

REVIEW

Internal ribosome entry sites in eukaryotic mRNA molecules

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Initiation of translation of most eukaryotic mRNAs commences with 5' end-dependent recruitment of 40S ribosomal subunits to the mRNA. The 40S subunit carrying the initiator methionine-tRNA and certain eukaryotic initiation factors (eIFs) is thought to scan the mRNA in a 5' to 3' direction until an appropriate start codon is encountered at which stage a 60S subunit joins to form an 80S ribosome that can decode the RNA into protein (Kozak 1989; Hershey and Merrick 2000). A subset of mRNAs contains internal ribosomal entry sites (IRESs), usually in the 5' NTR, that enable end-independent initiation to occur. IRES-containing mRNAs are not subjected to many of the regulatory mechanisms that control recruitment of most mRNAs to the translation apparatus. In this review, we briefly provide an introduction to the known mechanisms of translation initiation. Then, we discuss in detail the molecular mechanisms of IRES-mediated initiation and how they are used by specific mRNAs to permit translation under physiological circumstances such as mitosis, apoptosis, hypoxia, and some viral infections when translation of most mRNAs is repressed.

Mechanisms that mediate translation initiation

Most vertebrate mRNAs are functionally monocistronic and contain a 5'-terminal m⁷GpppN (where N can be any nucleotide) cap structure. The initiation codon used as the start site for protein synthesis is preceded by a 5' nontranslated region (NTR) in which length, nucleotide composition, and structure can determine the efficiency and the mechanism by which a given mRNA is translated (Hershey and Merrick 2000). For the most part, the factors that influence the binding of 40S subunits to the mRNA provide the limiting step in translation initiation. In recent years, it has been found that a particular initiation mechanism can be used preferentially when

one or more specific initiation factors are limiting. Thus, it is crucial to the understanding of translational regulation to know the requirements of each initiation mechanism for translation factors.

The canonical scanning mechanism of translation initiation

Genetic and biochemical evidence has shown that most mRNAs in eukaryotic cells recruit ribosomes by a mechanism in which a 43S complex, composed of a 40S subunit bound to eIF2-GTP/Met-tRNA_i, eIF1A, and eIF3 (Table 1), is recruited at the capped 5' end (Kozak 1989; Donahue 2000; Hershey and Merrick 2000). Binding of the 43S complex to mRNA involves recognition of the 5' capped mRNA by the eIF4E (cap-binding) subunit of eIF4F (Table 1) and is greatly enhanced by the poly(A)-binding protein (PABP) bound to the 3' poly(A) tail (for review, see Sachs and Varani 2000). The cap-binding protein complex eIF4F comprises eIF4E, eIF4A, and eIF4G subunits (Table 1). eIF4A is a DEAD-box RNA helicase/RNA-dependent ATPase that exists both free and as part of eIF4F. The helicase activities of eIF4A and eIF4F are strongly enhanced by eIF4B. eIF4G is a large polypeptide and acts as a molecular scaffold that binds eIF3, eIF4A, eIF4E, and PABP and coordinates their activities (Hentze 1997; Gingras et al. 1999).

The mechanism that recruits 43S complexes onto the mRNA is not known. One hypothesis is that the attachment of the 43S complex is mediated by interactions between the eIF4G subunit of cap-bound eIF4F and the eIF3 component of the 43S complex, as well as between the mRNA and eIF4G, eIF3 and the 40S subunit (Lamphear et al. 1995). After attachment, eIF1 and eIF1A act synergistically to enable scanning of the 43S complex in a 5' to 3' direction from its initial binding site to the initiation codon (Pestova et al. 1998a). In support of this model, genetic analyses in yeast have implicated eIF1, eIF2, and eIF5 in initiation codon recognition (Donahue 2000). The resulting ribosomal 48S complex in which base pairing between the initiation codon of mRNA and the anticodon of initiator tRNA has been established

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Table 1. Characteristics of mammalian translation initiation factors

Name	Subunits	Mass (kD)	Function
eIF1	1	12.6	Enables ribosomes to scan; destabilizes aberrant initiation complexes
eIF1A	1	16.5	Promotes binding of Met-tRNA to 40S subunit; promotes ribosomal scanning
eIF2	3	126	GTP-dependent binding of Met-tRNA to 40S subunit; GTPase
eIF2B	5	261	Guanine nucleotide exchange factor for eIF2
eIF3	11	~700	Ribosomal dissociation; promotes binding of mRNA and Met-tRNA to 40S subunit
eIF4A	1	44	RNA-dependent ATPase; RNA helicase
eIF4B	1	70	Promotes RNA helicase activity of eIF4A, eIF4F
eIF4E	1	26	m ⁷ G cap-binding subunit
eIF4G	1	154	Binds RNA, PABP, eIF4E, eIF4A, eIF3
eIF4F	3	223	eIF4E/4A/4G heterotrimer: binds m ⁷ G caps, RNA helicase
eIF5	1	49	Activates GTPase activity of eIF2
eIF5B	1	139	Ribosomal subunit joining; GTPase

contains eIF1, eIF1A, eIF3, and eIF2-GTP/Met-tRNA_i. eIF5 induces hydrolysis of eIF2-bound GTP, leading to displacement of eIF2-GDP; the inactive eIF2-GDP is recycled to the active eIF2-GTP by eIF2B, a guanine nucleotide exchange factor (Table 1; Hershey and Merrick 2000). Finally, eIF5B (Table 1) mediates joining of a 60S subunit to the 40S subunit, resulting in formation of an 80S ribosome in which initiator tRNA is positioned in the ribosomal P site and that is competent to begin protein synthesis (Pestova et al. 2000).

The scanning mechanism predicts that mRNAs are most efficiently translated if their 5' NTRs are relatively unstructured and at times when none of the eIFs is limiting. As discussed below, these requirements become simplified for other initiation mechanisms.

Leaky scanning and reinitiation

Initiation usually occurs at the AUG triplet that is proximal to the 5' end of an mRNA. Although the most efficiently used AUG triplets are embedded within the sequence ACCAUGG (the initiation codon is underlined; Kozak 1987), almost any AUG can be used. If pyrimidine residues occupy positions -3 or +4 (if the adenosine in the AUG triplet is designated as +1), the scanning ribosomal complex may bypass the embedded AUG by leaky scanning (Kozak 1989).

It is possible for ribosomes to reinitiate after translation of an upstream open reading frame (Morris and Geballe 2000). In the best characterized example, reinitiation on *GCN4* mRNA in *Saccharomyces cerevisiae* is regulated by amino acid availability that determines the level of ternary eIF2/GTP/initiator tRNA complexes and thus the rate at which ribosomes become competent to reinitiate after termination (Hinnebusch 1997).

A low proportion of eukaryotic mRNAs, mostly encoding regulatory proteins, have very long, highly structured 5' NTRs that contain multiple AUG triplets (Kozak 1991). Deletion of such highly structured or AUG-burdened 5' NTRs from mRNAs improves expression of the encoded protein (e.g., Marth et al. 1988; Arrick et al. 1991). However, certain viral and cellular mRNAs that contain long structured 5' NTRs can be

translated very efficiently at times when other leader-burdened mRNAs are not. Two mechanisms, both operationally distinct from the canonical scanning mechanism, have been discovered that can account for the translation of such mRNAs: ribosomal shunting and internal initiation.

Ribosomal shunting

Ribosomal shunting has been found to mediate translation initiation on a few viral mRNAs, including cauliflower mosaic virus 35S mRNA (Futterer et al. 1993) and adenovirus late mRNAs (Yueh and Schneider 1996) and on the cellular mRNA that encodes heat shock protein 70 (Yueh and Schneider 2000). In this mechanism, ribosomal subunits bind the mRNA in a 5' cap-dependent manner and scan downstream until a stable RNA structure is encountered that may arrest the scanning ribosomes or cause them to dissociate from the RNA. This arrest is followed by intramolecular shunting of the ribosomal subunit to a downstream landing site, bypassing the RNA structure. The ribosome resumes scanning until the next appropriate start codon is encountered. Although the mechanism of ribosomal shunting is not known, RNA sequences located between the hairpins and the initiation codon, some of which are complementary to sequences located near the 3' end of 18S ribosomal RNA, play an important role. It has been speculated that the 18S-complementary sequences interact directly with the 40S subunit to provide a landing site after it has bypassed the hairpins (Yueh and Schneider 2000). Whether shunting is commonly used as a means of efficiently translating mRNAs burdened with secondary structures in their 5' NTRs is not known.

Internal initiation

Twenty-two years ago, it was reported that prokaryotic, but not eukaryotic, ribosomes could bind circular RNA molecules, suggesting that eukaryotic ribosomes enter mRNAs exclusively via their free 5' ends (Kozak 1979; Konarska et al. 1981).

However, it was also known that RNA genomes of picornaviruses such as encephalomyocarditis virus (EMCV) and poliovirus have properties that are incompatible with initiation by 5' end-dependent scanning. In 1988, it was discovered that picornaviral mRNAs are translated by a mechanism, distinct from shunting, that enables ribosomes to initiate translation effectively on highly structured regions located within the 5' NTRs (Jang et al. 1988; Pelletier and Sonenberg 1988). These regions were named IRESs.

Internal ribosome entry site

Discovery of IRES elements

Picornaviral 5' NTRs can range in length from 610 to 1500 nucleotides, are highly structured, and contain multiple nonconserved AUG triplets upstream of the initiation codon that should act as strong barriers to scanning ribosomes. Although one could imagine that a sophisticated combination of leaky scanning and reinitiation guides a scanning 40S subunit to the start site codon, several lines of experimentation over the past decade have argued that this is not the case.

