Functional interactions in internal translation initiation directed by viral and cellular IRES elements

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Introduction

Initiation of protein synthesis involves a series of tightly regulated events that allow the recruitment of ribosomal subunits to the mRNA. Two distinct mechanisms have evolved in eukaryotic cells to initiate translation. In one of them, the methyl-7-G(5')pppN structure present at the 5' end of the mRNA, known as cap, is recognized by the initiation factor eIF4F, which is composed of eIF4E, eIF4G and eIF4A. Additionally, preinitiation complex formation requires, among others, the concerted action of initiation factor eIF2, responsible for binding to the initiator tRNA-Met, and eIF3, which interacts with the 40S ribosomal subunit (reviewed in Hershey & Merrick, 2000).

In the alternative mechanism, translation initiation occurs internally and is mediated by a cis-acting element, known as the internal ribosome entry site (IRES), that recruits the translational machinery to an internal initiation codon in the mRNA with the help of trans-acting factors (reviewed in Jackson, 2000). During many virus infections, as well as in other cellular stress conditions, changes in the phosphorylation state of eIF2, which lower the levels of the ternary complex eIF2–GTP–tRNA-Met, result in overall inhibition of protein synthesis. Conversely, specific shut-off of cap-dependent initiation depends upon modification of eIF4F functionality (reviewed in Thompson & Sarnow, 2000).

IRES elements bypass cap-dependent translation inhibition. Hence, IRES-driven translation initiation prevails during picornavirus infection (Macejak & Sarnow, 1991; Johannes & Sarnow, 1998; Johannes et al., 1999). Under these circumstances, cap-dependent initiation is severely compromised, due to the presence of small amounts of functional eIF4F. This is caused by cleavage or loss of solubility of eIF4G (Gradi et al., 1998; Cuesta et al., 2000), 4E-BP dephosphorylation (Gingras et al., 1996) or poly(A)-binding protein (PABP) cleavage (Joachims et al., 1999; Kerekatte et al., 1999).

Here, we discuss recent reports on the functional interactions between the key players in ribosome recruitment to internal sites in the mRNA: (i) the RNA sequences that constitute the cis-acting element, (ii) the eIFs required to link the RNA to the small ribosomal subunit and (iii) other trans-acting proteins that act as RNA chaperones and/or modulate IRES activity in response to physiological stimuli.

Occurrence of IRES elements in eukaryotic mRNAs

The development of bicistronic expression vectors containing the picornavirus 5' untranslated region (UTR) located in the intercistronic region led to the definitive demonstration that this region was able to confer internal initiation of translation on an mRNA, independent of the 5' end (Jang et al., 1988; Pelletier & Sonenberg, 1988). Since the discovery of IRES elements in picornavirus RNAs, functional IRES elements have been identified in an increasing number of mRNAs from animal cells and their pathogens (Table 1; reviewed in Carter et al., 2000). IRES-containing mRNAs encode a variety of proteins such as translation initiation factors, transcription factors, oncogenes, growth factors, homeotic genes and survival proteins, that are functional under acute cellular stress.

The presence of IRES elements in the mRNAs of plant cells and their viruses is still under study. Several plant viral mRNAs are naturally uncapped, hence their translation initiation is cap-independent (Niepel & Gallie, 1999; Skulachev et al., 1999; Wu & White, 1999). Interestingly, plant viral mRNAs often contain translational enhancers in their 3' UTRs, a strategy that also allows stimulation of translation initiation independent of the 5' cap (Wang et al., 1997, 1999b; Allen et al., 1999). It is likely that this strategy has been exploited in general by plant pathogens as an alternative to IRES elements.

The presence of IRES elements in yeast and fungal mRNAs is still unclear. Although yeast extracts support both IRES- and cap-dependent translation (Iizuka et al., 1994), none of the animal virus IRES elements seems to be active in yeast cells. An inhibitory activity exerted by a short inhibitor RNA (iRNA) has been reported to be responsible for the inactivation of several IRES elements when tested in yeast (Venkatesan et al., 1999).

