuD and HuR –members of the Hu family of RNA-binding proteins known to interact with AU-rich elements and the poly(A) tail– decrease p27 protein expression by reducing the p27^{Kip1} IRES activity, while PTB is known to enhance the p27^{Kip1} IRES activity (9, 37, 143). Interestingly, this interplay of RNA-binding proteins and the p27^{Kip1} IRES activities occurs in a cell-cycle-dependent fashion (37, 143).

IRES AND APOPTOSIS

IRES activity is crucial in determining the final cellular fate, namely survival or death by apoptosis (102). Induction of apoptosis is characterized by a general inhibition of protein synthesis that is attributed to the proteolytic cleavage of translation initiation factors (reviewed in (39)). Caspase-dependent as well as caspase-independent cleavage of eIF4G has been reported, and this event correlates with the shut down of protein synthesis. Interestingly, the apoptotic fragments of the eIF4Gs are capable of supporting IRES-mediated translation initiation. This overall phenomenon is not general to cell death. During necrosis, protein synthesis is sustained in the dying cell, up to the point where the cell loses its membrane integrity (231). Stringent control of caspase activity is thus critical for cellular homeostasis. Opposing the cellular destruction by caspases are two classes of cellular apoptotic inhibitors, members of the Bcl-2 and inhibitors of apoptosis (IAP) gene families. Whereas the Bcl-2 proteins can block only the mitochondrial branch of apoptosis by preventing the release of cytochrome c, the IAPs block both the mitochondria- and death-receptor-mediated pathways of apoptosis by directly binding to and inhibiting both the initiator and effector caspases (232). Cumulative data suggest that cell commitment to death by apoptosis depends on a delicate balance between the IRES-driven translation initiation of a number of mRNAs coding for both anti- and pro-apoptotic proteins (reviewed in 102). Indeed, mRNAs coding for IAPs, such as the cellular inhibitor of apoptosis proteins c-IAP1 and HIAP2 (264, 270), BCL-2 (240) and XIAP (101), as well as some pro-death proteins, including Apaf-1 (40), and the death-associated protein 5 (DAP5) (96) are translated via an IRES. DAP5 is a member of the eIF4G family and shares the central segment of eIF4G/II responsible for eIF3 and eIF4A binding but lacks the N-terminal domain responsible for interaction with the cap-binding protein eIF4E. DAP5 thus resembles the cleaved version of eIF4G/II, devoid of its N

terminus, which can result from infection by several members of the picornavirus family. Such cleaved C-terminal fragments of eIF4G/II fail to mediate cap-dependent translation but retain their ability to promote IRES-mediated translation initiation (95). The identification of DAP5 as a positive mediator of apoptosis complicates the correlation between the modulation of IRES activity and cell death.

While the exact molecular pathways that regulate the balance between IRES-mediated translation of pro-survival and pro-death mRNAs need to be further investigated, it has become clear that IRESs are critically involved in the regulation of the overall processes of apoptosis.

IRESs AND VIRAL REPLICATION

Viruses are obligate intracellular parasites and depend on cells for their replication. However, they have evolved mechanisms to ensure that their replication can be achieved in an efficient and, in some instances, a cell-type-specific manner. Yet during the early stages of infection, viral mRNA must compete with their host counterparts for the protein synthesis machinery, not for ribosomes as much as for the limited pool of eukaryotic translation initiation factors (eIFs) that mediate the recruitment of ribosomes to both viral and cellular mRNAs (201, 245). To circumvent this competition, we have described how viruses often modify certain eIFs within infected cells so that ribosomes can be recruited selectively to viral mRNAs. We also have outlined that this strategy implies that such viral mRNAs contain structural features such as the IRES that are distinct from most polymerase II-derived host mRNAs (116, 130, 204, 233). In the following section, we will discuss the relevance of viral IRESs in virus tropism and control of the viral replication cycle.