First, changing six of the seven AUG codons in the polioviral 5' NTR to UUG, a scenario that should affect the rate of reinitiation, did not alter the efficiency of initiation (Pelletier et al. 1988). Second, polysome-associated picornaviral RNAs have a 5' pUp terminus, instead of the commonly found 5' cap structure (Nomoto et al. 1977). This finding suggested that translation of the viral mRNA does not need the eIF4E cap-binding subunit of eIF4F that normally recruits 40S subunits to capped 5' ends. Indeed, picornaviral mRNA translation is not inhibited when eIF4E is sequestered by cap analogs in vitro (e.g., Canaani et al. 1976), by increased concentrations of eIF4E-binding proteins (eIF4-BPs) in vitro and in vivo (Pause et al. 1994a), or by treatment of cultured cells with rapamycin to induce the dephosphorylation of eIF4-BPs, which sequester eIF4E in eIF4E/eIF4-BP complexes (Beretta et al. 1996). Additional support that translation of all picornaviral mRNAs (except hepatitis A virus) was independent of an intact cap-binding protein complex (eIF4F) came from studies conducted to examine the mechanism by which picornaviruses rapidly inhibit translation of capped cellular mRNAs in infected cells. Some picornaviruses such as poliovirus and foot-and-mouth disease virus (FMDV), encode proteases that cleave both isoforms of eIF4G separating eIF4G into N-terminal eIF4G/eIF4E/PABP and C-terminal eIF4G/eIF4A/eIF3 complexes, thereby uncoupling the cap recognition from ribosome-binding and helicase functions of eIF4F (Lamphear et al. 1993, 1995; Gradi et al. 1998). In addition, sequestration of eIF4E by eIF4-BPs is increased as a result of EMCV and poliovirus infection of cells (Gingras et al. 1996). Both mechanisms strongly reduce the activity of eIF4F in cap-mediated initiation on host cell mRNAs during viral infections.

All of the above results indicated that picornaviral mRNAs must be translated by a cap-independent initia-

tion mechanism. This notion was verified when it was shown that the 5' NTRs of EMCV and poliovirus could mediate internal entry of ribosomes. Specifically, insertion of these viral 5' NTRs into the intercistronic spacer region of a dicistronic mRNA mediated translation of the second cistron (Jang et al. 1988; Pelletier and Sonenberg 1988). Translation of the downstream cistron occurred even when translation of the upstream cistron was abolished, but was dependent on the integrity of the inserted ~450-nt long IRES. Small deletions or insertions, and even substitution of single nucleotides in the IRES elements, severely reduced their activity (Svitkin et al. 1985; Kuge and Nomoto 1987; Trono et al. 1988).

Not surprisingly, the first IRES element in a cellular mRNA, encoding the immunoglobulin heavy chain binding protein BiP, was discovered by its continuing activity in poliovirus infected cells at a time when the bulk of host cell mRNA translation was inhibited (Sarnow 1989; Macejak and Sarnow 1991). In more recent studies, additional IRES-containing cellular mRNAs have been identified in poliovirus-infected cells (Johannes and Sarnow 1998; Johannes et al. 1999). Note that certain mRNAs can be translated under such conditions that do not contain IRESs, such as the late adenoviral mRNAs that are translated by a shunting mechanism (Dolph et al. 1988).

So far, all picornaviral mRNAs have been found to contain IRES elements. Table 2 provides a list of RNA viruses and one DNA virus, Kaposi's sarcoma-associated herpesvirus, whose genomes contain IRES elements.

Table 2. Internal ribosome entry sites in viral genomes

Virus	Reference
Poliovirus	Pelletier and Sonenberg 1988
Rhinovirus	Borman and Jackson 1992
Encephalomyocarditis virus	Jang et al. 1988
Foot-and-mouth disease virus	Kuhn et al. 1990
Hepatitis C virus	Tsuklyama-Kohara et al. 1992
Classic Swine Fever Virus	Rijnbrand et al. 1997
Bovine viral diarrhea virus	Poole et al. 1995
Friend murine leukemia virus <i>gag</i> mRNA	Berlioz and Darlix 1995
Moloney murine leukemia virus <i>gag</i> mRNA	Vagner et al. 1995b
Rous sarcoma virus	Deffaud and Darlix 2000
Human immunodeficiency virus <i>env</i> mRNA	Buck et al. 2001
<i>Plautia stali</i> intestine virus	Sasaki and Nakashima 1999
<i>Rhopalosiphum padi</i> virus	Domier et al. 2000
Cricket paralysis virus	Wilson et al. 2000b
Kaposi's sarcoma-associated herpesvirus	Grundhoff and Ganem 2001; Bielecki and Talbot 2001

Listed are IRES elements that passed at least two tests when located in the intergenic region in a dicistronic mRNA: IRES is intact after translation in a cell-free system or in cultured cells expressing the dicistronic gene. IRES-mediated translation of the second cistron is independent of the translation of the first cistron. (IRES) internal ribosomal entry site.

Table 3 lists cellular mRNAs that contain IRES activity. As will be discussed below, all viral IRES elements need to maintain a specific higher-ordered RNA structure to be functional. This feature contrasts with some cellular IRESs for which short, noncontiguous segments can mediate internal ribosome entry.

Experimental test for IRES activity

The recent flurry of newly discovered IRES elements in both viral and cellular mRNAs warrants a brief discussion about the experimental criteria that should be applied to an RNA sequence before it is termed an IRES. First, and most importantly, the integrity of the translated dicistronic mRNA, which contains a suspected IRES element in the intergenic region, has to be examined to evaluate other possible reasons for translation of the downstream cistron. Care must be taken if sensitive enzymatic assays are used to monitor translation of the second cistron because small amounts of broken dicistronic RNAs could yield functionally monocistronic RNA that, although uncapped, can be translated into active enzyme. In addition, it has to be determined whether intercistronic spacer regions can provide promoter activity or contain cryptic splice sites that could lead to synthesis of functionally monocistronic mRNA; the first of these scenarios is often encountered in *S. cerevisiae* in which TATA-like sequences can function as a promoter. Second, IRES-mediated translation of the second cistron must be independent of translation of the first cistron to rule out mechanisms such as termination-reinitiation as a cause of translation of the downstream cistron. A particular convincing demonstration of IRES-mediated initiation involves the insertion of a suspected IRES into a circularized RNA, engineered to contain a single continuous open reading frame, so that multiple rounds of translation of the circle can be used as a measure of its integrity (Chen and Sarnow 1995).

Mechanism of ribosome recruitment by picornaviral IRES elements

So far, the dicistronic mRNA translation assay has been used to identify IRES elements in viral genomes (Table 2) and in a broad range of cellular mRNAs from mammals to insects (Table 3). There are few, if any, convincing similarities between IRES elements in terms of sequence, size, or structure except for those from families of related viruses. The implication is that there is no universal mechanism of internal ribosomal entry, or that an elusive Shine-Dalgarno-like sequence, perhaps created by secondary or tertiary structure, cannot be easily detected in IRESs.

Much is known about the sequence and factors that recruit ribosomal subunits to picornavirus, hepatitis C virus, and cricket paralysis virus IRESs. Thus, we first discuss three fundamentally different mechanisms of initiation used by viral IRES elements.

Table 3. Internal ribosome entry sites in cellular mRNAs

Gene product	Reference
Growth factors	
Fibroblast growth factor 2 (FGF2)	Vagner et al. 1995a
Platelet-derived growth factor B (PDGF/c-sis)	Bernstein et al. 1997
Vascular endothelial growth factor (VEGF)	Akiri et al. 1998; Stein et al. 1998; Huez et al. 1998
Cyr61	Johannes et al. 1999
Transcription factors	
Antennapedia	Oh et al. 1992; Ye et al. 1997
Ultrabithorax	Ye et al. 1997
MYT2	Kim et al. 1998
NF- κ B repressing factor NRF	Oumard et al. 2000
AML1/RUNX1	Pozner et al. 2000
Gtx homeodomain protein	Chappell et al. 2000a
Oncogenes	
c-myc	Nabru et al. 1997; Stoneley et al. 1998
Pim-1	Johannes et al. 1999
Protein kinase p58 ^{PITSLRE}	Cornelis et al. 2000
Transporters/receptors	
Cationic amino acid transporter Cat-1	Fernandez et al. 2001
Nuclear form of Notch 2	Lauring and Overbaugh 2000
Translation factors	
Eukaryotic initiation factor 4G (eIF4G) ^a	Gan and Rhoads 1996
Eukaryotic initiation factor 4G1 (eIF4G1) ^a	Johannes and Sarnow 1998
Death-associated protein 5 (DAP5)	Henis-Korenblit et al. 2000
Activators of apoptosis	
Apoptotic protease activating factor Apaf-1	Coldwell et al. 2000
Dendritically localized proteins	
Activity-regulated cytoskeletal protein (ARC)	Pinkstaff et al. 2001
α subunit of calcium calmodulin-dependent kinase II dendrin	Pinkstaff et al. 2001
Microtubule-associated protein 2 (MAP2) neurogranin (RC3)	Pinkstaff et al. 2001
Others	
Immunoglobulin heavy chain binding protein (BiP)	Macejak and Sarnow 1991
La autoantigen	Carter and Sarnow 2000
β subunit of mitochondrial H ⁺ -ATP synthase	Izquierdo et al. 2000
Ornithine decarboxylase	Pyronnet et al. 2000

^amRNAs contain different 5' noncoding regions. Listed are IRES elements that passed at least two tests when located in the intergenic region in a dicistronic mRNA: IRES is intact after translation in a cell-free system or in cultured cells expressing the dicistronic gene. IRES-mediated translation of the second cistron is independent of the translation of the first cistron. (IRES) internal ribosomal entry site.