Comparison of the IRES elements of picornaviruses indicates a lack of conservation of primary sequences, with the exception of a short polypyrimididine tract (Pestova et al., 1991). Moreover, when this comparison is extended to distantly
related viral and cellular IRES elements, it is clear that not only the sequence but also the length varies among different IRES elements. IRES elements of around 200 nt are present in insect RNA viruses (Sasaki & Nakashima, 1999; Wilson \textit{et al.}, 2000), whereas picornavirus RNAs have IRES elements of about 450 nt (Jang & Wimmer, 1990; Kühn \textit{et al.}, 1990). This large variation is indicative of differences in (i) structural organization and (ii) the strategies used to interact with the translational machinery. These aspects of the IRES biology are discussed below.

**Structure–function relationships in IRES elements**

The phylogenetic conservation of IRES secondary structure is one of the strongest arguments in support of the structural requirements that determine IRES activity. Studies on the genetic variability within the IRES regions of infectious, highly variable RNAs, such as foot-and-mouth disease virus (FMDV) and hepatitis C virus (HCV), reveal that most of the substitutions cause compensatory changes in the secondary structure of the IRES. Moreover, IRES variability selected in nature is usually accompanied by a change in activity within a narrow range (Martínez-Salas \textit{et al.}, 1993; Kamoshita \textit{et al.}, 1997; Collier \textit{et al.}, 1998; Sáiz \textit{et al.}, 1999; Lerat \textit{et al.}, 2000), suggesting a restriction imposed by natural selection on the translation efficiency of viable viruses.

Nevertheless, the structural requirements for initiation of translation seem to be different in IRES elements from distant picornaviruses, defining two main groups of IRES structures (entero- and rhinoviruses versus cardio- and aphthoviruses), this difference being more remarkable when the comparison is extended to the HCV or classical swine fever virus (CSFV) IRES elements (Jackson, 2000). Hence, the same function can be accomplished by a different structural organization.

**Identification of regions essential for IRES activity**

On the assumption that conserved motifs often correspond to essential parts of the molecule, mutational analysis has been carried out on many IRES elements to define the precise sequences required for activity. In addition to studies performed with infectious picornavirus clones (Haller & Semler, 1992; Pilipenko \textit{et al.}, 1992), an exhaustive mutational analysis has taken advantage of the IRES property of directing internal entry in bicistronic expression vectors (reviewed in Jackson, 2000). The latter approach offers the possibility of testing the IRES in the absence of virus infection, thus eliminating interference with other functions of the virus cycle where the IRES may also be involved, such as replication or encapsidation (Borman \textit{et al.}, 1994; Ishii \textit{et al.}, 1999; Johansen & Morrow, 2000).

With this experimental approach, parts of the secondary structure of IRES elements have been associated with activity. Some essential sequences were found to form part of double-stranded regions (Jang & Wimmer, 1990; Hoffman & Palmenberg, 1996; Honda \textit{et al.}, 1996, 1999; Martínez-Salas \textit{et al.}, 1996), whereas others, located in apical or internal loops,