Factors related to both the host and the infectious agents determine pathogenesis of virus-induced disease. In this sense, there is significant genetic evidence that IRESs contain determinants of cell specificity supporting the notion that viral tropism can be modulated at the level of viral protein

synthesis. In the case of poliovirus (PV), the efficiency of viral mRNA translation is a major determinant of neurovirulence and disease pathogenesis (160-162, 255). In about 1% of humans infected with PV the neurovirulent phenotype is expressed, resulting in paralytic poliomyelitis. Repeated passages of PV strains in animals and cultured cells generated the corresponding attenuated vaccine strains (Sabin types 1-3). Thus, the improved ability of these PV variants to grow in non-nervous tissue compromised their ability to grow in the nervous system, as demonstrated by the decreased neurovirulence in monkeys (162). The live, attenuated Sabin vaccine strains of PV were shown to contain single point mutations within the IRES resulting in compromised translation efficiency specifically in neuronal cells (7, 32, 161, 162, 165, 182, 253, 272). This reduction is mediated by impaired binding of eIF4G, eIF4B and PTB to the IRES leading to an impaired association of ribosomes with the viral RNA (183). In agreement with these findings, the reversion of the Sabin strains towards a pathogenic phenotype, a major cause of vaccine-associated paralytic poliomyelitis, is associated with compensatory mutations in the IRES with a concomitant recovery in secondary structure and translational activity (61, 75, 165).

The ability of PV to adapt to different cell types also correlates with IRES-specific mutations. Most PV strains only infect primates. Since transgenic mice are made PV sensitive by introducing the human PV receptor into their genome, it was assumed that the host range of PV was primarily determined by a cell surface molecule that functions as virus receptor (58, 107). However, the PV-sensitive transgenic (PV-Tg) mouse model led to the characterization of a number of adaptive mutations which allowed PV to replicate in primate cells and the central nervous system (CNS) of monkeys but not in mouse cells or in the CNS of Tg mice (277). The failed capacity to replicate in both PV-Tg mice and in mouse cells was due to an impediment at the level of translation initiation, suggesting that not only the viral receptor but also interactions between the

viral IRES and host factors are important determinants of virus host specificity (241). Further genetic evidence correlating IRES activity with virus cell specificity came from the study of a mutant poliovirus in which the IRES had been substituted by the rhinovirus IRES. This chimeric virus replicated as well as wild-type poliovirus in HeLa cells, but replication of the mutant (but not wild-type) viruses was completely restricted in neuronal cells (85).

Another example is the Hepatitis A virus (HAV), sole member of the hepatovirus genus of the picornaviridae. HAV is characterized by its lack of sequence relatedness with other members of the picornavirus family and by several unique biological characteristics, including slow non-cytopathic growth in cell culture and an inability to shut down host-cell protein synthesis (20, 51). HAV possesses an IRES in its 5' UTR, and translation is the rate-limiting step for virus replication (28, 29, 67). However, and in sharp contrast to the major types of picornavirus IRESs, the activity of the HAV IRES requires intact eIF4F (4, 20, 23). In common with PV, highly replicating HAV was recovered following successive passage in cells that normally allowed poor virus replication only. HAV was shown to have acquired mutations in its IRES that enhance replication by facilitating cap-independent translation in a cell-type-specific fashion (33, 65, 66, 234). Interestingly, passage of HAV in different cell types engendered different sets of mutations; however, all adaptive events were clustered within the IRES (33, 65, 66, 234).

The activity of the HCV IRES also varies depending on the cell type (129, 151, 276), and studies comparing the efficiency of the IRES element from different HCV genotypes have established differential translation initiation capabilities (31, 41, 104, 105). Interestingly, the biological differences among HCV genotypes in terms of quantity of virus in serum or sensitivity to antiviral drugs directly correlates with the translation efficiency of the IRES (31). Furthermore, recent studies correlated *in vivo* tropism of HCV with the ability of the viral IRES to support translation initiation

(276). Taken together, these observations support the notion of IRES-dependent virus tropism and stress the role of IRESs in virus pathogenesis.