The mechanism of initiation on picornavirus IRESs: the EMCV paradigm

The picornavirus IRES elements are divided into two major groups by sequence and structural similarities (Pilipenko et al. 1989a,b). One group contains EMCV, FMDV, and Theiler's murine encephalomyelitis virus (TMEV), and the other group contains poliovirus and rhinovirus. A third minor group contains hepatitis A virus (HAV). Sequence variation within a group can exceed 50%, but many substitutions are covariant, resulting in conservation of secondary structure (Jackson and Kaminski 1995). Mutations that disrupt the integrity of structural elements or of conserved sequence motifs can cause general or cell type-specific loss of IRES function. These properties of picornavirus IRESs are also characteristic of all other major IRES groups discussed below.

The ribosomal entry sites on various picornavirus IRESs have been mapped. All picornavirus IRESs contain an AUG triplet at their 3' border, 25 nucleotides downstream from the beginning of a pyrimidine-rich tract. In EMCV and TMEV (GDVII strain), this AUG triplet is the initiation codon and ribosomes bind directly to it without scanning (Kaminski et al. 1990, 1994; Pilipenko et al. 1994). In FMDV, initiation occurs at this AUG triplet but also, more frequently, at the next downstream AUG triplet (Sangar et al. 1987). Very little initiation occurs at the AUG at the 3' border of the poliovirus IRES and the initiation codon is ~160 nucleotides downstream from it. The mechanism by which ribosomal subunits reach the start codon is not known. It could involve scanning or shunting of subunits to the initiation codon from the 3' border of the IRES, or IRES-mediated loading of the ribosome onto the mRNA anywhere between the 3' border of the IRES and the initiation codon, followed by scanning (Belsham 1992; Hellen et al. 1994; Pestova et al. 1994; Ohlmann and Jackson 1999).

The minimum set of factors required for recruitment of the 40S subunit to the EMCV initiation codon has been determined by biochemical reconstitution of this process (Pestova et al. 1996a,b). The formation of 48S complexes (i.e., binding of 43S to the mRNA) is ATP-dependent and requires the same factors as the 5' end-dependent initiation mechanism except that there is no requirement for eIF4E, PABP, or large N-terminal and C-terminal fragments of eIF4G (Fig. 1). Specifically, the cap-binding protein complex eIF4F can be replaced by a complex of eIF4A and the central domain of eIF4G, eIF4G₆₉₇₋₉₄₉ (Lomakin et al. 2000). Both eIF4A and the function of the central domain of eIF4G are essential for 48S complex formation (Fig. 1), exemplified by the profound inhibition of EMCV translation by dominant-negative mutant forms of eIF4A, which sequester the eIF4A/4G complex in an inactive form (Pause et al. 1994b). Recent data indicate that eIF4A plays its role in initiation on this IRES as part of a complex with eIF4G rather than as a singular polypeptide (Lomakin et al. 2000). Together, eIF2, eIF3, eIF4B, and 4G/4A promote 48S complex formation equally at AUG₈₃₄, the authentic initiation codon, and at AUG₈₂₆, which is virtually un-

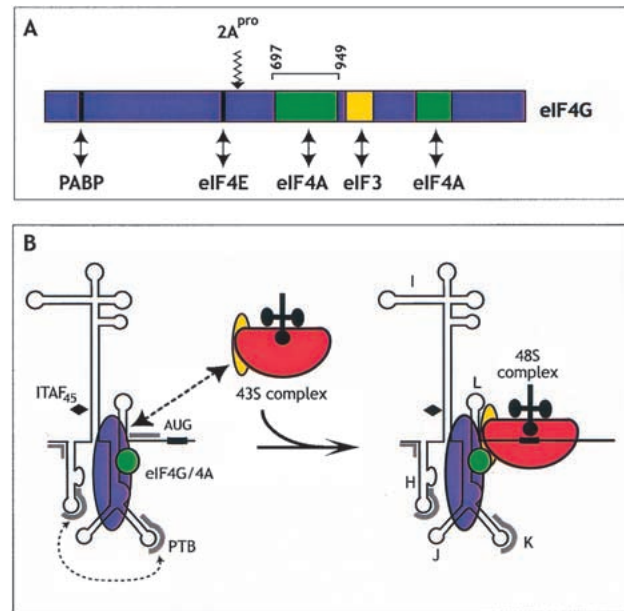


Figure 1. (A) Schematic diagram of human eIF4G1 protein. Shown are the binding sites for PABP, eIF4E, eIF4A, and eIF3 and the target site for picornaviral proteinase 2A^{pro}. The minimum eIF4G1 fragment that binds specifically to the EMCV IRES and supports 48S complex formation corresponds to amino acid residues 697–949. (B) Model for assembly of 48S complexes on EMCV-like IRESs. Structural domains of the IRES and regions of contact with the following factors as determined by footprinting are shown: eIF4G/4A complex (blue/green), ITAF 45 (diamonds), PTB (gray). PTB contains four RRM domains and binds multiple sites on EMCV-like IRESs; such binding (indicated by a dotted line) therefore may stabilize a specific conformation of the IRES. The recruitment of a 40S ribosomal subunit (red) carrying initiator tRNA and eIF3 (yellow) is shown. See text for details.

used *in vivo*. Remarkably, eIF1 promotes dissociation of the ribosomal complex at AUG₈₂₆, consistent with the previously noted function of this factor in enhancing the fidelity of initiation codon selection (Pestova et al. 1998a).

The essential eIF4G₆₉₇₋₉₄₉/4A core of eIF4F binds specifically to the JK domain of the EMCV IRES, upstream of the initiation codon, and this interaction is necessary for initiation (Fig. 1; Pestova et al. 1996b; Kolutpaeva et al. 1998). The structure of the central domain of eIF4G recently has been solved by X-ray crystallography, revealing a crescent-shaped domain that contains five α -helical HEAT-repeats (Marcotrigiano et al. 2001). The identification of adjacent, partially overlapping binding sites for eIF4A and the EMCV IRES on this domain of eIF4G is consistent with the enhancement by eIF4A of eIF4G's affinity for the IRES (Lomakin et al. 2000).

It is not known whether the requirement for eIF4A and ATP reflects a requirement for local unwinding of mRNA near the initiation codon or structural rearrangement of subdomains of the IRES to allow ribosomal attachment. The eIF4G/4A complex likely also recruits

other eIFs to the IRES such as eIF4B and eIF3 (Meyer et al. 1995; Pestova et al. 1996b; Lomakin et al. 2000). Although it is likely that these interactions contribute to promoting ribosomal attachment to the IRES, details of the mechanism by which ribosomal loading occurs are not known. Note also that the model depicted in Figure 1 does not invoke a role for the 230 nucleotide domain I of the EMCV IRES, which plays an unknown but essential role in initiation. It is possible that this domain makes weak contacts with eIF3 or even the 40S subunit that so far have escaped detection.

Similar reconstitution experiments have not been conducted to determine the canonical factors required for initiation on poliovirus or rhinovirus IRESs. A requirement for the eIF4G/eIF4A complex is strongly suggested for the poliovirus IRES by the strong inhibition of the IRES by the dominant-negative form of eIF4A and by the insensitivity to the overexpression of eIF4E-binding proteins, causing sequestration of eIF4E (Pause et al. 1994b). Curiously, both the poliovirus and rhinovirus IRESs are stimulated to a much greater extent by cleavage of eIF4G by picornaviral proteases than the EMCV and FMDV IRESs (Hambidge and Sarnow 1992; Borman et al. 1995). The reason for this difference is not known. Remarkably, the activity of the HAV IRES is strongly reduced by cleavage of eIF4G, but can be restored by addition of eIF4F to *in vitro* translation reactions (Whetter et al. 1994; Borman and Kean 1997). Infection of cells by HAV does not lead to cleavage of eIF4G, and it therefore is possible that the HAV IRES differs from EMCV-like and poliovirus-like IRESs in that it has not acquired the ability to function when eIF4G is cleaved.

Although PABP is not necessary for the formation of 48S/EMCV IRES complexes, it enhances EMCV IRES-mediated initiation and, to a much greater extent, poliovirus IRES-mediated initiation on mRNAs with 3' polyadenosine tails (Bergamini et al. 2000; Michel et al. 2000). These findings may indicate a function for PABP in IRES-mediated initiation independent of its interaction with the N terminus of eIF4G.

Cellular RNA-binding proteins that stimulate picornavirus IRES elements

The activity of several picornavirus IRESs is subject to cell type-specific restriction. For example, the attenuated neurovirulence of Sabin vaccine strains of poliovirus is in large part due to a defect in translation initiation in neuronal cells caused by substitutions in the IRES (Svitkin et al. 1985; Stewart and Semler 1998). It is possible that a noncanonical translation initiation factor that is required for IRES-mediated initiation is limiting in neurons. This hypothesis is consistent with observations that translation mediated by poliovirus and rhinovirus IRESs is also very weak in rabbit reticulocyte lysates (RRL) but can be enhanced by addition of the ribosomal salt wash fraction from HeLa cells (e.g., Brown and Ehrenfeld 1979). Initiation mediated by these, and possibly other, IRESs therefore requires IRES *trans*-acting

factors (ITAFs) that are either absent in RRL or significantly less abundant in RRL than in permissive cells. Ultraviolet cross-linking assays initially were used to identify IRES-binding proteins, but it has become apparent that proteins can bind specifically to an IRES yet play no role in promoting initiation on it, so that confirmation of a role in IRES-mediated initiation for a suspected ITAF requires a direct functional assay. A handful of ITAFs have been discovered in recent years that play important roles in modulating the efficiencies of various picornavirus IRES elements.