### Table 1. Examples of IRES elements

<table>
<thead>
<tr>
<th>Virus/gene type</th>
<th>Virus/gene type</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Viral RNAs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Picornaviruses</td>
<td>Poliovirus (PV)</td>
<td>Pelletier &amp; Sonenberg (1988)</td>
</tr>
<tr>
<td>Flaviviruses</td>
<td>Hepatitis C virus (HCV)</td>
<td>Reynolds et al. (1995)</td>
</tr>
<tr>
<td>Pestiviruses</td>
<td>Classical swine fever virus (CSFV)</td>
<td>Pestova et al. (1998)</td>
</tr>
<tr>
<td>Retroviruses</td>
<td>Murine leukaemia virus (MLV)</td>
<td>Berloz &amp; Darlix (1995)</td>
</tr>
<tr>
<td>Lentiviruses</td>
<td>Simian immunodeficiency virus (SIV)</td>
<td>Ohlmann et al. (2000)</td>
</tr>
<tr>
<td>Insect RNA virus</td>
<td>Cricket paralysis virus (CrPV)</td>
<td>Wilson et al. (2000a)</td>
</tr>
<tr>
<td><strong>Cellular mRNAs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Translation initiation factors</td>
<td>eIF4G</td>
<td>Johannes &amp; Sarnow (1998)</td>
</tr>
<tr>
<td>Transcription factors</td>
<td>c-Myc</td>
<td>Stoneley et al. (2000)</td>
</tr>
<tr>
<td>Transcription factors</td>
<td>NF-xB-repressing factor (NRF)</td>
<td>Oumard et al. (2000)</td>
</tr>
<tr>
<td>Growth factors</td>
<td>Vascular endothelial growth factor (VEGF)</td>
<td>Huez et al. (1998)</td>
</tr>
<tr>
<td>Growth factors</td>
<td>Fibroblast growth factor 2 (FGF-2)</td>
<td>Creancier et al. (2000)</td>
</tr>
<tr>
<td>Homeotic genes</td>
<td>Antennapedia</td>
<td>Bernstein et al. (1997)</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>BiP</td>
<td>Coldwell et al. (2000)</td>
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<tr>
<td></td>
<td></td>
<td>Macejak &amp; Sarnow (1991)</td>
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required a specific primary sequence to allow IRES activity (López de Quinto & Martínez-Salas, 1997; Psaridi et al., 1999; Robertson et al., 1999; Jubin et al., 2000). Disruption of some of these regions is associated with the modification of essential RNA–protein interactions (López de Quinto & Martínez-Salas, 2000). However, other motifs such as the conserved GNRA tetraloop located at a distal loop in the central domain of FMDV IRES (Fig. 1) are candidates to determine long-range RNA interactions. Taken together, there is experimental evidence that not only point substitutions but also small deletions and/or insertions inactivate the IRES, indicating that the overall RNA structure needs to be maintained in order to retain activity.

The structural organization of cellular IRES elements is poorly defined. Mutations found in the c-myc IRES identified a C-to-U transition within this element that modified the predicted secondary structure and led to enhanced activity in multiple myeloma cell lines (Chappell et al., 2000b). In the case of vascular endothelial growth factor (VEGF) mRNA, two IRES elements seem to occupy non-overlapping sequences (Huez et al., 1998), each of them directing initiation at a different codon. On the other hand, a modular organization, consisting of repetitions of a short sequence, has been proposed for the Gtx IRES (Chappell et al., 2000a).

**IRES tertiary structure interactions**

Experimental evidence in support of tertiary structure generated by RNA–RNA interactions in IRES elements is available for FMDV and HCV (Fig. 1). A pseudoknot structure in the HCV IRES was shown to be absolutely required for IRES activity, as mutations that destabilized the tertiary interactions between residues of loop IIIb and those complementary in domain IV were accompanied by a strong reduction in translation initiation (Wang et al., 1995; Honda et al., 1996). Similarly, the IRES located in the intergenic region of an insect virus RNA also contains a pseudoknot-like structure, as shown by phylogenetic and mutational studies (Wilson et al., 2000a; Sasaki & Nakashima, 2000).

Long-range RNA–RNA interactions have been shown to occur in vitro between functional domains of the FMDV IRES (Ramos & Martínez-Salas, 1999). These interactions are strand-specific and depend on RNA concentration, ionic conditions and temperature, suggesting a dynamism in the tertiary structure of the IRES that may play an important role in the biology of this element. The RNA–RNA interactions observed in vitro between separated domains of the FMDV IRES, in the absence of proteins, suggest that the IRES adopts a specific folding, depending upon environmental conditions. A similar situation has been described for the HCV IRES, which adopts different folding patterns in response to increased ion concentrations (Kieft et al., 1999). Since the intracellular concentration of cations varies with time in picornavirus-infected cells (Carrasco, 1995), it is likely that changes in the tertiary structure of the IRES occur readily during infection and, as a consequence, modulate translation efficiency.

Interestingly, the central region (domain 3) of the FMDV IRES is unique in its ability to interact with all the other domains (see Fig. 1), including the whole IRES (Ramos & Martínez-Salas, 1999). This result has two implications: the first is that domain 3 acts as a scaffold structure that holds together the remaining domains of the IRES and the second is that domain 3 is necessary to determine intermolecular interactions with other IRES molecules. These interactions can account for reports of complementation between defective IRES elements (Drew & Belsham, 1994; Roberts & Belsham, 1997). In the context of a virus infection where replication occurs in precise cellular regions (Bollen et al., 1998), the local RNA concentration can reach the levels required to allow intermolecular interactions.