Upon establishment of a competent infection, IRESs also can play other pivotal roles during viral replication. For example, positive-stranded IRES containing viruses such as PV and HCV utilize the genomic RNA (gRNA) as a common template for translation and RNA replication. Both processes cannot occur simultaneously on a unique RNA template, as they proceed in opposing directions. Consequently, the viral polymerase is unable to use the gRNA as a template for RNA synthesis, while it is being used by translating ribosomes (73, 74, 280). Modulation of IRES activity by viral and cellular factors is required to coordinate these two antagonistic processes. In PV, the binding of the cellular protein PCBP to the IRES enhances viral translation, while the binding of the viral protein 3CD represses translation and facilitates negative-strand synthesis (73, 74). In HCV, the viral core protein down-regulates translation allowing initiation of viral RNA transcription (280). A similar mechanism directed at modulating translation and gRNA encapsidation also has been proposed for retroviruses (26, 48, 184), and a correlation between inhibition of translation and gRNA encapsidation has been reported for the Rous sarcoma virus (RSV) (16, 246). Even though the case for complex retroviruses such as HIV-1 has not been demonstrated experimentally, similar phenomena may be at work, as supported by a number of studies that clearly establish that the full-length HIV-1 5' leader region that contains the IRES element can adopt two mutually exclusive secondary structures (the branched multiple hairpin conformation, BMH, and the long-distance conformation, LDI) that may be functionally different (1). Interestingly, the two conformations differ in their ability to form RNA dimers, structures required for gRNA encapsidation. Moreover, the RNA region, including the first start codon, folds differently in each of these conformations. This region forms an extended hairpin structure in the LDI conformation while

creating a long-distance interaction with upstream sequences that occludes the start codon of the viral protein open reading frame in the BMH conformation (1). The conformational switch from LDI to BMH would be favored by the viral protein Gag. This model has been reviewed recently by Darlix et al. (48).

Enormous efforts have been directed at understanding the mechanism underlying viral IRES-mediated translation initiation and the involvement of these elements in virus replication. A better knowledge of the mechanism by which viral-IRES activity is regulated may lead to the design and validation of drug candidates that specifically inhibit virus replication by targeting translation initiation. In the case of HCV, this notion already has received attention (50, 69, 124). Indeed, a number of reports have described specific HCV IRES inhibitors (92, 122, 164, 229). Moreover, at least one phase I dose-escalation clinical study using an HCV IRES inhibitor has been reported (244). Protein synthesis inhibitors are well known in antibacterial therapy, however, to date no antiviral agents have been identified that target viral protein synthesis despite the fact that several viruses of extreme medical significance (e.g. HCV and HIV) possess unique *cis*-acting RNA elements, such as IRESs, that are essential for mRNA translation. Therefore, understanding the molecular mechanisms underlying viral IRES function will prove instrumental in the development of novel antiviral strategies that specifically target viral protein synthesis.

CONCLUDING REMARKS

Much remains to be learned in the exciting field of translational control. Since the elucidation of the scanning model for eukaryotic translation initiation, alternative hypotheses such as IRES-mediated translation initiation have gained support. Indeed the cap and 5' end-independent mechanism of ribosome recruitment and protein synthesis initiation is now widely accepted for picornavirus. However, the notion of IRES-mediated translation

initiation continually has expanded to include other viral families and a growing number of cellular mRNAs. Experimental evidence suggests that IRESs have evolved as a strategy to ensure the synthesis of certain proteins under physiological stress conditions, where cap-mediated initiation is repressed. The existence of a functional link between disease and regulation of IRESs has been proposed, however, direct evidence remains elusive. Clearly, a more detailed understanding of the molecular mechanisms underlying IRES-mediated initiation of protein synthesis will impact not only on our understanding of gene expression as a whole but also on the development of treatment strategies for certain diseases. As discussed herein, several oncogenes, growth factors and proteins involved in the regulation of programmed cell death contain IRES elements in their 5' UTRs. A growing body of evidence supports the hypothesis that selective IRES-mediated translation of these genes may contribute to the survival of cancer cells under conditions of stress (such as nutrient deprivation, hypoxia or therapy-induced DNA damage) to the development and progression of cancer and to the establishment of cancer cells that are resistant to conventional therapies. Moreover, the mRNA of some viruses of extreme medical significance such as HCV and HIV-1 are also capable of IRES-mediated translation initiation. Together, these observations highlight IRESs and their ITAFs as potential targets for the development of novel agents that specifically target IRES-mediated translation initiation. The diversity in length, primary sequence and structural requirements of IRESs, together with the observed variety of trans-acting proteins required for their activity suggests that the possibility of developing a broad-spectrum drug to target general IRES activity is remote. However, these very characteristics may instead permit the development of strategies capable of specifically targeting a subpopulation of IRESs, or hopefully a single IRES, increasing the potential therapeutic benefits of targeted inhibition of translation initiation.

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