The pyrimidine tract-binding protein (PTB) contains four RNA recognition motif-like domains and binds to all picornavirus IRESs, most likely to noncontiguous sites on each, as has been determined for EMCV and FMDV by footprinting (Kolupaeva et al. 1996; Pilipenko et al. 2000). Translation initiation on the wild-type EMCV IRES was enhanced less than twofold by PTB (Pestova et al. 1996a; Kaminski and Jackson 1998), but the PTB dependence of initiation was significantly increased either by a single adenosine nucleotide insertion in the eIF4G-binding site in the IRES or by alteration of the coding sequence adjacent to the initiation codon (Kaminski and Jackson 1998). The interaction of PTB with the EMCV IRES was not affected by these changes. These observations are more consistent with a model in which ITAFs such as PTB bind the IRES (Fig. 1) and help it to attain or maintain an active conformation in which it is able to bind essential factors and the 43S complex in a productive manner rather than, for example, recruiting them directly (Kaminski and Jackson 1998).

Very recently, a novel ITAF was discovered that influenced the binding of the eIF4G/4A complex to the FMDV IRES. The TMEV (GDVII strain) and the FMDV (01K) IRESs are ~40% identical and are closely related to the EMCV IRES. Initiation on all three IRESs involves binding of the eIF4G/4A complex to the J-K domain (see Fig. 1). However, initiation on the TMEV IRES was strongly dependent on PTB and initiation on the FMDV IRES required both PTB and a 45-kD ITAF (ITAF45) that is expressed in proliferating cells (Pilipenko et al. 2000). PTB and ITAF45 bound to nonoverlapping sites on the FMDV IRES and together caused both localized RNA conformational changes adjacent to the eIF4G/4A-binding site and enhanced binding of eIF4G/4A to the IRES. The EMCV, FMDV, and TMEV IRESs all bind to both PTB and ITAF45, but only the FMDV IRES requires ITAF45 for function. Therefore, functional requirement for ITAFs by certain IRESs rather than the ability of the ITAFs to bind to the IRES is important for their role in IRES efficiency. Significantly, the neurovirulent GDVII strain of TMEV can be completely attenuated by substituting its IRES with FMDV O₁K IRES even though growth of the chimeric virus in nonneuronal BHK cells was not impaired (Pilipenko et al. 2000). Differences in the expression of ITAF45 in neurons and in proliferating nonneuronal cells could explain the failure of the chimeric virus containing the FMDV IRES to grow in murine brain cells.

Last, we briefly discuss three ITAFs that regulate the poliovirus and rhinovirus IRESs. It has been known for a long time that the nuclearly located La autoantigen binds to the poliovirus IRES, stimulates its activity *in vitro*, and enhances the accuracy of initiation codon selection; however, these effects required concentrations of La far above those present in HeLa cell extracts (Meerovitch et al. 1993). Recently, it has been reported that the La autoantigen is cleaved by a virus-encoded protease in virally infected cells and that the IRES stimulatory fragment of La is retained in the cytoplasm in infected cells (Shiroki et al. 1999). Thus, it is possible the ITAF-containing fragment of La exists in infected cells at a higher local intracellular concentration than was assumed previously.

In a second line of investigation, biochemical fractionation of HeLa cytoplasmic lysates was used to identify ITAFs that synergistically stimulate the rhinovirus IRES. This study resulted in the identification of PTB as well as a complex between the *unr* (upstream of *N-ras*) protein and an associated WD-repeat protein (*unrip*; Hunt et al. 1999). Curiously, neither *unr* nor the *unr/unrip* complex stimulated the poliovirus IRES.

Finally, the poliovirus IRES is greatly stimulated by poly (rC) binding protein-2 (PCBP-2); curiously PCBP-2 binds both to the viral IRES and the 5' terminal cloverleaf-type structure that is an important signal in viral RNA replication (Blyn et al. 1996, 1997; Gamarnik and Andino 1997; Walter et al. 1999; Gamarnik and Andino 2000). It is not yet apparent whether the requirement for PTB and PCBP-2 for poliovirus translation can begin to provide an explanation for the attenuated neurovirulence of Sabin strains of poliovirus. Like PTB, PCBP-2, and *unr* also contain multiple RNA-binding domains and therefore have the potential to make multipoint interactions with IRESs to stabilize their folding into an active conformation. Overall, these findings suggest that the poliovirus/rhinovirus and EMCV/FMDV/TMEV IRESs display a diversity in sequence and structure that can lead to specific requirement for a variety of ITAFs. These specific requirements could lead to differential IRES regulation.

Initiation of translation by factor-independent binding of ribosomes to the hepatitis C virus IRES

A landmark discovery in the eukaryotic translation field was the finding that the hepatitis C virus (HCV) IRES can bind 40S subunits in the absence of any eIF, including the eIF2/GTP/initiator tRNA ternary complex. Remarkably, toeprinting experiments showed that the AUG initiation codon is positioned at or near the P site of the bound 40S subunits (Fig. 2; Pestova et al. 1998b). Together with the recent cryo-electron microscopic reconstruction at 20 Å resolution of 40S/HCV IRES complexes (Spahn et al. 2001), these findings have provided a spectacular insight into molecular interactions that take place in an IRES–40S complex. We describe and discuss these major findings in the following sections.

Comparison of IRES elements between HCV and related flaviviruses

The 5' NTRs of HCV and the related classical swine fever virus (CSFV), bovine viral diarrhea virus (BVDV), and GB virus B (GBV-B) all promote internal initiation of translation (Tsukiyama-Kohara et al. 1992; Wang et al. 1993; Poole et al. 1995; Rijnbrand et al. 1997, 2000; Grace et al. 1999). These 5' NTRs contain IRESs that are ~330–390 nucleotides in length and have related structures, even though their sequences differ significantly from one another (Lemon and Honda 1997). Sequence differences between the IRESs of different genotypes of any one of these viruses consist mostly of compensatory nucleotide changes within structural elements that serve to maintain base pairing (e.g., Smith et al. 1995). As in the picornavirus IRESs, mutations that disrupt helical regions in HCV-like IRESs can substantially reduce their initiation activity, but this can be restored by compensatory second-site mutations (Rijnbrand et al. 2000). These observations are consistent with a model for IRES function in which helices form a structural scaffold to orient conserved unpaired regions that act as binding sites for factors and ribosomes so that they can assemble functional initiation complexes.

Structural features of the HCV IRES

Mutagenesis studies combined with biochemical probing analysis revealed that the HCV IRES consists of three major structural domains (II, III, and IV) radiating from a complex pseudoknot (Fig. 2). Reconstructions of the IRES at ~20 Å resolution produced by cryo-electron microscopy have shown that these domains form an extended structure in which the pseudoknot and domain IV are centrally located (Spahn et al. 2001).

Domain I comprises the extreme 5' end of the 5' NTR and is not part of the IRES. Domain II contributes significantly to IRES function, but deletion of this domain does not lead to complete loss of activity (Tsukiyama-Kohara et al. 1992; Reynolds et al. 1996; Kolupaeva et al. 2000a,b). Domain III contains several essential elements, including a large four-way junction (IIIabc) and two smaller stem-loop structures (IIIc and IIId). The structures of IIIc and IIId have been solved by NMR spectroscopy (Klinck et al. 2000; Lukavsky et al. 2000). Subdomain IIIc contains an internal asymmetric E-loop motif (Klinck et al. 2000; Lukavsky et al. 2000) that is also present in the sarcin/ricin loop in 28S rRNA (Correll et al. 1998; Seggerson and Moore 1998). In the HCV IRES, this E-loop motif contains two unusual S-turns involving GAGU_{256–259} and GGUC_{267–270}, located on the same site of the hairpin, presenting a unique feature of a narrowed major groove and a distorted phosphodiester backbone. By analogy with the sarcin/ricin loop, the HCV E-loop may be involved in interactions with other IRES sequences or components of the 40S subunit (Klinck et al. 2000; Lukavsky et al. 2000). Subdomain IIId adopts a novel tetraloop fold in which the loop bases of the A296 and U297 point toward the major groove and thus are