Is the rearrangement of IRES structure relevant to biological activity? Physiological changes in the intracellular milieu
affecting ionic conditions, pH gradients, temperature, free radical formation, expression of specific RNA-binding proteins and so on are likely to induce reorganization of the IRES structure, which may have important consequences for recruiting \textit{trans}-acting factors. Consistent with this, artificial reorganization of the spatial RNA structure has strong effects on the binding of cellular factors to the HCV IRES (Odreman-Macchioli \textit{et al}., 2000). Additionally, mutations in loop IIIId cause structural reorganization of the HCV IRES, as measured by RNase T1 sensitivity and Fe(II) EDTA cleavage, concomitant with a reduction in IRES activity (Kieft \textit{et al}., 1999; Jubin \textit{et al}., 2000). Taken together, the experimental evidence indicates that the overall RNA structure of the IRES has an active role in internal initiation.

\section*{Interaction of viral IRES elements with the translational machinery}

Soon after the first reports of IRES activity in the picornavirus 5' UTR, specific RNA–protein interactions were described (Jang \& Wimmer, 1990; Luz \& Beck, 1991; reviewed in Stewart \& Semler, 1997; Andino \textit{et al}., 1999). Surprisingly, none of the first proteins reported was catalogued as a translation eIF. Hence, the question was: do IRES elements represent a different strategy for recruiting ribosomes and, then, do all IRES elements use the same mechanism to interact with the translational machinery? RNA–protein interaction assays have been exploited extensively, with cellular extracts as well as purified proteins, to study the mechanism of ribosome recruitment by viral IRES elements. However, binding per se does not necessarily mean a functional requirement for IRES activity. A correlation between lack of activity and absence of binding should be followed by the restoration of binding upon gain of IRES function. In recent years, it has become increasingly clear that eIFs play an essential role in IRES-dependent initiation. According to their functional requirements for RNA–protein interactions (Table 2), viral IRES elements can be separated into different categories, which are discussed below.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{IRES} & \textbf{Translation initiation factors} & \textbf{Trans-acting factors} \\
\hline
EMCV & eIF4G-Ct, eIF4A, eIF3, eIF2 & PTB* \\
FMDV & eIF4G-Ct, eIF4A, eIF3, eIF2 & PTB, ITAF13 \\
Poliovirus & NK & PTB, PCBP2, La, unr \\
Rhinovirus & NK & PTB, PCBP2, La, unr \\
HCV & eIF3, eIF2 & PTB, PCBP2, La \\
CrPV & None & NK \\
\hline
\end{tabular}
\caption{Functional RNA–protein interactions in viral IRES elements}
\end{table}

\textbf{eIF4G-dependent IRES: encephalomyocarditis virus (EMCV) and FMDV}

The canonical initiation factors eIF4A, eIF4G, eIF2 and eIF3 were reported to be required for 48S complex formation in a reconstituted 40S ribosome-binding assay with the EMCV IRES (Pestova \textit{et al}., 1996; Kolupaeva \textit{et al}., 1998). This assay reveals the minimal requirements for assembly of the initiation complex \textit{in vitro}.

A mutational analysis in conjunction with UV cross-linking, immunoprecipitation, Western blot and competition assays led to the identification of a structural region in the base of domain 4 as an essential component of the FMDV IRES involved in binding to eIF4G (López de Quinto \& Martínez-Salas, 2000) (see Fig. 2A). In agreement with this observation, eIF4G interacts with the related EMCV IRES in a similar position, as shown by toeprint as well as footprint analysis (Kolupaeva \textit{et al}., 1998). Binding of eIF4G to a transcript encoding FMDV domain 4 alone was as effective as binding to the full-length IRES, suggesting strongly that no additional sites for recognition of this protein in the FMDV IRES are required. Furthermore, the C-terminal region of the proteolytically processed form of eIF4G (eIF4G-Ct), present in cells transfected with the FMDV Lb protease, binds as efficiently to the FMDV IRES as does the unprocessed protein (López de Quinto \& Martínez-Salas, 2000). The strong correlation found between eIF4G–RNA interaction and IRES activity in transfected cells demonstrates that eIF4G binding is an essential step in recruitment of the translational machinery \textit{in vivo}.