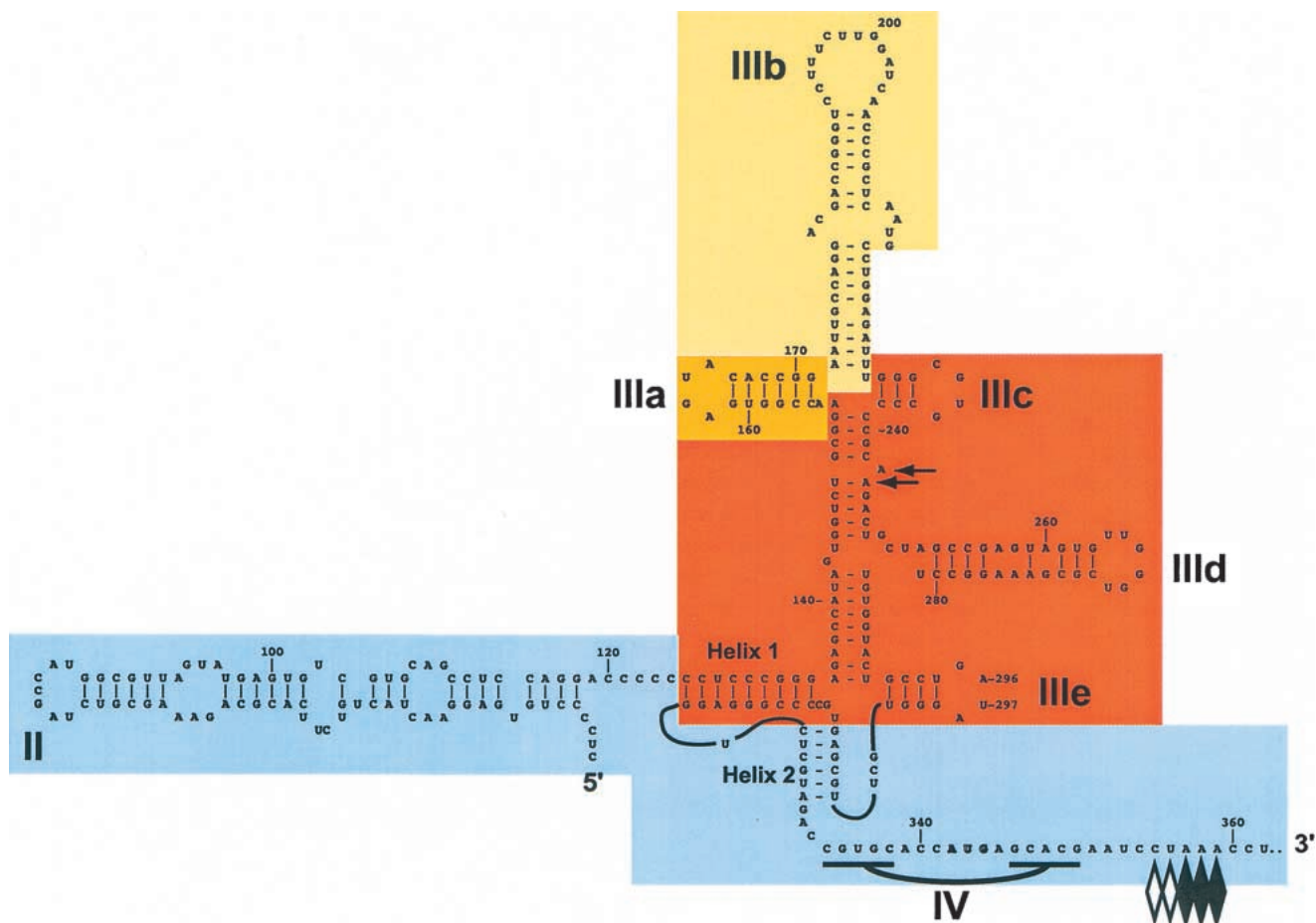


Figure 2. Protein–RNA interactions on the HCV IRES. The secondary structures of domains II, III, and IV of the HCV IRES are shown schematically (adapted from Honda et al. 1999). Binding sites for eIF3 (yellow) and 40S subunits (red) are shown. A subdomain that is required for binding of eIF3 and the 40S subunit is shown in orange, and elements of the IRES that interact with 40S subunits and are required for accurate placement of the coding region in the decoding site of the 40S subunit are colored light blue. The toeprints detected at the leading edge of bound eIF3 are indicated by arrows. The toeprints at the leading edge 40S subunits in binary IRES/40S subunit complexes are indicated by unfilled diamonds; toeprints formed on eIF2/GTP/Met-tRNA_i/40S subunits are indicated by filled diamonds.

exposed and not in contact with the RNA backbone (Lukavsky et al. 2000).

Domain IV contains the initiation codon and is base-paired in the HCV and GBV-B IRESs, but not in those from BVDV or CSFV. HCV coding sequences per se are not essential for internal initiation, because they can be replaced completely by heterologous sequences (Tsukiyama-Kohara et al. 1992; Wang et al. 1993). However, there has been some controversy as to whether HCV sequences located downstream from the initiation codon influence the efficiency of IRES-mediated initiation (Reynolds et al. 1995; Lu and Wimmer 1996). If one considers that ribosomal entry occurs at the initiation codon without prior scanning (Reynolds et al. 1996; Rijnbrand et al. 1997), and that mutations in the IRES that stabilize structures containing the initiation codon inhibit ribosomal attachment and consequently IRES-mediated initiation (Honda et al. 1996; Myers et al. 2001), it then seems likely that the negative influence of some heter-

ologous coding sequences on IRES function may be because they interfere sterically with ribosomal attachment.

HCV IRES can recruit 40S subunits without any eIF

Biochemical reconstitution of the initiation process on HCV, CSFV, and BVDV IRESs has revealed that it occurs by a mechanism that differs fundamentally from initiation on EMCV-like IRESs (Pestova et al. 1998b; Pestova and Hellen 1999). The 40S subunit binds specifically and stably to the HCV-like IRESs 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 (Pestova et al. 1998b). Subsequent addition of the ternary eIF2/GTP/initiator tRNA complex to IRES/40S subunit complexes is necessary and sufficient for formation of 48S complexes in which the anticodon of initiator tRNA is base-paired with the initiation codon.

This base pairing is associated with the appearance of three new toeprints (Fig. 2, filled diamonds) immediately downstream from those detected for binary IRES/40S subunit complexes (Fig. 2, open diamonds).

Although eIF3 is not needed for 48S complex formation, it binds specifically to these IRESs and is likely to be a constituent of 40S/IRES complexes *in vivo*. Human eIF3 has a molecular mass of ~600 kD and contains at least 11 subunits; four of these interact directly with the IRES (Sizova et al. 1998), but it is not yet known which subunit(s) are primary determinants of this interaction. The binding site for eIF3 has been mapped by toeprinting and footprinting to the terminal half of domain III (Fig. 2; arrows; Buratti et al. 1998; Pestova et al. 1998b; Sizova et al. 1998; Pestova and Hellen 1999; Odreman-Macchioli et al. 2000; Kieft et al. 2001). The role eIF3 in HCV IRES translation is not yet known. eIF3 may destabilize incorrectly assembled 48S complexes (Kolupaeva et al. 2000b) and play a role in stages in initiation after 48S complex formation (Pestova et al. 1998b). The independent interaction of eIF3 and eIF2/GTP/initiator tRNA with the HCV-like IRESs is likely to enhance the affinity and specificity of binding and to stabilize the resulting 48S IRES/mRNA complexes.

Significantly, initiation on HCV-like IRESs has no requirement for ATP or any factor associated with ATP hydrolysis, including eIF4A, eIF4B, or eIF4F, and is resistant to inhibition by dominant negative eIF4A mutants (Pestova et al. 1998b; Pestova and Hellen 1999). Indeed, these eIFs probably are unable to gain access to the region flanking the initiation codon of HCV-like IRESs to which ribosomes bind. Certainly, RNA unwinding by eIF4A is not observed, because initiation on these IRESs is repressed by local secondary structures in this region that would be readily unwound during cap-mediated initiation (Honda et al. 1996; Myers et al. 2001). Several other proteins such as La, PTB, and hnRNPL interact with the HCV IRES (Ali and Siddiqui 1995, 1997; Hahn et al. 1998), but they are not essential for 48S complex formation *in vitro*. Evidence for the involvement of PTB in HCV IRES-mediated translation initiation has been disputed by experiments conducted in RRL depleted of PTB (Kaminski et al. 1995). However, it is possible that isoforms of PTB may enhance the HCV IRES in cultured cells (Anwar et al. 2000; Gosert et al. 2000).

A prokaryotic-like mode of 40S recruitment to the HCV-like IRESs

The principal difference between initiation on HCV-like IRESs from all previously characterized modes of initiation is the ability of these IRESs to bind directly to 40S subunits in such a way that the initiation codon is placed directly in the ribosomal decoding region. This ability enables initiation on these IRESs to dispense with a requirement for several canonical factors including eIF4A, eIF4B, and eIF4F.

Although there are analogies between factor-independent binding of 30S ribosomal subunits to Shine-Dalgarno sequences in prokaryotic mRNAs (Shine and Dal-

garno 1975) and factor-independent binding of 40S eukaryotic subunits to HCV-like IRESs, there are also significant differences. Toeprinting and deletion analyses indicated that 40S subunits bind to multiple noncontiguous RNA sequences in these IRESs rather than to a single linear sequence such as the prokaryotic Shine-Dalgarno sequence. Furthermore, the discrete 40S binding sites are not close together in the folded RNA structure; therefore, ribosomal attachment involves multiple sites on the 40S subunit (Pestova et al. 1998b; Pestova and Hellen 1999).

Cryo-electron microscopy analysis reveals an intricate network of 40S/HCV IRES interactions

Recent results obtained by Spahn and colleagues using cryo-electron microscopic analysis of 40S/HCV IRES complexes have yielded exciting new insights into the structure of the 40S/HCV IRES complex (Spahn et al. 2001). First, the IRES assumes a single conformation when bound to the 40S subunit. Second, the 40S subunit makes numerous contacts with the IRES. Specifically, the IRES binds to the solvent side of the 40S subunit near the channel through which mRNA exits the ribosome, such that domain IIIb is positioned close to the site on the 40S subunit to which eIF3 binds. Third, domain II loops out from the 40S surface and the region of contact partially overlaps the exit (E) site of the ribosome, normally occupied by deacylated tRNA before it detaches from the ribosome. Excitingly, association of the HCV IRES with 40S induces a dramatic conformational change in the 40S subunit. This change is dependent on domain II. The positions of the observed changes on 40S indicated the altered 40S may function as a clamp that helps to hold the coding mRNA in the decoding cleft (Spahn et al. 2001). Such changes are consistent with the reduced intensity of 40S toeprints associated with mRNA bound in this cleft when domain II of HCV and CSFV were removed (Kolupaeva et al. 2000a,b).

Positioning of initiation-competent 40S subunits to the HCV IRES occurs in at least two separable steps

The functional importance of these observed sites of ribosomal interaction in recruiting 40S subunits to the initiation codon are supported by the results of extensive mutational analyses (Pestova et al. 1998b; Kieft et al. 1999, 2001; Jubin et al. 2000; Kolupaeva et al. 2000a,b; Lukavsky et al. 2000). Toeprinting of 40S subunits bound to CSFV and HCV IRESs has shown that they contain one set of determinants that is required for initial ribosomal attachment, and a second set of determinants that promotes accurate placement of the initiation codon in the ribosomal P site. The set of determinants required for ribosomal attachment includes the apical residues GGG_{266–268} in HCV IIIId and analogous residues in CSFV IIIId1, and the 5' proximal helix 1 of the pseudoknot. In contrast, helix 2 of the pseudoknot is not required for ribosomal binding, but substitutions in this helix impair

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binding of 40S to sequences flanking the initiation codon in the mRNA-binding cleft. Similarly, deletion of domain II and substitutions or deletions in subdomain IIIa did not impair the formation of binary IRES/40S subunit complexes but also impaired binding of the initiation codon region to the ribosomal mRNA-binding cleft. The observation that the initiation codon region can be deleted without impairing ribosomal attachment to the IRES even though it constitutes a major site of interaction between these IRESs and the 40S subunit is consistent with the conclusion that ribosomal attachment occurs in at least two separable steps.

Insect cricket paralysis-like viruses: initiation of translation without initiator tRNA

That notion that RNA has the ability to function as a catalyst in a variety of biological reactions without the participation of proteins recently has entered the IRES field. Although eIF-independent formation of 40S/HCV IRES complexes was heretical, the recent discovery that IRES elements in cricket paralysis-like viruses cannot only perform eIF-independent formation of 40S/IRES complexes but do not need eIF2, initiator tRNA, eIF5B, or GTP hydrolysis to form an 80S/IRES complex has been astounding.

The initiation codon-less IRES in cricket paralysis-like viruses

The recent sequencing of several insect picorna-like RNA virus genomes, epitomized by cricket paralysis virus (CrPV) has revealed that they are dicistronic (for references see <http://www.iah.bbsrc.ac.uk/virus/picorna-like>). The upstream open reading frame (ORF) that encodes the nonstructural protein precursor is separated by an intergenic region (IGR) from the downstream ORF encoding the structural protein precursor (Fig. 3). Translation of both ORFs is mediated by distinct IRESs (Sasaki and Nakashima 1999; Domier et al. 2000; Wilson et al. 2000b). Most interest has focused on the structurally conserved IGR IRES, ~180 nucleotides in length, because nucleotide and protein sequence analyses have shown that 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. The N-terminal residue of the capsid protein precursor is either alanine (encoded by GCU or GCA) or glutamine (encoded by CAA). This coding triplet is immediately adjacent to an ~42 nucleotide pseudoknot that is essential for IRES function determined by mutations that disrupt (Fig. 3, mutant 1) or restored the pseudoknot (Fig. 3, mutant 2; Sasaki and Nakashima 1999; Domier et al. 2000; Wilson et al. 2000b). Nucleotides upstream of this domain may form an extensive triple-nested pseudoknot (Kanamori and Nakashima 2001). As in the other classes of IRES described above, helical regions of CrPV-like IRESs appear primarily to play a structural role: analysis of seven CrPV-like viral genomes has indicated that co-

variant substitutions that maintain base pairing complementarity of helices have at most minor effects on IRES function. Most invariant nucleotides (shown in blue in Fig. 3) are located in unpaired loops and bulges in the triple pseudoknots and therefore may be involved in interactions with the ribosome.

IGR-IRES mediate translation is methionine independent

In a landmark study, Sasaki and Nakashima provided convincing evidence that the IGR-IRES in *Plautia stali* intestine virus (PSIV) mediates translation initiation at a glutamine-encoding CAA codon (Sasaki and Nakashima 2000). These authors showed that the PSIV IGR-IRES mediated translation of a protein lacking an N-terminal methionine residue; in clever control experiments, they excluded the possibility that methionine aminopeptidases removed the N-terminal methionine residue on the newly synthesized proteins (Sasaki and Nakashima 2000). This finding raised the possibility that if translation initiation from PSIV IGR-IRES did not need an initiator tRNA methionine, perhaps it could dispense with the entire ternary eIF2/GTP/initiator tRNA complex for initiation.

The IGR-IRES can assemble 80S complexes without eIFs, position the initiation start codon in the ribosomal A site, and translocate without formation of a peptide bond

Direct binding experiments showed that 40S subunits can bind stably to the CrPV IGR-IRES in the absence of ternary complex or any other eIF. Toeprint analysis performed to determine the position of the ribosome on the IGR-IRES indicated that the first decoded triplet, GCU, was in the ribosomal A site with the preceding CCU triplet in the P site (Wilson et al. 2000a). The CCU triplet is not decoded and can be substituted by any codon, even with a stop codon, without effect on IRES function if the pseudoknot structure is maintained (Sasaki and Nakashima 1999; Wilson et al. 2000b).

Surprisingly, addition of purified 60S subunits to the 40S/IGR-IRES complexes resulted in the formation of 80S/IGR-IRES complexes without any requirement for eIF5, eIF5B, or hydrolysis of GTP. Furthermore, translation assays in RRL showed that the aminoacyl-tRNA is delivered to the empty ribosomal A site by elongation factor 1 (EF1), after which the 80S ribosome undergoes a pseudotranslocation (i.e., without formation of a peptide bond) moving the GCU codon to the ribosomal P site (Wilson et al. 2000a). This translocation event does not depend on prior peptide bond formation and is resistant to both the nonhydrolyzable GTP analog GMP-PNP and the elongation inhibitor cycloheximide, indicating that translocation does not involve the enzymatic activity of EF2. Pseudotranslocation depends on delivery of aminoacyl-tRNA to the A site, as it is inhibited by antibiotics such as sparsomycin (Wilson et al. 2000a). 80S ribosomes

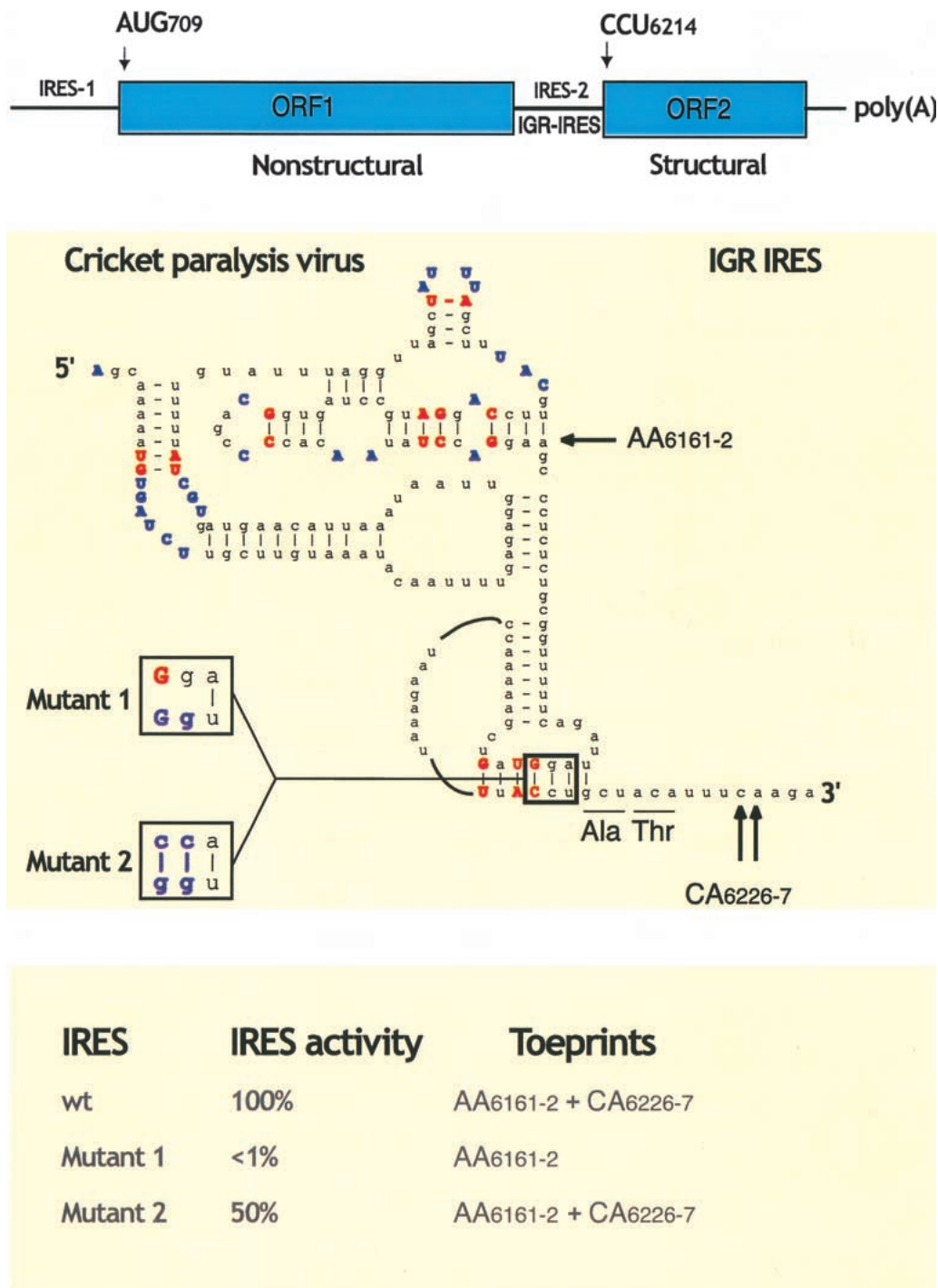


Figure 3. Features of the cricket paralysis virus (CrPV) IRES. A diagram depicting the genome structure of CrPV with its two IRES elements is shown on the top (see text for details). The predicted secondary structure of the CrPV IRES is remodeled after the *Plautia stali* intestine virus IRES (Kanamori and Nakashima 2001). Conserved base-paired and unpaired residues are shown in red and blue, respectively. The locations and genotypes of mutant 1 and mutant 2 are indicated. The bottom panel shows the IRES activities and toeprinting patterns in the mutated IRES elements.

therefore can move on the CrPV IGR-IRES in a way that has not been observed for any other mRNA. The mechanism by which translocation is induced has not yet been determined, but occupancy of the P site by the pseudo-knot as well as other interactions of upstream elements

of the IRES with the ribosome may potentiate ribosomal movement.