The C-terminal region of eIF4G that interacts with the IRES contains the binding sites for eIF3 and eIF4A (Gingras \textit{et al}., 1999). Accordingly, eIF4A stimulates binding of the central part of eIF4GI (aa 746–949) to the EMCV and FMDV IRES elements (Lomakin \textit{et al}., 2000; Pilipenko \textit{et al}., 2000). Although eIF4GI contains two non-canonical RNA-binding motifs in this central region (Gingras \textit{et al}., 1999), it is not yet known which residues in eIF4G are responsible for IRES binding. Consistent with the interaction of eIF4G-Ct with picornavirus IRES elements, internal initiation of translation promoted by these
elements is efficient under conditions of eIF4G cleavage (Ohlmann et al., 1996). However, the hepatitis A virus IRES requires intact eIF4G (Borman & Kean, 1997).

The initiation factor eIF4B stimulates 48S complex formation on EMCV (Pestova et al., 1996) and also binds to sequences within the 3’ end of the FMDV IRES (Meyer et al., 1995). However, in contrast to eIF4G, eIF4B binding to the FMDV IRES requires sequences within domain 5 (López de Quinto & Martínez-Salas, 2000), suggesting that both eIFs interact with the 3’ region of the IRES but do so by using different residues (Fig. 2A). Although strong eIF4B–RNA binding has been shown, the biological significance of this interaction in IRES-dependent initiation has yet to be resolved.

Several non-canonical initiation factors have been shown to interact functionally with picornavirus IRES elements (Table 2). In contrast to Théier’s murine encephalitis virus and EMCV, the FMDV IRES requires both the polypyrimidine tract-binding protein (PTB) (Kolupaeva et al., 1996; Niepmann et al., 1997) and the proliferation-associated factor ITAF45 for 48S complex formation in vitro (Pilipenko et al., 2000). Therefore, even closely related IRES elements that share secondary structure and primary sequence in essential regions behave differently in terms of functional RNA–protein associations.

The other group of IRES elements in the picornaviruses (poliovirus and rhinovirus) interacts with La, PCBP2 (hnRNP E2), unr and PTB (hnRNP l) (Table 2) (Meerovitch et al., 1993; Blyn et al., 1997; Gamarnik & Andino, 1997; Hunt et al., 1999; Walter et al., 1999). Most of these are cellular proteins with previously assigned functions. Additionally, specific stimulation of poliovirus and rhinovirus IRES activity is exerted by 2A and Lb picornavirus proteases (Ziegler et al., 1995; Ohlmann et al., 1996) by as yet unknown mechanisms.

Some of the factors mentioned above (PTB, ITAF45 and PCBP2) contain several RNA-binding motifs and display multiple interactions with the IRES molecule and their capacity to dimerize has been described (Blyn et al., 1997; Conte et al., 2000; Gamarnik & Andino, 2000; Kim et al., 2000; Pilipenko et al., 2000). Hence, it is likely that these proteins act as RNA chaperones, directing or stabilizing the tertiary folding of the RNA.

eIF4G-independent, eIF2-dependent IRES elements: HCV and CSFV

The results of toeprinting analysis indicate that 48S initiation complex formation driven by the HCV and CSFV IRES elements requires only eIF2–GTP/Met-tRNAi, eIF3 and 40S subunits (Pestova et al., 1998; see Table 2). The binding site of eIF3 has been mapped to residues in stem–loop IIIb (Fig. 2B) (Buratti et al., 1998; Sizova et al., 1998). The 40S ribosomal subunit seems to interact at multiple sites, including sequences in loop IId, the helix between IIIc and IId and residues flanking the pseudoknot as well as the initiator AUG (Kolupaeva et al., 2000). To date, HCV is the only IRES for which a ribosomal protein, S9, has been shown to interact with IRES sequences (Fukushi et al., 1999). Recently, eIF2B γ and eIF2 γ have been identified as cofactors of HCV IRES-mediated translation (Kruger et al., 2000).
In support of the biological relevance of the interactions mentioned above, mutation of the regions that constitute the binding site for these proteins is detrimental to IRES activity (Honda et al., 1996; Jubin et al., 2000). As in the case of the picornaviruses, the HCV IRES also binds trans-acting factors other than the canonical eIFs (Table 2) (Ali & Siddiqui, 1997; Kamoshita et al., 1997; Hahm et al., 1998; Spangberg & Schwartz, 1999).