In examples discussed above, the EMCV IRES is able to bypass the requirement for eIF4E, and the HCV IRES does not require eIF4E, eIF4A, eIF4B, or eIF4F. The model

for CrPV IRES function presented here indicates that it has eliminated the requirement for initiator tRNA and all initiation factors and is able to bind ribosomes and to induce them to enter the elongation phase of translation directly. There is no evidence that any cellular mRNAs use this mode of IRES-mediated initiation, but any that were to do so would be expected to be translated efficiently in circumstances in which eIF2-GTP is limiting, for example, by phosphorylation of eIF2. Indeed, it has been shown that the activity of the CrPV IRES is greatly enhanced during the induction of the unfolded protein response, a scenario that results in the phosphorylation of eIF2 (Wilson et al. 2000a).

Properties of cellular IRES elements

Although the list of cellular mRNAs containing IRES elements is growing (Table 3), little is known about the mechanism by which naturally occurring cellular IRES elements capture 40S subunits. On the other hand, substantial evidence has been accumulated that points to the translational regulation of IRES-containing mRNAs during the cell cycle and during various stress situations that can lead to cell death. We address these findings in the following sections.

Cellular IRES elements can comprise multiple noncontiguous sequences that display IRES activity

The structural features of cellular IRES elements remain largely unknown. Le and Maizel have predicted that a Y-shaped double-hairpin structure followed by a small hairpin constitutes an RNA motif that can be found upstream of the start site codon in a variety of cellular IRES elements (Le and Maizel 1997). However, there is no experimental evidence for a functional role for this motif in internal initiation.

As described above, IRES elements in viral RNA genomes contain higher ordered structures whose integrities are essential for IRES activity. Thus, it was surprising that the IRES elements in BiP (Yang and Sarnow 1997), vascular endothelial growth factor (Huez et al. 1998), and c-myc (Stoneley et al. 1998) contained several noncontiguous sequence elements that displayed IRES activities on their own. These findings have suggested that elusive SD-like sequences may exist in IRES elements and that they can be isolated by functional means from complex IRES elements. A series of experiments by Mauro and colleagues has substantiated this notion. First, Mauro and Edelman noted that many eukaryotic mRNAs contain rRNA-like sequences in both sense and antisense orientations in their NTRs, coding and intron sequences (Mauro and Edelman 1997). Next, a functional role for these rRNA-like sequences was examined in the mRNAs encoding ribosomal protein S15 (Tranque et al. 1998) and the homeodomain GTX (Hu et al. 1999), both of which contain several sequence motifs with complementarity to the 3' end of 18S rRNA. Crosslinking of these sequence to 40S subunits could be accomplished

and cell-free translation assays showed that the strength of the mRNA-rRNA interactions was correlated inversely with mRNA translation efficiency. Further examination of the 196-nucleotide 5' NTR of GTX revealed the presence of an IRES (Table 3) that could be dissected into four nonoverlapping RNA sequences, each displaying IRES activity (Chappell et al. 2000a). A nine-nucleotide sequence element with complementarity to 18S rRNA also displayed IRES activity in the dicistronic assay that was approximately threefold over background; however, 10 linked copies of this mini-IRES stimulated second cistron translation by ~500-fold (Chappell et al. 2000a). The curious finding that the same nine nucleotide sequence element also can function as a translational repressor (see above) when present in the 5' NTR of a capped monocistronic mRNA is puzzling. Although there is no immediate answer to explain this apparent discrepancy, it is known from studies on 40S/HCV IRES complexes that the translational efficiency of mutated IRES elements can vary greatly even when the binding affinity of 40S to the mutated IRES was unaffected (Kieft et al. 2001). Thus, events subsequent to 40S binding might dictate in some manner the efficiency of the IRES.

Factors that regulate cellular IRES elements

It is likely that the canonical eIFs that recruit 40S subunits to picornaviral IRES elements are used in the recruitment of 40S subunits to most cellular IRES elements. However, besides the report that overexpression of eIF4G in cultured cells stimulated both cap-dependent translation as well as the IRES present in ornithine decarboxylase (Hayashi et al. 2000) nothing is known about the role of canonical eIFs in internal initiation.

The role of noncanonical ITAFs only recently has been explored for cellular IRES elements. Elroy-Stein and colleagues provided evidence that a phosphorylated form of hnRNP C interacts with the differentiation-induced IRES in PDGF2 mRNA (Table 3; Sella et al. 1999). Similarly, Holcik and colleagues found that the La autoantigen stimulated the IRES in X-linked inhibitor of apoptosis mRNA (Holcik and Korneluk 2000). Jang and collaborators showed that PTB inhibits the BiP IRES (Table 3) both in vitro and in cultured cells (Kim et al. 2000), suggesting that PTB can be both a negative and a positive (for the picornaviral IRESs) ITAF. More recently, Mitchell and colleagues showed that the activity of the Apaf-1 (apoptotic protease-activating factor 1) IRES was activated in rabbit reticulocyte lysates supplemented with PTB and unr (Mitchell et al. 2001). Similarly, Apaf-1 IRES activity was enhanced in cell lines deficient in these ITAFs after overexpression of PTB and unr. Moreover, this IRES bound unr, but bound PTB only in the presence of unr (Mitchell et al. 2001). From what is known about initiation on EMCV-like IRESs, ITAFs therefore likely exert their function on cellular IRESs as chaperones that aid in the folding of RNA motifs.

Although the auxiliary roles of ITAFs is unclear, it is intriguing that some of the ITAFs such as PTB, La, and hnRNP C can shuttle between the nucleus and cyto-

plasm during the cell cycle (Ghetti et al. 1992) and in poliovirus-infected cells (Gustin and Sarnow 2001), indicating that they may regulate the activity of IRES elements at particular stages of the cell cycle, during apoptosis or during viral infection.

A striking property of many cellular IRESs is that their activity shows strong cell type-specific variation and in some instances is developmentally controlled (Ye et al. 1997; Creancier et al. 2000; Stoneley et al. 2000b). The inability of an IRES to mediate initiation in specific cells or under specific physiological conditions could be due to the positive or negative regulatory ITAFs that influence IRES function but not cap-mediated initiation.

Growth regulatory genes and gene transcribed in response to stress contain IRES elements

To identify mRNAs that require reduced concentrations of intact cap-binding complex eIF4F, the polyribosomal association of mRNAs was examined by a cDNA microarray in poliovirus-infected cells at a time when both isoforms of eIF4G were significantly proteolyzed (Johannes et al. 1999). This analysis was expected to reveal classes of mRNAs that were simply overexpressed as a consequence of viral infection, in which translational elongation rates were slowed, that required low concentrations of intact eIF4F such as the shunting-promoting late leader of adenovirus (see above), or that contained IRES elements. Approximately 3% of the mRNAs examined remained on polysomes in infected cells; some of those have been shown to harbor IRES elements (Table 3). These IRES-containing mRNAs encode proteins that are produced as a response to a variety of stress situations, such as inflammation, angiogenesis, and response to serum (Johannes and Sarnow 1998; Johannes et al. 1999). Although these studies have the caveat of being performed in virus-infected cells, it is noteworthy that some of the IRES-containing mRNAs were identified by different experimental approaches as well. For example, the c-myc IRES has been shown to be active both during mitosis (Pyronnet et al. 2000) and during the induction of apoptosis (Stoneley et al. 2000a).

Regulation of IRES elements during cell growth and programmed cell death

It is known that global cellular protein synthesis is regulated during the G₂/M phase of the cell cycle (Bonneau and Sonenberg 1987; Huang and Schneider 1991) during programmed cell death (for review, see Holcik et al. 2000) and during a variety of stress situation such as nutritional deprivation, heat shock, or the unfolded protein response (for review, see Kaufman 1999). Declines in overall protein synthesis has been found to correlate with the inactivation of certain eIFs. Thus, it was of interest to test whether IRES-mediated translation was refractory to translation inhibition in these circumstances, especially in cases when the activity of eIF4F was diminished.