HCV IRES-dependent initiation resembles the mechanism used by the Shine–Dalgarno region in prokaryotic mRNAs (Pestova et al., 1998; reviewed in Jackson, 2000). It was proposed long ago that the IRES–ribosome interaction may take place by base-pair complementarity between a purine-rich sequence close to the 3′ end of the 18S rRNA and the conserved polypyrimidine tract present in the picornavirus IRES (Beck et al., 1983; Pestova et al., 1991). However, this possibility has never been documented experimentally.

eIF2-independent IRES: cricket paralysis virus (CrPV)

It has been shown that the intergenic region of CrPV has IRES activity when tested in bicistronic expression vectors (Wilson et al., 2000b). Surprisingly, toeprinting assays carried out in the presence of drugs that interfere with AUG recognition by the 40S–eIF2–GTP/Met-tRNA<sub>i</sub> complex demonstrated that this peculiar IRES recruits 40S ribosomes in vitro in the absence of eIFs (Wilson et al., 2000a; see Table 2). This is indicative of a molecular mimicry mechanism, by which the IRES itself substitutes functionally for the role played by the initiator Met-tRNA in the eIF2–GTP ternary complex (Wilson et al., 2000a). In agreement with this, the IRES present in the closely related Plautia stali intestinal virus (PSIV) is also able to direct protein synthesis initiation at a CAA codon in the absence of eIF2, Met-tRNA or GTP (Sasaki & Nakashima, 2000).

Modular organization of IRES elements: distribution of functions in structural domains

Is a core structural motif required for basal IRES activity? An IRES could be envisaged as a regulatory element having modular organization, in which the activity of a core region, able to interact with the translational machinery, is modulated, either stimulated or repressed, by other IRES subunits. This model theoretically could apply to EMCV and FMDV, where the domains located at the 3′ end of the IRES (I–K or 4–5, respectively) (Fig. 2A) mediate the interaction with eIFs required for 48S initiation complex formation in vitro (Kolupaeva et al., 1998; Pilipenko et al., 2000) and establish RNA–eIF4G interactions, which are essential for IRES activity in vitro (López de Quinto & Martínez-Salas, 2000). Assuming that this hypothetical model is true, basal IRES activity should be detected when this region alone is used to promote internal initiation. To date, there is no experimental evidence to indicate that this region is active by itself in promoting internal initiation in vivo.

In contrast, there is extensive evidence in support of the requirement for the whole length of the picornavirus IRES for detecting full activity. Several point substitutions, outside domains 4–5, abolish IRES activity completely by altering essential motifs that are candidates to mediate RNA folding (López de Quinto & Martínez-Salas, 1997; Robertson et al., 1999). Furthermore, precise deletions removing each of the five predicted stem–loops of FMDV were shown to reduce IRES activity (Drew & Belsham, 1994). Hence, IRES–eIF4G binding (achieved by domain 4 in FMDV or domains J–K in EMCV) is necessary, but not sufficient, for fully efficient internal initiation.

Thus, we favour an alternative model of RNA functional compartmentalization, which takes into account the distribution of functions among the different regions of the IRES molecule. Hence, whereas the main function of domains 4–5, in the 3′ region of the IRES, is to interact with eIFs (Fig. 2A), the 5′ and central regions (domains 1–2 and 3) are involved in the organization of the IRES architecture (Fig. 1), directing intramolecular RNA–RNA interactions (Ramos & Martínez-Salas, 1999). In agreement with the latter model, the PTB protein that seems to act as a RNA chaperone has its main binding site in the 5′ end of the IRES, but it also interacts with sequences in the 3′ end (Luz & Beck, 1991; Kolupaeva et al., 1996).

Cellular IRES elements: specific stimulation

A characteristic feature of cellular mRNAs that contain IRES elements is that they are also translated in a cap-independent manner, although quite inefficiently, often leading to the production of a different version of the protein (Huez et al., 1998; Henis-Korenblit et al., 2000; Cornelis et al., 2000). Hence, cellular IRES elements represent a mechanism of gene expression regulation that is functional during nuclear inactivity, as in the case of homeotic genes (Oh et al., 1992), and responds quickly to acute stress conditions.