Certain viral and cellular IRES elements are active during the G₂/M phase of the cell cycle

The eIF4E subunit of eIF4F is dephosphorylated during the G₂/M phase of the cell cycle, correlating with diminished cap-dependent translation (Bonneau and Sonenberg 1987; Huang and Schneider 1991). However, the unstructured adenovirus late leader-containing mRNAs (Huang and Schneider 1991) and the IRES elements of poliovirus (Bonneau and Sonenberg 1987) and HCV (Honda et al. 2000) are able to mediate translation initiation efficiently during G₂/M, suggesting that these mRNAs require little or no phosphorylated eIF4F for ribosome recruitment. Very recently, G₂/M-regulated IRES elements have been identified in the mRNAs encoding ornithine decarboxylase (Pyronnet et al. 2000) and protein kinase p58^{PITSLRE} (Cornelis et al. 2000); in addition, the c-myc IRES was found to be active during G₂/M (Pyronnet et al. 2000). These experiments showed that inhibition of 5' end-dependent translation by dephosphorylation of eIF4F correlates with the selective translation of some IRES-containing mRNAs. However, cDNA microarray experiments have shown that several other IRES-containing mRNAs are not selectively translated during G₂/M (Qin and P. Sarnow, unpubl.), suggesting that other events that take place during G₂/M contribute to the translation of selected IRES-containing mRNAs.

IRES-regulated expression of the cationic amino acid transporter cat-1 during amino acid deprivation

It has been known from studies in *S. cerevisiae* that amino acid deprivation can lead to the phosphorylation of the α -subunit of eIF2 (see above), causing the enhanced translation of *GCN4* mRNA by a reinitiation mechanism (for review, see Hinnebusch 1997). Recent studies by Hatzoglou and colleagues have shown that amino acid starvation of mammalian cells also results in a rapid phosphorylation of eIF2 α (leading to a reduced concentration of ternary complex) and dephosphorylation of eIF4E (leading to a reduced concentration of active eIF4F), events that result in an overall decrease in host cell mRNA translation (Fernandez et al. 2001). The starved cells respond to this stressful situation with the synthesis of proteins involved in amino acid biosynthesis and of amino acid transporters, including the cationic amino acid transporter cat-1 that mediates the uptake of lysine and arginine (Hyatt et al. 1997). It was unclear how newly transcribed cat-1 mRNA could be translated when overall translation was inhibited. It was found that by 2 h after amino acid starvation phosphorylation of eIF2 declined substantially; by 9 h after starvation, a time when cap-dependent translation was still inhibited, translation of cat-1 mRNA was greatly enhanced (Fernandez et al. 2001). It was found that cat-1 contained an IRES that was significantly enhanced during amino acid deprivation (Fernandez et al. 2001). It will be very interesting to test whether the time lag observed is due to synthetic events: for example, phosphorylation of eIF2 α could promote the synthesis of an ITAF that then would enhance cat-1 IRES-mediated translation.

IRES-mediated translation during apoptosis

Table 3 lists IRES-containing cellular mRNAs that are translated during apoptosis, such as c-myc (Stoneley et al. 2000a), apoptotic protease activating factor (Apaf-1; Coldwell et al. 2000) and death-associated protein 5 (DAP5; Henis-Korenblit et al. 2000) mRNAs. It is known that the p38 mitogen-activated protein kinase pathway, which is activated during apoptosis, regulates internal initiation mediated by the c-myc IRES (Stoneley et al. 2000a). Thus, the mechanism and the outcome of the translation of these mRNAs has been under intense scrutiny because it is not immediately clear why proteins that both inhibit (DAP5) and activate (Apaf-1) apoptosis should be synthesized at the onset of programmed cell death.

Insight into this puzzle has come from studies that have examined the fate of the canonical eIFs after the onset of apoptosis. It was found that caspase 3 mediates the proteolysis of eIF2, eIF4B, eIF4GI, eIF4GII, the p35 subunit of eIF3, and eIF4E-BP both in vitro and in cells induced to enter apoptosis by a variety of treatments (Clemens et al. 1998; Marissen and Lloyd 1998; Satoh et al. 1999; Bushell et al. 2000b; Marissen et al. 2000a,b). Interestingly, caspase 3-mediated cleavage of eIF4GI in apoptotic lymphoma cells generates a stable, 76-kD M-FAG (middle fragment of apoptotic cleavage of eIF4G) protein that retains the binding sites for eIF4A, eIF3, and eIF4E but loses the binding site for PABP and Mnk1 (Bushell et al. 2000a). The elimination of a role for PABP in translation initiation during apoptosis was supported further by the finding that eIF4B is rapidly cleaved by caspase 3; mammalian eIF4B recently has been found to interact with PABP (Bushell et al. 2001). Overall, these findings suggest that M-FAG could mediate cap-dependent translation, although with less efficiency than eIF4GI, without the aid of PABP in apoptotic cells.

Thus, it was surprising that DAP5/p97 was found to be translated by internal initiation during apoptosis. It is a member of the eIF4G family that inhibits both cap-dependent and IRES-dependent translation (Imataka et al. 1997; Yamanaka et al. 1997) and that lacks the amino-terminal binding sites for PABP and eIF4E. The p97 form of DAP5 was cleaved by caspase 3 near its C terminus to yield a p86 protein (Henis-Korenblit et al. 2000); interestingly, p86 was found to function as a specific ITAF for the IRES in DAP5/97 (Henis-Korenblit et al. 2000). Therefore, both positive (i.e., M-FAG, DAP/p86) and negative (i.e., DAP5/p97) eIF4G-like molecules and complexes are expected to be present in apoptotic cells. Overall, these findings suggest that the affinity of IRES elements in mRNAs encoding pro- and antiapoptotic proteins for a variety of eIFs and ITAFs might dictate which IRES recruits ribosomal subunits most efficiently at a given time during the onset of apoptosis.

IRES regulation in human disease

Aberrant IRES-mediated translation can be correlated with the pathogenesis of two human diseases, multiple

myeloma and Charcot-Marie-Tooth disease (CMTX). On analysis of several cell lines obtained from patients with multiple myeloma, Willis and colleagues noted that c-myc protein levels were greatly enhanced, without a concomitant increase in the intracellular levels of c-myc mRNA. Inspection of c-myc mRNA revealed a single C to U change in the c-myc IRES in all of the cell lines (Paulin et al. 1996). The mutated c-myc IRES was more active than the wild-type IRES in cell lines derived from patients with multiple myeloma and this activity correlated with the enhanced binding of a distinct set of proteins (Paulin et al. 1998; Chappell et al. 2000b). More recently, the same C to U change in the IRES was found in 42% of bone marrow samples collected from patients with multiple myeloma; this change was not detected in control cells (Chappell et al. 2000b). Thus, there is a correlation between enhanced activity of the c-myc IRES and the development of disease. Whether mutations in other oncogenes have occurred in these cell lines is not yet known.

In a second example, Hudder and Werner showed that down-regulation of nerve-specific connexin 32 mRNA translation correlated with the CMTX phenotype, a neurodegenerative disease (Hudder and Werner 2000). Connexin 32 is a gap junction protein that is expressed by tissue-specific promoters both in the liver and in Schwann cells. Although most mutations in CMTX patients are found in the coding region of connexin 32, Hudder and Werner identified a C to U change in the 5' NTR of connexin 32 that lead to a tremendous inhibition of mRNA translation. Using dicistronic mRNAs and Northern analysis (R. Werner, pers. comm.), it was found that the connexin 5' NTR contained an IRES element. Using transgenic mice, it was shown that the C to U change in the connexin 32 IRES silenced the translation of a mutant IRES-containing reporter mRNA (Hudder and Werner 2000). The reason why connexin 32 mRNA translation is mediated by an IRES not clear. The authors speculated that the synthesis of the small amounts of connexin 32 needed might be fine-tuned to ensure that the gap junction protein is not present at the wrong place in the membrane when large excess of myelin that wraps around the axon is being synthesized (Hudder and Werner 2000).

Summary

Viral IRES elements with very different structures have revealed that 40S ribosomes can be recruited by at least three different mechanisms. As exemplified by the EMCV IRES, interaction of eIF4G/eIF4A with the IRES, aided by canonical eIFs 2, 3, and 4B, is necessary to recruit 40S subunits to the IRES. The HCV IRES can bind eIF-free 40S subunits without helicase eIF4A or other eIFs, yet recruits the ternary complex to position the initiator tRNA into the ribosomal P site. In contrast, the CrPV-like IRES elements can assemble 80S ribosomes without any eIF including the initiator tRNA; in fact, parts of the IRES itself occupy the ribosomal P site. These findings suggest that structural features in IRES

elements dictate the need for certain eIFs, such as helicase eIF4A, that aid in 40S recruitment. Thus, in each of these three mechanisms, ribosomal recruitment involves noncanonical interactions with canonical components of the translation apparatus. Notwithstanding the HAV IRES, even IRESs that require almost all canonical eIFs for 40S recruitment can function without eIF4E. Thus, most IRESs should be refractory to mechanisms that control 5' end-dependent translation via dephosphorylation of eIF4E or sequestration of eIF4E by eIF4E-binding proteins.

In contrast with viral IRES elements, some cellular IRES elements seem to function in a modular fashion. Certain large IRES elements can be dissected into smaller elements, each of which can display IRES activity. The failure of viral IRES elements to operate in modules may reflect their multifunctional roles in translation, replication, or packaging of the viral genome. Both viral and cellular IRES elements can be regulated by ITAFs in a cell-specific manner, adding complexity to the regulation of IRES elements. Equally important, the continuous activity of certain IRES elements when certain eIFs are limiting, for example, during various situations of cell stress and cell death, points to the biological importance of internal initiation. Translational regulation in the nervous system just recently has become appreciated as playing a crucial role in events such as activity-dependent synaptic changes (Scheetz et al. 2000). The recent finding that certain synaptically located mRNAs contain IRES elements raises the possibility that internal initiation may be used to synthesize proteins that are involved in regulation of synaptic plasticity (Scheetz et al. 2000; Pinkstaff et al. 2001).

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