It is known that several cellular IRES elements are activated by physiological stimuli (Table 3) including, among others, hypoxia, vascular lesions, serum deprivation, γ-irradiation, as well as factors that induce apoptosis, growth arrest and angiogenesis (Huez et al., 1998; Stein et al., 1998; Holcik et al., 2000; Stoneley et al., 2000a). Activation of IRES elements present in the ornithine decarboxylase and p58IPITSLRE protein kinase mRNAs is observed during specific phases of the cell cycle or in a proliferation-dependent manner (Cornelis et al., 2000; Pyronnet et al., 2000). On the other hand, tissue-specific regulation of fibroblast growth factor (FGF-2) (Creancier et al., 2000), c-myc (Stoneley et al., 2000b) or platelet-derived growth factor (PDGF B/c-sis) (Bernstein et al., 1997; Sella et al., 1999) is likely related to the involvement of IRES elements in the fine tuning of gene expression. However, the activating factors have not yet been clearly established, and
Table 3. RNA–protein interactions in cellular IRES elements

<table>
<thead>
<tr>
<th>IRES</th>
<th>Trans-acting factors</th>
<th>Stimulatory conditions</th>
</tr>
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<tbody>
<tr>
<td>DAP5</td>
<td>DAP5</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>XIAP</td>
<td>hnRNP C, La</td>
<td>Apoptosis, γ-irradiation</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>PTB, unr</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>c-Myc</td>
<td>PTB, IRP, unr, DAP5</td>
<td>Apoptosis, Bloom syndrome</td>
</tr>
<tr>
<td>VEGF</td>
<td>PTB</td>
<td>Vascular lesions, hypoxia</td>
</tr>
<tr>
<td>PDGF2</td>
<td>hnRNP C</td>
<td>Megakaryocytic differentiation</td>
</tr>
<tr>
<td>Antennapedia</td>
<td>NK</td>
<td>Third-instar larvae</td>
</tr>
</tbody>
</table>

NK, Not known.

it is not known whether different IRES elements share activator molecules.

RNA–protein interaction studies conducted on cellular IRES elements have identified a number of proteins (Table 3). With the exception of the apoptotic translation-initiation factor DAP5, none of the currently known cellular IRES-binding proteins is a canonical eIF and in most cases (hnRNP C, PTB, PCBP2, La) they have a role in nuclear RNA metabolism (Sella et al., 1999; Holcik & Korneluk, 2000; Stoneley et al., 2000b). In contrast to viral IRES elements, several cellular IRES elements function only when expressed from within the nucleus (Stoneley et al., 2000b), suggesting that the nuclear compartment tags the RNA, although the nature of this tagging is unknown.

It remains to be elucidated whether or not cellular IRES elements require canonical eIFs to initiate translation. As the studies carried out are still very limited, it is too early to include these IRES elements in any of the categories defined above for viral IRES elements. In one example, complementarity of a 9 nt repeat in the Gtx homeodomain protein mRNA and the 18S rRNA has been suggested to direct RNA–RNA interaction between the IRES and the rRNA (Chappell et al., 2000a).

Start-codon recognition in IRES-dependent initiation

Discrimination of the authentic initiation site is achieved by both the IRES element and functional sequences surrounding the AUG codon (López de Quinto & Martínez-Salas, 1998; Ohlmann & Jackson, 1999; reviewed in Martínez-Salas, 1999; Belsham & Jackson, 2000). The parameters that influence initiation site recognition in cellular IRES elements are as yet poorly characterized.

The initiation site in aphthovirus RNAs is of particular interest. In contrast to poliovirus and EMCV, which use one AUG codon to start translation (Nicholson et al., 1991; Hellen et al., 1994; Kaminski et al., 1994), FMDV translation initiation occurs at two in-frame AUG codons separated by 84 nt, with the second being used most frequently (Cao et al., 1995; López de Quinto & Martínez-Salas, 1999; reviewed in Belsham & Jackson, 2000). Studies conducted recently on equine rhinitis A virus, another member of the genus Aphthovirus, that also contains two initiator AUGs suggested that the second AUG is not reached by leaky scanning (Hinton et al., 2000). Hence, the experimental evidence to explain aphthovirus start codon selection is compatible with two possibilities. One is the existence of a unique ribosome-loading site in front of the first AUG, followed by transfer to the vicinity of the second AUG. The second possibility is that two ribosome-loading sites exist in the FMDV RNA. In both of these models, ribosomes initiate translation at the second site in the vast majority of the viral RNAs (López de Quinto & Martínez-Salas, 1999).

Synergism between the IRES and 3’ mRNA regions

Cap-dependent translation initiation is strongly enhanced by poly(A) tails (Gallie, 1991; reviewed in Sachs, 2000). This synergism is due to the physical interaction of PABP with eIF4G which, in turn, respectively tether the ends of the mRNA through their specific recognition of the poly(A) tail and the cap–eIF4E complex (Tarun & Sachs, 1996; Preiss & Hentze, 1998).

The evidence for RNA circularization during IRES-dependent initiation is still limited. A small enhancement of IRES-driven translation by poly(A) tails in vitro has been reported recently (Michel et al., 2000). However, the PABP–eIF4G–RNA interaction is disrupted in picornavirus-infected cells by the action of 2A or Lb proteases. Although eIF4G-Ct supports IRES-dependent translation and binds efficiently to the IRES (Kolupaeva et al., 1998; López de Quinto & Martínez-Salas, 2000), the binding motif for PABP is retained in the N-terminal fragment of the processed protein (Gingras et al., 1999). PABP, eIF4G and eIF4B are also cleaved during apoptosis (reviewed in Clemens et al., 2000), another circumstance in which IRES-dependent initiation is induced. Hence, the stimulation observed in vitro could not be extrapolated to living cells unless the proteolytic fragments are able to interact with each
other or there are other proteins involved in such an interaction.

In agreement with the notion that interactions among hnRNP E2, I, K and L, in concert with their RNA-binding preferences, could mediate interactions between the IRES and the 3' end of the mRNA, protein–protein–hnRNP interactions have recently been found (Kim et al., 2000). Accordingly, PTB, which binds both the HCV 3' end and the IRES, has been proposed to stimulate the HCV IRES (Hahn et al., 1998; Ito et al., 1998). Other possibilities to close the circle could be the interactions eIF4A–PAIP–PABP (Craig et al., 1998), eIF4B–PABP (Le et al., 2000) or PCBP–PABP (Wang et al., 1999a), but none of these has yet been reported to stimulate IRES activity.

Concluding remarks

The RNA that constitutes the IRES plays a pivotal role in determining its activity. The IRES seems to substitute functionally for one (eIF4E) or more (eIF4F, eIF3 and eIF2) components of the initiation machinery necessary for cap-dependent initiation.

At present, there are several possibilities to account for the differences in the mechanism of IRES-driven protein synthesis. One is that it may be a fail-safe strategy that has evolved from, or co-evolved with, cap-dependent initiation that allows synthesis of proteins required under stress conditions. On the other hand, the IRES may be reminiscent of the Shine–Dalgarno sequences that govern translation initiation in prokaryotic cells. In the case of the picornavirus IRES, the features shared with cap-dependent initiation in terms of requirements for eIFs favour the first possibility. With the exception of eIF4E, the rest of the eIFs are required, and non-canonical eIFs exert a regulatory role on IRES activity. However, the mechanism used by the HCV IRES favours the second explanation.

The diversity of IRES sequences, in terms of length, primary sequence and structural requirements, together with the variety of trans-acting proteins found in the IRES elements described so far, suggests that they have probably appeared at different points in evolution, being positively selected for by their ability to interact with factors that are essential for recruitment of ribosomal subunits. On the other hand, the differences found in the molecular mechanisms used by IRES elements to recruit the translational machinery indicate that internal initiation is not simply an alternative mechanism, rather it represents a fail-safe strategy to ensure the synthesis of certain proteins under physiological stress conditions.

In view of the rapidly increasing amount of information available on IRES structural organization and their exploitation of particular functional interactions to recruit ribosomes internally, we should look forward to an exciting future in understanding fully the biological relevance of IRES elements.

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